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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE TRADEMARK TRIAL AND APPEAL BOARD

Proceeding	91194218
Party	Plaintiff Illumina, Inc.
Correspondence Address	SUSAN M NATLAND KNOBBE MARTENS OLSON & BEAR LLP 2040 MAIN ST, 14TH FL IRVINE, CA 92614 UNITED STATES efiling@knobbe.com
Submission	Plaintiff's Notice of Reliance
Filer's Name	Brian C. Horne
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Signature	/Brian C. Horne/
Date	11/06/2014
Attachments	Opposer's Notice of Reliance ILLINC.266M.pdf(51008 bytes) Exhibit 1.pdf(62587 bytes) Exhibit 2.pdf(1029997 bytes) Exhibit 3.pdf(151728 bytes) Exhibit 4.pdf(471447 bytes) Exhibit 5.pdf(195981 bytes) Exhibit 6.pdf(298385 bytes) Exhibit 7.pdf(1991226 bytes) Exhibit 8.pdf(985354 bytes) Exhibit 9.pdf(3514264 bytes) Exhibit 10.pdf(286249 bytes) Exhibit 11.pdf(911536 bytes) Exhibit 12.pdf(679552 bytes) Exhibit 13.pdf(279376 bytes) Exhibit 14.pdf(908217 bytes) Exhibit 15.pdf(215086 bytes) Exhibit 16.pdf(393873 bytes) Exhibit 17.pdf(259992 bytes) Exhibit 18.pdf(749607 bytes) Exhibit 19.pdf(515780 bytes) Exhibit 20.pdf(329444 bytes) Exhibit 21.pdf(481499 bytes) Exhibit 22.pdf(501051 bytes) Exhibit 23.pdf(283473 bytes) Exhibit 24.pdf(781425 bytes) Exhibit 25.pdf(4207616 bytes) Exhibit 26.pdf(983537 bytes) Exhibit 27.pdf(411124 bytes) Exhibit 28.pdf(354054 bytes) Exhibit 29.pdf(379575 bytes) Exhibit 30.pdf(518008 bytes) Exhibit 31.pdf(380679 bytes) Exhibit 32.pdf(1096053 bytes) Exhibit 33.pdf(313829 bytes) Exhibit 34.pdf(751947 bytes) Exhibit 35.pdf(2421609 bytes) Exhibit 36.pdf(304870 bytes)

	Exhibit 37.pdf(256781 bytes) Exhibit 38.pdf(110770 bytes) Exhibit 39.pdf(281628 bytes) Exhibit 40.pdf(1891479 bytes) Exhibit 41.pdf(3712615 bytes) Exhibit 42.pdf(1166486 bytes) Exhibit 43.pdf(3994991 bytes) Exhibit 44.pdf(1248335 bytes) Exhibit 45.pdf(193250 bytes) Exhibit 46.pdf(594622 bytes) Exhibit 47.pdf(79694 bytes) Exhibit 48.pdf(847023 bytes) Exhibit 49.pdf(323873 bytes) Exhibit 50.pdf(680363 bytes) Exhibit 51.pdf(1773466 bytes) Exhibit 52.pdf(561703 bytes) Exhibit 53.pdf(168595 bytes) Exhibit 54.pdf(467998 bytes) Exhibit 55.pdf(1412735 bytes) Exhibit 56.pdf(424554 bytes) Exhibit 57.pdf(520159 bytes) Exhibit 58.pdf(149679 bytes) Exhibit 59.pdf(181474 bytes) Exhibit 60.pdf(197961 bytes) Exhibit 61 CONFIDENTIAL.pdf(430394 bytes) Exhibit 62 CONFIDENTIAL.pdf(42099 bytes) Exhibit 63 CONFIDENTIAL.pdf(42475 bytes) Exhibit 64 CONFIDENTIAL.pdf(936856 bytes) Exhibit 65 CONFIDENTIAL.pdf(150128 bytes) Exhibit 66 CONFIDENTIAL.pdf(64381 bytes) Exhibit 67.pdf(309563 bytes) Exhibit 68.pdf(111384 bytes) Exhibit 69.pdf(359659 bytes) Exhibit 70.pdf(101939 bytes) Exhibit 71.pdf(375749 bytes) Exhibit 72.pdf(1258124 bytes) Exhibit 73.pdf(262165 bytes) Exhibit 74.pdf(145998 bytes) Exhibit 75.pdf(203693 bytes) Exhibit 76.pdf(305430 bytes) Exhibit 77.pdf(141147 bytes) Exhibit 78.pdf(61571 bytes)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE TRADEMARK TRIAL AND APPEAL BOARD

Illumina, Inc.,	)	Opposition No.: 91194218
	)	
Opposer,	)	
	)	
v.	)	
	)	
Meridian Bioscience, Inc.,	)	
	)	
Applicant.	)	
	)	

OPPOSER'S NOTICE OF RELIANCE

Commissioner for Trademarks
P.O. Box 1451
Alexandria, VA 22313-1451

Dear Sir or Madam:

Opposer Illumina, Inc., pursuant to 37 C.F.R. §§ 2.120 and 2.122, and the parties' July 16, 2014, stipulation pursuant to 37 C.F.R. §2.121(a)(1), makes the following of record:

Exhibit 1

Opposer's Registration Certificate No. 4053668 for the mark ILLUMINADX, and corresponding printout from the Trademark Status and Document Retrieval ("TSDR") database showing ownership. Exhibit 1 is relevant generally to Opposer's Illumina Marks and to the similarity between Opposer's Illumina Marks and Applicant's ILLUMIPRO, ILLUMIPRO-10, ILLUMIGENE MOLECULAR SIMPLIFIED, and ILLUMIGENE marks.

Exhibit 2

Applicant's Answers to Opposer's Interrogatory Nos. 12, 16, and 21, dated March 14, 2011.

Exhibit 3

GenomeWeb article published online on May 15, 2006, titled "Decode, Illumina to Co-develop, Sell Molecular Diagnostics." Exhibit 3 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's

and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 4

Article published in issue 1217, page 10 in *Clinica: World Medical Device News* on August 4, 2006, titled "Lab Diagnostics: Competition Hots Up In Bead-Based Technology." Exhibit 4 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 5

Bio-IT World article with perspective from Jay Flatley published online on March 12, 2007, titled "Champions 2.0." Exhibit 5 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 6

GenomeWeb article published online on March 21, 2007, titled "Illumina, Solexa, MDS, Molecular Devices, Thermo Fisher, FDA, Cepheid, Applied Biosystems, Luminex, US Air Force, CombiMatrix." Exhibit 6 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 7

GenomeWeb article published online on November 28, 2007, titled "Gates Foundation Awards Md. Vaccine Center $5.6M for Pathogen Dx; Illumina, Ibis Tapped as Subcontractors." Exhibit 7 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 8

Journal of Clinical Microbiology article published online on July 24, 2013, titled "Survey of Culture, GoldenGate Assay, Universal Biosensor Assay, and 16S rRNA Gene Sequencing as Alternative Methods of Bacterial Pathogen Detection." Exhibit 8 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 9

GenomeWeb article published online on November 18, 2009, titled "EraGen Partners with Illumina to Develop Molecular Dx Assays." Exhibit 9 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 10

Summary of GenomeWeb article by Justin Petron published online on June 17, 2008, titled "At Goldman Sachs Confab, Illumina Predicts Future Growth in AgBio, Diagnostic Sectors." Exhibit 10 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 11

Summary of GenomeWeb article by Justin Petrone published online on November 18, 2008, titled "Illumina Aims to Have FDA-Cleared Dx, CLIA Lab in '09; CEO Says Arrays Have Dx Future." Exhibit 11 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 12

Genoneweb.com article published online on January 21, 2009, titled "Illumina Unveils Strategy to Enter Molecular Diagnostics Market." Exhibit 12 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 13

TiE South Coast article published online on September 30, 2009, titled "Illumina: Revolutionizing Healthcare through Personalized Medicine and Genomics." Exhibit 13 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 14

GenomeWeb article by Julia Karow published online on January 18, 2011, titled "Illumina Starts Validating Ovarian, Gastric Cancer Genes from Discovery Project; Adds Colorectal Cancer." Exhibit 14 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 15

Genoneweb.com article published online on August 3, 2011, titled "Illumina sees Clinical Applications for MiSeq; Plans FDA Submission for 2012." Exhibit 15 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 16

Yahoo Finance article published online on October 31, 2011, titled "Illumina Names Dr. Daniel S. Grosu Chief Medical Officer." Exhibit 16 is relevant generally to show Opposer's

expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 17

IVD Technology web article published online on November 2, 2011, titled "Siemens, Illumina Partner on Infections Disease Testing Using Next-Generation Sequencing." Exhibit 17 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 18

Genoneweb.com article published online on March 14, 2012, titled "Q&A: Matt Posard on Illumina's Clinically Focused Business Unit" regarding the Translational and Consumer Genomics business unit. Exhibit 18 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 19

Genoneweb.com article published online on April 25, 2012, titled "Illumina in Discussions with 100 Potential Clinical Customers for MiSeq, Plans Cancer Diagnostics." Exhibit 19 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 20

Genoneweb.com article published online on June 20, 2012, titled "Illumina Sees Diagnostics as Largest Growth Opportunity; Dx strategy to Focus on Cancer." Exhibit 20 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due

to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 21

SelectScience article published online on September 12, 2012, titled "Illumina Alliance Introduces Clinical Next Generation Sequencing Tools to Pathologists." Exhibit 21 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 22

Genoneweb article published online on September 12, 2012, titled "Illumina Solidifies Clinical Strategy with TruSight Products, Collaboration with Partners HealthCare." Exhibit 22 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 23

Cambridgebluegnome.com press release published online on September 19, 2012, titled "Illumina Acquires BlueGnome." Exhibit 23 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 24

Genoneweb.com article published online on January 4, 2013, titled "New Players, Clinical Applications, and the Lure of Commercialization among 2012 Bioinformatics Trends." Exhibit 24 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 25

NY Times.com article published online on January 7, 2013, titled "Illumina Buys Maker of Down Syndrome Test." Exhibit 25 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 26

Xconomy article published online on January 15, 2013, titled "Illumina CEO Jay Flatley on Diagnostics, the $1K Genome and China." Exhibit 26 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 27

Genoneweb.com article published online on July 24, 2013, titled "Illumina's Clinical Business Driven by HiSeq 2500 Upgrades, Partnerships, Acquisitions." Exhibit 27 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 28

Genoneweb.com article published online on October 2, 2013, titled "Illumina 'Optimistic' About Speedy FDA Clearance of MiSeq; Pitches Companion Dx Program to Pharmas." Exhibit 28 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 29

Genoneweb.com article published online on October 16, 2013, titled "Organizational Changes at Illumina Aimed at Growth, New Market Entries." Exhibit 29 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This

demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 30

Fierce Medical Devices article published online on October 23, 2013, titled "Illumina dazzles with Q3 diagnostics expansion and revenue growth." Exhibit 30 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 31

Genoneweb.com article published online on October 23, 2013, titled "Ilumina Continues Push into Clinic with MiSeq Panels, HiSeq 2500, Reorganization." Exhibit 31 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 32

Seeking Alpha article published online on October 25, 2013, titled "What Shutdown? Illumina Rises Above The Noise." Exhibit 32 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 33

Website printout from the Penn Medicine's Center for Personal Diagnostics webpage. Exhibit 33 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 34

UT San Diego article published online on November 26, 2013, titled "Illumina scores medical sequencing breakthrough." Exhibit 34 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 35

GEN Genetic Engineering & Biotechnology News article published online on January 16, 2014, titled "Illumina, Amgen to Develop CDx for Colorectal Cancer." Exhibit 35 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 36

Website printout from U.S. Food and Drug Administration's 510(k) Premarket Notification database regarding Illumina BeadXpress System and Illumina VeraCode Genotyping Test for Factor V and Factor II. Exhibit 36 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 37

iStock Analyst article published online on May 4, 2010, titled "Illumina Receives FDA 510(k) Clearance for Its BeadXpress® Multiplex Analysis System." Exhibit 37 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 38

Business Wire press release article published online on May 18, 2010, titled "Illumina Receives FDA 510(k) Clearance for VeraCode® Genotyping Test for Factor V (Leiden) and

Factor II (Prothrombin)." Exhibit 38 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 39

FDA press release published online on November 19, 2013, titled "FDA allows marketing of four 'next generation' gene sequencing devices." Exhibit 39 is relevant generally to show Opposer's expansion into and presence in the molecular diagnostics market. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 40

Clinical Chemistry Review, 55:4 (2009), pages 659-669, titled "Microarray-Based Genomic DNA Profiling Technologies in Clinical Molecular Diagnostics." Exhibit 40 is relevant generally to show the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 41

Clinical Chemistry Review article, 55:4 (2009), pages 641-658, titled "Next-Generation Sequencing: From Basic Research to Diagnostics." Exhibit 41 is relevant generally to show the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 42

BMJ Open article published online on May 2, 2014 titled "A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance." Exhibit 42 is relevant generally to show the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the

similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 43

Washington University School of Medicine article published online on January 1, 2013, titled "Diagnosis of *Clostridium difficile* infection: An ongoing conundrum for clinicians and for clinical laboratories." Exhibit 43 is relevant generally to show the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 44

The New England Journal of Medicine article published online on September 26, 2013, titled "Diverse Sources of *C. difficile* Infection Identified on Whole-Genome Sequencing." Exhibit 44 is relevant generally to show the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 45

Printouts from Becton, Dickenson, and Company ("BD") website regarding BD's worldwide business segments. Exhibit 45 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 46

Printouts from the Gen-Probe website regarding Gen-Probe's products and services. Exhibit 46 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a

likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 47

Printout from the Life Technologies website. Exhibit 47 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 48

Printouts from the PerkinElmer website. Exhibit 48 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 49

Printouts from the Qiagen website regarding Qiagen's sample and assay technologies. Exhibit 49 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 50

Printouts from the Roche website regarding Roche's products available in the United States. Exhibit 50 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the

similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 51

Printouts from the Agilent Technologies website. Exhibit 51 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 52

Printouts from the Beckman Coulter website regarding Beckman Coulter's products and services. Exhibit 52 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 53

Printouts from the Siemens website regarding Siemens' molecular diagnostics products. Exhibit 53 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 54

Printouts from the Luminex website regarding Luminex's products. Exhibit 54 is relevant generally to show that companies often operate in both the research and diagnostic fields. This demonstrates the natural progression from offering goods for use in research to offering those or similar goods for use in diagnostics. This in turn demonstrates a likelihood of confusion as to

the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 55

MedCity News article by Mary Vanac published online on February 1, 2010, titled "MedCity News Q&A: Meridian Bioscience CEO Jack Kraeutler." Exhibit 55 is relevant generally to show that Opposer and Applicant market and sell their goods to the same types of customers. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 56

Printout from the American Association for Clinical Chemistry ("AACC") regarding AACC 2006 Annual Meeting OEM Lecture Series. Exhibit 56 is relevant generally to show that Opposer and Applicant market and sell their goods to the same types of customers. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 57

Sign On San Diego article published online on February 17, 2011, titled "Illumina ranks 4th on new Forbes tech list." Exhibit 57 is relevant generally to show the fame of Opposer's Illumina Marks.

Exhibit 58

Forbes.com article published online on January 29, 2009, titled "The 25 Fastest-Growing Technology Companies in America." Exhibit 58 is relevant generally to show the fame of Opposer's Illumina Marks.

Exhibit 59

Seeking Alpha article published online on March 4, 2009, titled "Illumina Dominating the Sequencing Market." Exhibit 59 is relevant generally to show the fame of Opposer's Illumina Marks.

Exhibit 60

Forbes.com article published online on January 29, 2009, titled "Illumina: Shining In Dreary Times." Exhibit 60 is relevant generally to show the fame of Opposer's Illumina Marks.

Exhibit 61

Document produced by Applicant "Meridian Bioscience Inc., Illumigene P&L, FY 2010 and FY 2011, BASE CASE." Exhibit 61 is relevant generally to show that Applicant advertises its goods in similar journals as Opposer. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 62

Document produced by Applicant titled "ILLUMIGENE™ Lead generation/Advertising Plan Design Brief – Economic Buyer." Exhibit 62 is relevant generally to show the purchasers of Applicant's goods. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 63

Document produced by Applicant titled "ILLUMIGENE™ Lead Generation Plan Design Brief – Technical/Tech-Economic Buyer." Exhibit 63 is relevant generally to show the purchasers of Applicant's goods. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 64

Excerpts of a document produced by Applicant listing the customers of Applicant's goods. Exhibit 64 is relevant generally to show the customers of Applicant's goods. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 65

Compilation of documents produced by Applicant listing additional customers of Applicant's goods. Exhibit 65 is relevant generally to show the customers of Applicant's goods.

This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 66

Document produced by Applicant titled "One-on-one Topics: November 8, 2010." Exhibit 66 is relevant generally to show Applicant's marketing strategies. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 67

Document produced by Applicant titled "Letters, CAP TODAY, July 2010, *C. difficile* detection." Exhibit 67 is relevant generally to show use of Applicant's goods to detect *C. difficile*. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 68

CAP TODAY article published online in November 2010 titled "C. Diff Molecular Testing with Less Time and Effort." Exhibit 68 is relevant generally to show use of Applicant's goods to detect *C. difficile*. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 69

Applicant's 510(k) Summary for ILLUMIGENE *C. difficile*, cited by Applicant in response to Opposer's Interrogatory No. 21. Exhibit 69 is relevant generally to describe Applicant's goods and the intended use thereof. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 70

FDA 510(k) Substantial Equivalence Determination Decision Summary for Applicant's ILLUMIGENE *C. difficile* assay, cited by Applicant in response to Opposer's Interrogatory No.

21. Exhibit 70 is relevant generally to describe Applicant's goods and the intended use thereof. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 71

Applicant's Revised 510(k) Summary for ILLUMIGENE *C. difficile*, cited by Applicant in response to Opposer's Interrogatory No. 21. Exhibit 71 is relevant generally to describe Applicant's goods and the intended use thereof. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 72

Website printouts from Applicant's webpage, including product advertisements and information sheets available on Applicant's webpage. Exhibit 72 is relevant generally to describe Applicant's goods. This demonstrates a likelihood of confusion as to the source of Applicant's goods due to the similarity of Opposer's and Applicant's goods and the similarity of trade channels in which Opposer's and Applicant's goods travel.

Exhibit 73

Opposer's Application for Serial No. 86373206 for the mark ILLUMINOTES, and corresponding printout from the Trademark Status and Document Retrieval ("TSDR") database showing ownership. Exhibit 73 is relevant generally to Opposer's Illumina Marks and to the similarity between Opposer's Illumina Marks and Applicant's ILLUMIPRO, ILLUMIPRO-10, ILLUMIGENE MOLECULAR SIMPLIFIED, and ILLUMIGENE marks.

Exhibit 74

Website printout from TiE South Cost website. Exhibit 74 is relevant generally to describe the TiE South Cost website and the readers thereof, who include both Opposer's and Applicant's customers and potential customers.

Exhibit 75

Website printout from SelectScience website. Exhibit 75 is relevant generally to describe the SelectScience websites and the readers thereof, who include both Opposer's and Applicant's customers and potential customers.

Exhibit 76

Website printouts form Xconomy website. Exhibit 76 is relevant generally to describe the Xconomy website and the readers thereof, who include both Opposer's and Applicant's customers and potential customers.

Exhibit 77

Website printouts from Fierce Medical Devices website. Exhibit 77 is relevant generally to describe the Fierce Medical Devices website and the readers thereof, who include both Opposer's and Applicant's customers and potential customers.

Exhibit 78

Website printouts from Seeking Alpha website. Exhibit 78 is relevant generally to describe the Seeking Alpha website and the readers thereof, who include both Opposer's and Applicant's customers and potential customers.

The above described documents are filed concurrently herewith.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: November 6, 2014

By:_____

Susan M. Natland
Brian C. Horne
Marissa Calcagno
Knobbe Martens Olson & Bear, LLP
2040 Main Street, 14th Floor
Irvine, California 92614
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Tel: (949) 760-0404
Fax: (949) 760-9502
Attorneys for Opposer, Illumina, Inc.

CERTIFICATE OF SERVICE

I hereby certify that I served a copy of the foregoing Opposer's Notice of Reliance upon Applicant's counsel by depositing one copy thereof in the United States Mail, first-class postage prepaid, on November 6, 2014, addressed as follows:

J. Michael Hurst
Keating Muething & Klekamp PLL
One East 4th Street
Suite 1400
Cincinnati, OH 45202

Sarah Beno Couvillion

19126989

Exhibit 1

United States of America

United States Patent and Trademark Office

ILLUMINADX

Reg. No. 4,053,668

Registered Nov. 8, 2011

Int. Cl.: 5

TRADEMARK

PRINCIPAL REGISTER

ILLUMINA, INC. (DELAWARE CORPORATION)
9885 TOWNE CENTRE DRIVE
SAN DIEGO, CA 92121

FOR: CLINICAL DIAGNOSTIC REAGENTS, REAGENT KITS, AND BEADS WITH ATTACHED BIOMOLECULES, COMPRISED PRIMARILY OF OLIGONUCLEOTIDES AND OTHER NUCLEIC ACIDS, NATURAL AND MODIFIED NUCLEOTIDES, BUFFERS, LABELS, AND SUBSTRATES, FOR CLINICAL DIAGNOSTIC PURPOSES, IN CLASS 5 (U.S. CLS. 6, 18, 44, 46, 51 AND 52).

FIRST USE 3-19-2010; IN COMMERCE 3-19-2010.

THE MARK CONSISTS OF STANDARD CHARACTERS WITHOUT CLAIM TO ANY PARTICULAR FONT, STYLE, SIZE, OR COLOR.

OWNER OF U.S. REG. NOS. 2,471,539, 2,632,507, AND 2,756,703.

SN 77-982,582, FILED 5-28-2009.

CIMMERIAN COLEMAN, EXAMINING ATTORNEY

David J. Kappos

Director of the United States Patent and Trademark Office

Generated on: This page was generated by TSDR on 2014-11-04 19:45:05 EST

Mark: ILLUMINADX

ILLUMINADX

US Serial Number: 77982582

Application Filing Date: May 28, 2009

US Registration Number: 4053668

Registration Date: Nov. 08, 2011

Register: Principal

Mark Type: Trademark

Status: Registered. The registration date is used to determine when post-registration maintenance documents are due.

Status Date: Nov. 08, 2011

Publication Date: Jun. 08, 2010

Notice of Allowance Date: Aug. 03, 2010

Mark Information

Mark Literal Elements: ILLUMINADX

Standard Character Claim: Yes. The mark consists of standard characters without claim to any particular font style, size, or color.

Mark Drawing Type: 4 - STANDARD CHARACTER MARK

Related Properties Information

Claimed Ownership of US Registrations: 2471539, 2632507, 2756703, 77747038

Child Of: 77747038

Goods and Services

Note: The following symbols indicate that the registrant/owner has amended the goods/services:

- Brackets [..] indicate deleted goods/services;
- Double parenthesis ((..)) identify any goods/services not claimed in a Section 15 affidavit of incontestability; and
- Asterisks *..* identify additional (new) wording in the goods/services.

For: Clinical diagnostic reagents, reagent kits, and beads with attached biomolecules, comprised primarily of oligonucleotides and other nucleic acids, natural and modified nucleotides, buffers, labels, and substrates, for clinical diagnostic purposes

International Class(es): 005 - Primary Class

U.S Class(es): 006, 018, 044, 046, 051, 052

Class Status: ACTIVE

Basis: 1(a)

First Use: Mar. 19, 2010

Use in Commerce: Mar. 19, 2010

Basis Information (Case Level)

Filed Use:	No	**Currently Use:**	Yes	**Amended Use:**	No
Filed ITU:	Yes	**Currently ITU:**	No	**Amended ITU:**	No
Filed 44D:	No	**Currently 44D:**	No	**Amended 44D:**	No
Filed 44E:	No	**Currently 44E:**	No	**Amended 44E:**	No
Filed 66A:	No	**Currently 66A:**	No		
Filed No Basis:	No	**Currently No Basis:**	No		

Current Owner(s) Information

Owner Name: Illumina, Inc.

Owner Address: 5200 Illumina Way
San Diego, CALIFORNIA 92122
UNITED STATES

Legal Entity Type: CORPORATION

State or Country Where Organized: DELAWARE

Attorney/Correspondence Information

Attorney of Record

Attorney Name:	Lisa M. Martens	**Docket Number:**	39319-003500
Attorney Primary Email Address:	tmdoctc@fr.com	**Attorney Email Authorized:**	Yes

Correspondent

Correspondent Name/Address:	Lisa M. Martens Fish & Richardson P.C. P.O. Box 1022 Minneapolis, MINNESOTA 55440-1022 UNITED STATES		
Phone:	(858) 678-5070	**Fax:**	(858) 678-5099
Correspondent e-mail:	tmdoctc@fr.com	**Correspondent e-mail Authorized:**	Yes

Domestic Representative - Not Found

Prosecution History

Date	Description	Proceeding Number
Jul. 31, 2014	ATTORNEY REVOKED AND/OR APPOINTED	
Jul. 31, 2014	TEAS REVOKE/APPOINT ATTORNEY RECEIVED	
Mar. 08, 2012	APPLICANT/CORRESPONDENCE CHANGES (NON-RESPONSIVE) ENTERED	88888
Mar. 08, 2012	TEAS CHANGE OF OWNER ADDRESS RECEIVED	
Nov. 08, 2011	REGISTERED-PRINCIPAL REGISTER	
Oct. 07, 2011	NOTICE OF ACCEPTANCE OF STATEMENT OF USE E-MAILED	
Oct. 06, 2011	LAW OFFICE REGISTRATION REVIEW COMPLETED	73797
Sep. 30, 2011	ALLOWED PRINCIPAL REGISTER - SOU ACCEPTED	
Sep. 20, 2011	NOTICE OF PSEUDO MARK MAILED	
Sep. 19, 2011	STATEMENT OF USE PROCESSING COMPLETE	61813
Sep. 16, 2011	USE AMENDMENT FILED	61813
Sep. 19, 2011	DIVISIONAL PROCESSING COMPLETE	
Sep. 16, 2011	DIVISIONAL REQUEST RECEIVED	
Sep. 16, 2011	TEAS REQUEST TO DIVIDE RECEIVED	
Sep. 16, 2011	TEAS STATEMENT OF USE RECEIVED	
Aug. 16, 2011	NOTICE OF APPROVAL OF EXTENSION REQUEST E-MAILED	
Aug. 15, 2011	EXTENSION 2 GRANTED	61813
Aug. 02, 2011	EXTENSION 2 FILED	61813
Aug. 15, 2011	CASE ASSIGNED TO INTENT TO USE PARALEGAL	61813
Aug. 02, 2011	TEAS EXTENSION RECEIVED	
Feb. 03, 2011	NOTICE OF APPROVAL OF EXTENSION REQUEST E-MAILED	
Feb. 01, 2011	EXTENSION 1 GRANTED	98765
Feb. 01, 2011	EXTENSION 1 FILED	98765
Feb. 01, 2011	TEAS EXTENSION RECEIVED	
Aug. 03, 2010	NOA E-MAILED - SOU REQUIRED FROM APPLICANT	
Jun. 08, 2010	OFFICIAL GAZETTE PUBLICATION CONFIRMATION E-MAILED	
Jun. 08, 2010	PUBLISHED FOR OPPOSITION	
Apr. 30, 2010	LAW OFFICE PUBLICATION REVIEW COMPLETED	73797
Apr. 30, 2010	APPROVED FOR PUB - PRINCIPAL REGISTER	
Apr. 30, 2010	EXAMINER'S AMENDMENT ENTERED	88888
Apr. 30, 2010	NOTIFICATION OF EXAMINERS AMENDMENT E-MAILED	6328
Apr. 30, 2010	EXAMINERS AMENDMENT E-MAILED	6328
Apr. 30, 2010	EXAMINERS AMENDMENT -WRITTEN	76635
Apr. 23, 2010	ASSIGNED TO EXAMINER	76635
Apr. 23, 2010	PREVIOUS ALLOWANCE COUNT WITHDRAWN	
Apr. 16, 2010	WITHDRAWN FROM PUB - OG REVIEW QUERY	60756

Apr. 07, 2010	LAW OFFICE PUBLICATION REVIEW COMPLETED	73797
Apr. 07, 2010	ASSIGNED TO LIE	73797
Mar. 21, 2010	APPROVED FOR PUB - PRINCIPAL REGISTER	
Feb. 24, 2010	TEAS/EMAIL CORRESPONDENCE ENTERED	88889
Feb. 23, 2010	CORRESPONDENCE RECEIVED IN LAW OFFICE	88889
Feb. 23, 2010	TEAS RESPONSE TO OFFICE ACTION RECEIVED	
Sep. 04, 2009	NOTIFICATION OF NON-FINAL ACTION E-MAILED	6325
Sep. 04, 2009	NON-FINAL ACTION E-MAILED	6325
Sep. 04, 2009	NON-FINAL ACTION WRITTEN	76635
Aug. 27, 2009	ASSIGNED TO EXAMINER	76635
Jun. 02, 2009	NOTICE OF PSEUDO MARK MAILED	
Jun. 01, 2009	NEW APPLICATION OFFICE SUPPLIED DATA ENTERED IN TRAM	
Jun. 01, 2009	NEW APPLICATION ENTERED IN TRAM	

TM Staff and Location Information

TM Staff Information - None

File Location

Current Location: PUBLICATION AND ISSUE SECTION **Date in Location:** Oct. 06, 2011

Proceedings

Summary

Number of Proceedings: 1

Type of Proceeding: Opposition

Proceeding Number: 91211615 **Filing Date:** Jul 17, 2013

Status: Pending **Status Date:** Jul 17, 2013

Interlocutory Attorney: GEORGE POLOGEORGIS

Defendant

Name: Boston Scientific Neuromodulation Corporation

Correspondent Address: LOUIS T PERRY
FAEGRE BAKER DANIELS LLP
2200 WELLS FARGO CENTER , 90 SOUTH SEVENTH STREET
MINNEAPOLIS MN , 55402
UNITED STATES

Correspondent e-mail: james.steffen@faegrebd.com , trademarkmpls@faegrebd.com , louis.perry@faegrebd.com , tmindy@faegrebd.com

Associated marks

Mark	Application Status	Serial Number	Registration Number
ILLUMINA 3D	Opposition Pending	85789767	

Plaintiff(s)

Name: Illumina, Inc.

Correspondent Address: SUSAN M NATLAND
KNOBBE MARTENS OLSON & BEAR LLP
2040 MAIN STREET, 14TH FLOOR
IRVINE CA , 92614
UNITED STATES

Correspondent e-mail: efiling@knobbe.com

Associated marks

Mark	Application Status	Serial Number	Registration Number
ILLUMINA	Renewed	76072152	2471539
ILLUMINA	Renewed	75982227	2632507
ILLUMINA	Renewed	76112547	2756703
ILLUMINADX	Abandoned - No Statement Of Use Filed	77747038	
ILLUMINADX	Registered	77982582	4053668
ILLUMINA	Registered	85648539	4502341
ILLUMINA			

ILLUMINA	Registered	85648638	4268307
ILLUMINA	Registered	85791910	4335004

Prosecution History

Entry Number	History Text	Date	Due Date
1	FILED AND FEE	Jul 17, 2013	
2	NOTICE AND TRIAL DATES SENT; ANSWER DUE:	Jul 17, 2013	Aug 26, 2013
3	PENDING, INSTITUTED	Jul 17, 2013	
4	CHANGE OF CORRESP ADDRESS	Aug 22, 2013	
5	ANSWER	Aug 22, 2013	
6	P REQ TO W/DRAW AS ATTORNEY	Nov 21, 2013	
7	RESPONSE DUE 30 DAYS (DUE DATE)	Dec 20, 2013	Jan 19, 2014
8	P APPEARANCE / POWER OF ATTORNEY	Jan 21, 2014	
9	PROCEEDINGS RESUMED; TRIAL DATES RESET	Feb 25, 2014	
10	STIP FOR EXT	Apr 15, 2014	
11	EXTENSION OF TIME GRANTED	Apr 16, 2014	
12	STIP FOR EXT	Jul 23, 2014	
13	EXTENSION OF TIME GRANTED	Jul 23, 2014	
14	STIP FOR EXT	Sep 22, 2014	
15	EXTENSION OF TIME GRANTED	Sep 22, 2014	
16	STIP FOR EXT	Oct 20, 2014	
17	EXTENSION OF TIME GRANTED	Oct 20, 2014	

Exhibit 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE TRADEMARK TRIAL APPEAL BOARD

ILLUMINA, INC.,	)	
	)	
Opposer,	)	Opposition No. 91194218 (parent)
	)	Opposition No. 91194219
-v-	)	
	)	Ser. No. 77768176
MERIDIAN BIOSCIENCE, INC.,	)	Ser. no. 77775316
	)	
Applicant.	)	

APPLICANT'S ANSWERS TO OPPOSER'S FIRST SET OF INTERROGATORIES

Applicant, Meridian Bioscience, Inc. ("Applicant") hereby submits its response to Opposer, Illumina, Inc.'s ("Opposer") First Set of Interrogatories, as follows:

GENERAL OBJECTIONS & STATEMENTS

1. Applicant objects to Opposer's interrogatories in their entirety on the grounds that they are overbroad, unduly burdensome, oppressive, duplicative, and impose an undue expense upon Applicant.

2. Applicant objects to Opposer's interrogatories to the extent that they are vague or ambiguous.

3. Applicant objects to Opposer's interrogatories on the grounds that they seek information which is not relevant to the subject matter of this case nor reasonably calculated to lead to the discovery of admissible evidence.

4. Applicant objects to Opposer's interrogatories to the extent they seek discovery or production of attorney-work product or attorney-client privileged material, and Applicant expressly refuses to disclose or produce same.

5. Applicant expressly reserves any and all objections to the introduction or use at trial of any information or documentation disclosed herein, including, but not limited to, objections upon the grounds of relevancy or materiality.

6. Applicant expressly reserves the continuing right to supplement these responses as discovery and investigation progresses.

7. Applicant objects to Opposer's interrogatories to the extent that they purport to impose upon Applicant any obligations which are greater than, or inconsistent with, those imposed by the Federal Rules of Civil Procedure and/or the Trademark Rules of Practice.

8. Applicant refers to documents produced, where applicable, in responding to certain interrogatories in accordance with Federal Rules of Civil Procedure 33(d).

9. Applicant objects to Opposer's interrogatories to the extent they improperly seek private, confidential and/or proprietary information beyond the scope of discovery in this proceeding. Notwithstanding the foregoing, Applicant will provide such information pursuant to the Protective Order entered into by and between the parties.

10. All of the foregoing General Objections are incorporated in the following responses to Opposer's discovery requests, whether or not any specific general objection is referenced therein.

INTERROGATORIES

Interrogatory No. 1: Describe in reasonable detail Applicant's creation, conception, selection, and adoption of Applicant's Marks including all steps to clear Applicant's Marks, describe all research conducted, and explanation of how and why Applicant's Marks were

In addition to the foregoing General Objections, Applicant objects to this Interrogatory because it is vague and ambiguous. In addition, Applicant objects to this Interrogatory to the extent that it seeks information that is confidential or trade secret in nature. Further, Applicant objects to this Interrogatory to the extent that it seeks information about Applicant's activities outside the United States as such activities are irrelevant for the purposes of, and beyond the scope of, the instant proceeding. Further, Applicant objects to this Interrogatory to the extent that it seeks information about goods, services, and trademarks not at issue in, and therefore irrelevant to, this proceeding. Further, Applicant objects to this Interrogatory because it is overbroad and would require a lengthy, all-inclusive narrative response.

Subject to and without waiving the foregoing objections, and pursuant to the Protective Order entered into between the parties, Applicant states that no such business establishments exist or are planned to exist.

Interrogatory No. 12: Describe the manner in which Applicant intends to or does distribute Applicant's Goods in connection with Applicant's Marks, identifying all channels of trade through which Applicant's Goods travel or will travel.

Answer:

In addition to the foregoing General Objections, Applicant objects to this Interrogatory because it is vague, ambiguous, and improperly compound. In addition, Applicant objects to this Interrogatory to the extent that it seeks information that is confidential or trade secret in nature. Further, Applicant objects to this Interrogatory to the extent that it seeks information about Applicant's activities outside the United States as such activities are irrelevant for the purposes of, and beyond the scope of, the instant proceeding. Further, Applicant objects to this Interrogatory to the extent that it seeks information about goods, services, and trademarks not at issue in, and therefore irrelevant to, this proceeding. Further, Applicant objects to this Interrogatory because it is overbroad and would require a lengthy, all-inclusive narrative response.

Subject to and without waiving the foregoing objections, and pursuant to the Protective Order entered into between the parties, Applicant states that Applicant distributes Applicant's Goods bearing Applicant's Marks to hospitals and reference labs through a sales force of approximately 25 sales representatives. Applicant's sales representatives interact with Applicant's customers directly via the telephone or via in-person sales meetings. Applicant's sales representatives may interact on these sales calls with the following individuals employed by the above-referenced hospitals and reference labs: microbiology technicians, lab managers, molecular supervisors, purchasing department personnel, infectious disease doctors, and pathologists.

Interrogatory No. 13: Identify the classes of customers for Applicant's Goods.

Answer:

See Applicant's response to Interrogatory No. 12.

Interrogatory No. 14: Identify the classes of users for Applicant's Goods.

Answer:

See Applicant's response to Interrogatory No. 12. Applicant's Goods are used by microbiology technicians.

Interrogatory No. 15: Identify the person(s) with primary responsibility for marketing, distribution or sale of Applicant's Goods in connection with Applicant's Marks.

Answer:

Richard Connors
Associate Marketing Director
Meridian Bioscience, Inc.
3471 River Hills Drive
Cincinnati, Ohio 45244

Susan Ward
Assistant Product Manager - Molecular Marketing
Meridian Bioscience, Inc.
3471 River Hills Drive
Cincinnati, Ohio 45244

See Applicant's response to Interrogatory No. 12.

Interrogatory No. 16: Identify the media through which Applicant has advertised or promoted Applicant's Goods under Applicant's Marks.

Answer:

In addition to the foregoing General Objections, Applicant objects to this Interrogatory because it is vague and ambiguous. In addition, Applicant objects to this Interrogatory to the extent that it seeks information that is confidential or trade secret in nature. Further, Applicant objects to this Interrogatory to the extent that it seeks information about Applicant's activities outside the United States as such activities are irrelevant for the purposes of, and beyond the scope of, the instant proceeding. Further, Applicant objects to this Interrogatory because it is overbroad and would require a lengthy, all-inclusive narrative response.

Subject to and without waiving the foregoing objections, and pursuant to the Protective Order entered into between the parties, Applicant states that prior to receiving FDA approval, Applicant advertised and/or promoted Applicant's Goods under Applicant's Marks through in-person meetings and presentations at the following conferences: Clinical Virology Symposium, April 2009; ASM (American Society of Microbiology), May 2009; AMP (Association of Molecular Pathology), November 2009; SHEA (Society of Healthcare Epidemiology of America), March 2010; Clinical Virology Symposium, April 2010; ASM, May 2010; AACC (American Association of Clinical Chemistry), July 2010; AMP, November 2010.

Following FDA approval, Applicant advertised and/or promoted Applicant's Goods under Applicant's Marks via the internet; through in-person meetings and presentations at the AACC (American Association of Clinical Chemistry) conference in July 2010; and in CAP Today (College of American Pathologists), July 2010.

Interrogatory No. 17: Identify any business plans or projections, revenue projections, cost projections, product plans or proposals concerning Applicant's Marks or Applicant's Goods.

Answer:

In addition to the foregoing General Objections, Applicant objects to this Interrogatory because it is vague, ambiguous, and improperly compound. In addition, Applicant objects to this Interrogatory to the extent that it seeks information that is confidential or trade secret in nature. Further, Applicant objects to this Interrogatory to the extent that it seeks information about Applicant's activities outside the United States as such activities are irrelevant for the purposes of, and beyond the scope of, the instant proceeding. Further, Applicant objects to this Interrogatory to the extent that it seeks information about goods, services, and trademarks not at issue in, and therefore irrelevant to, this proceeding. Further, Applicant objects to this Interrogatory because it is overbroad and would require a lengthy, all-inclusive narrative response.

vs. BD/GeneOhm	@ Hershey Medical Center	2010
vs. Culture	@ Summa Health	2010
vs. Prodesse	@ Nationwide Children's Hospital	2010

Interrogatory No. 21: Identify all requests for government approval to market or sell Applicant's Goods.

Answer:

In addition to the foregoing General Objections, Applicant objects to this Request to the extent that it seeks to require Applicant to produce documents and information not within Applicant's possession or control or which are more readily obtainable from another source.

Subject to and without waiving the foregoing objections, Applicant states that a 510(k) application was submitted to the U.S. Food & Drug Administration on June 21, 2009. See Applicant's responses to Opposer's Document Requests. See also:

http://www.accessdata.fda.gov/cdrh_docs/pdf10/K100818.pdf

http://www.accessdata.fda.gov/cdrh_docs/reviews/K100818.pdf

A Special 510(k) application was submitted to the U.S. Food & Drug Administration on December 31, 2010. See Applicant's responses to Opposer's Document Requests. See also:

http://www.accessdata.fda.gov/cdrh_docs/pdf11/K110012.pdf

Interrogatory No. 22: Identify each third party mark or name that you contend is relevant to this proceeding and describe in reasonable detail the impact of each such mark or name.

Answer:

In addition to the foregoing General Objections, Applicant objects to this Interrogatory because it is vague, ambiguous, and improperly compound. In addition, Applicant objects to this

Richard Connors
Associate Marketing Director
Meridian Bioscience, Inc.
3471 River Hills Drive
Cincinnati, Ohio 45244

Susan Ward
Assistant Product Manager - Molecular Marketing
Meridian Bioscience, Inc.
3471 River Hills Drive
Cincinnati, Ohio 45244

Linda DeRose
Marketing Communications Coordinator
Meridian Bioscience, Inc.
3471 River Hills Drive
Cincinnati, Ohio 45244

Interrogatory No. 29: Identify all documents relating to the subject matter of the foregoing interrogatories and the preparation of Applicant's responses thereto.

Answer:

See Applicant's Responses to Opposer's Request for Production of Documents.

Dated this 14th day of March, 2011.

Respectfully submitted,

J. Michael Hurst (0070828)
KEATING MUETHING & KLEKAMP PLL
One East Fourth St.
Suite 1400
Cincinnati, Ohio 45202
Phone: (513) 562-1401

Fax: (513) 579-6457
mhurst@kmklaw.com

Attorney for Applicant,
Meridian Bioscience, Inc.

CERTIFICATE OF SERVICE

I hereby certify that a copy of the foregoing APPLICANT'S ANSWERS TO OPPOSER'S FIRST SET OF INTERROGATORIES was served upon by ordinary mail this 14th day of March, 2011.

Carolyn Hunter

Carolyn Hunter

Exhibit 3

Drupal.behaviors.print = function(context) {window.print();window.close();}>

genomeweb

Decode, Illumina to Co-develop, Sell Molecular Diagnostics

May 15, 2006

Decode, Illumina to Co-develop, Sell Molecular Diagnostics

By a GenomeWeb staff reporter

NEW YORK, May 15 (GenomeWeb News) - Decode Genetics and Illumina plan to co-develop and commercialize DNA-based diagnostics in several major disease areas, the firms said today.

According to the firms, the partnership will use Illumina's platform for high-multiplex SNP-genotyping to develop diagnostics for gene variants that Decode has shown to be risk factors for various diseases.

Under the terms of the agreement, Illumina will gain access to disease-related biomarkers, which will be jointly validated as diagnostic panels. Illumina will market and sell these on its forthcoming BeadXpress platform, the partners said.

Illumina will also install its SNP-genotyping platform at Decode's labs in Iceland.

Initially, the companies will focus on the gene encoding leukotriene A4 hydrolase, which has been linked to heart attack; the gene encoding transcription factor 7-like 2, which is linked to type II diabetes; and the gene encoding BARD1, which is linked to breast cancer.

The companies will share development costs and split the profits from sales of the diagnostics tests.

Exhibit 4

Lab diagnostics: competition hots up in bead-based technology

Two high-flying publicly traded competitors that use bead technology in their multiplex arrays both revealed plans for enhancing their diagnostic portfolios at the annual scientific meeting of the American Association for Clinical Chemistry (AACC) in Chicago.

Illumina, a $73.5m company with a market cap of $1.6bn, has been marketing the genotyping services of its bead array technologies since 1998. But with the acquisition of privately-held CyVera in April 2005, the San Diego-based company now has bead-based technology that offers opportunities for in vitro and molecular diagnostic development.

VeraCode uses cylindrical glass microbeads measuring 240x28 microns. Each bead contains a digital holographic element that, when excited by a laser, emits a unique code image that allows identification of the bead type.

Its digital coding can be used to identify the sample, the laboratory and the reagent kit, the company said.

The VeraCode technology will form the basis of the company's BeadXpress diagnostic platform, which is scheduled for market introduction before the end of the year.

On July 20, Illumina announced it was forming a diagnostic collaboration with the Bangalore, Indian company ReaMetrix, which will develop, validate and market diagnostic panels based on this new platform. ReaMetrix will hold rights in India, Illumina in the rest of the world.

Analysts and journalists dominated the company's July 25 dinner presentation on this latest technology, after Paolo Fortina, of Thomas Jefferson University in Philadelphia declared Illumina to be a company with "great potential" at an AACC briefing for analysts earlier that day.

The smaller of the two competitors is Luminex, with net sales of $42.3m and a market cap of $512m. Its x-Map technology performs discrete bioassays on the surface of colour-coded beads, known as microspheres, which are then read in a compact analyser. Using lasers and high-speed digital-signal processors, its analysers are capable of multiplexing up to 100 analytes in a single well simultaneously, identifying the bioassay and measuring the results, all in real time.

Since its foundation in 1995, Luminex has licensed its beads and systems to more than 50 strategic partners, including Digene, PerkinElmer, Tm Bioscience, Celera Diagnostics Abbott, Bio-Rad and Bayer. As a result, its technology has featured in 34 FDA-cleared products. Now the Austin, Texas, company has begun to develop its own test kits, as president Patrick Balthrop explained during a press conference at the AACC.

> ***"There's plenty of opportunity available for both companies to compete in this market"***

The company's first product, a 14-plex pneumococcal assay, used to determine immune responses to common pneumococcal vaccines, will be sold to reference labs later this year. The multiple, esoteric tests now available for this purpose are cumbersome to administer, Mr Balthrop explained. A second diagnostic line, a microRNA assay licensed from the Massachusetts Institute of Technology, will be marketed to research laboratories in the second half of this year for use in research on cancer, fat metabolism and for other purposes. Finally, the company just received a $300,000 research grant from the Defense Advanced Research Projects Agency, a branch of the US Army, to develop a chip-scale x-Map technology for bio-defence applications. Mr Balthrop could offer few details about the research, but did admit that the chip could eventually be leveraged for non-defence related uses.

Mr Balthrop said he does not feel that Illumina's move into bead-based diagnostics is a threat to his company. "We believe it is more of a validation event," he said. "It shows we're on the right track," he said. "There's plenty of opportunity available for both companies to compete in this market," he added.

Last week, Luminex announced that revenue for the second quarter of 2006 was $13.3m, a 25% increase compared with $10.7m on in the second quarter of 2005.

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Siemens' move to become first full-service diagnostic firm has rivals taking note

Siemens Medical Solutions formally entered the in vitro diagnostics industry on July 27, when it finalised its $1.86bn acquisition of the immunoassay company, Diagnostic Products Corporation (DPC).

The DPC deal is only the first – and smaller – of the two steps the German company is taking to create the world's first full-service diagnostics company. Later this year, the Erlangen company hopes to complete its $5.3bn deal to buy Bayer HealthCare's diagnostics division from the Leverkusen-based group, which recently acquired pharmaceutical rival Schering.

Siemens is already a giant in diagnostic imaging and health information systems. Bayer Diagnostics, based in Tarrytown, New York, and Los Angeles, California-based DPC, now gives the company a bridge between in vivo and in vitro solutions, explained Mohammad Naraghi, the senior vice-president for global business development, who engineered the two deals.

Siemens is especially excited about what the acquisitions bring it in the field of molecular medicine, enabling early detection and pharmacogenomics, and expanding the potential for screening programmes.

As Dr Naraghi explained, currently, when doctors identify a potentially cancerous lesion on an MRI or CAT scan, they must rely on a lab to test the biopsy sample. That might not be necessary under a combined Siemens company with access to DPC products, said Dr Naraghi, who trained as a cardiologist, worked on neural systems and earned two PHDs, one in physiology and another in biophysics.

DPC has 3% of the total IVD market, Bayer 10% (excluding blood glucose monitoring and blood screening sales), putting Siemens behind the No 2 player Abbott, with its 14% market share – but still well below Roche, the market leader with a 22% of the market. Both companies have posted far better-than-average sales growth for the diagnostics industry – 8% for Bayer and 10%, projected for this year, for DPC.

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Exhibit 5

Champions 2.0

March 12, 2007

Jay Flatley

Illumina

Jay Flatley
President and CEO

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How has your company adapted and responded to the changing economic climate in the past five years when so many other companies did not?

We learned early on that evolving and continuously innovating our product and service offerings was critical. In 2006, Illumina launched over 25 products as part of its growing portfolio of genetic analysis tools. To do this, we listened closely to our customers and the scientific community, and anticipated where the industry was headed. Our success can be described by the researchers who have used our technology and completed studies much faster than they ever thought possible.

What is your vision for the future of the life sciences market over the next several years?

On a daily basis, the life sciences industry moves closer to understanding the genetic basis for human disease. Researchers using Illumina's technologies and platforms have recently identified genes that play a role in the development of complex diseases such as Parkinson's disease, Crohn's disease, type-2 diabetes and Lou Gehrig's disease. Additionally, the recent MicroArray Quality Consortium (MAQC) project announced that microarrays will be more widely adopted in clinical and regulatory settings in order to advance the rate of drug development.

We know that translational research will play an increasingly critical role in bridging disease discovery to clinical utility. We envision a market that will become increasingly consolidated and focused on delivering direct value to the patient from genetic discoveries.

What products and services does your company provide and what special capabilities do they offer the life sciences market?

Illumina is developing next-generation tools for genetic analysis. Since we completed our merger with Solexa, we are now the only company with genome-scale technology for genotyping, gene expression and sequencing -- the three cornerstones of modern genetic analysis. Combined with our commitment to provide support and service long after a system is installed, we offer a level of collaboration and confidence that is unique to other life science companies.

Partnerships are an effective way to track life science advances and ensure that your company delivers timely products and services. Which life sciences companies or organizations have you partnered with or invested in and why?

The life science community consists of a variety of organizations -- commercial, academic, pharmaceutical, and government. Illumina has partnered with a number of these institutions so that we can help researchers understand, prevent and ultimately cure disease.

Academic, pharmaceutical and government partners include the Children's Hospital of Pennsylvania (CHOP), the Center for Inherited Disease Research (CIDR), Genizon, Genome Quebec, the Welcome Sanger Trust Institute, deCODE genetics, Amgen and GlaxoSmithKline. We also have developed strong third party software and commercial service provider partnerships with groups such as Asuragen, Service Xpress, and GoldenHelix.

What are your most exciting products and initiatives in development, and how will they improve life science research?

Earlier this year we unveiled the industry's most comprehensive whole-genome DNA analysis solution -- the Human 1M BeadChip. This BeadChip will profile over 1 million diverse genetic variants on a single microarray. Combined with copy number variation content, this solution addresses applications beyond just traditional whole-genome association studies, and will set the new standard as being the most comprehensive and powerful tool available for genetic analysis.

In diagnostics, the company is excited to launch its VeraCode technology, which will be offered on the BeadXpress platform. Organizations like the Children's Hospital of Eastern Ontario and the Mayo Clinic have already initiated collaborations with Illumina to develop molecular diagnostics tests for complex diseases.

Illumina's Solexa Sequencing technology will also offer a powerful new approach to some of today's most important applications for genetic analysis and functional genomics, including sequencing and resequencing, digital gene expression, and small RNA identification.

ILLUM-1111

Where do you see your company in five years?

This year Forbes ranked Illumina #1 among 2,200 U.S. high-tech companies, naming us the fastest growing company in 2006. In five years, we envision a company with a continued commitment to be the premier genetic analysis tools provider, with a substantial business in molecular diagnostics. We have shown tremendous growth this year, and will continue to deliver value for the life science community, including our customers, our investors, and our employees.

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Exhibit 6

Drupal.behaviors.print = function(context) {window.print();window.close();}>

genomeweb

Illumina, Solexa, MDS, Molecular Devices, Thermo Fisher, FDA, Cepheid, Applied Biosystems, Luminex, US Air Force, CombiMatrix

March 21, 2007

Illumina, Solexa, MDS, Molecular Devices, Thermo Fisher, FDA, Cepheid, Applied Biosystems, Luminex, US Air Force, CombiMatrix

Accounting Rules Prevent Illumina from Recognizing Revenue on Some Solexa Sequencers

Accounting rules will prevent Illumina from booking most of the revenue from the sale of at least 13 Genome Analyzers that Solexa shipped to early-access customers, Illumina President and CEO Jay Flatley said at an investor conference last week.

A few weeks before it was acquired by Illumina in late January, Solexa said it had begun invoicing early-access customers in the fourth quarter, and that it expected to begin recording revenue from these placements in the current quarter.

"Due to the wonders of acquisition accounting, much of the revenue that occurred [before the merger was completed], even though it was not recognized as revenue for Solexa ... will not be recognizable by us," said Flatley, who spoke at the Cowen and Company 27th Health Care Conference in Boston. "So that revenue will disappear, it will not be recognized either by Solexa or by Illumina."

The rule will affect all 13 units that Solexa shipped in 2006, as well as any additional systems shipped between Jan. 1 and Jan. 26, the date Illumina completed the acquisition, a company spokeswoman told *BioCommerce Week* sister publication *In Sequence*.

Also at the conference, Flatley said although the integration of Solexa is "going quite well," R&D has not been consolidated yet. "We think it will take a little bit longer for us to put together all of

the key R&D teams," Flatley said.

Illumina had been conducting internal research in the area of sequencing, he added, which will be combined with Solexa's R&D groups.

He also reiterated that as of early February, the company had received 40 orders for the instrument, including the 13 systems it had shipped to early-access customers by the end of 2006.

Illumina Launches BeadXpress Platform

Illumina this week launched its BeadXpress system, the platform at the base of its molecular diagnostics strategy.

The BeadXpress is based on the VeraCode digital microbead technology the firm gained through its $17.5 million acquisition of CyVera in April 2005.

Illumina CFO Christian Henry said at the Lehman Brothers Global Healthcare Conference in Miami this week that the firm has already made its first shipments of the BeadXpress system.

According to Illumina, the BeadXpress system allows researchers to assay tens to hundreds of analytes in a single sample at one time. In addition to molecular diagnostics, Illumina anticipates the system will be used for biomarker research and validation, pharmaceutical development, industrial testing, and agricultural research.

"What this platform does is it enables us to move all the way down into lower and mid-multiplex ranges, which we really believe are going to be critical in the molecular diagnostics space," said Henry. "We're believers that microarrays are not the right approach to molecular diagnostics, in that they're not flexible enough, the reaction times are too long, and they're too expensive."

The firm expects 2007 revenue of between $295 million and $315 million, said Henry.

MDS Acquires 92 Percent of Molecular Devices' Shares

Molecular Devices' shareholders this week tendered 92 percent of the firm's shares in the acquisition offer from MDS.

In January, the companies had agreed to a deal under which MDS would buy Molecular Devices for $615 million in cash, or $35.50 a share for all of Molecular Devices' common stock (see *BioCommerce Week 1/31/2007*).

MDS said it would make an additional offering for the remaining 8 percent of shares outstanding. That offering is at the same price of $35.50 per share and will expire on March 23.

Upon completion of the acquisition, Molecular Devices and MDS' Sciex unit will be combined into a business unit called MDS Analytical Technologies. The new unit will be led by MDS Sciex President Andy Boorn.

According to MDS, Molecular Devices and MDS Sciex combined brought in 2006 revenue of $432 million.

Thermo Fisher Expands Asset Management Services in Europe

Thermo Fisher Scientific said last week that it has expanded its asset management services capabilities in Europe.

In addition to a new management team, new services are being launched to help customers in the pharmaceutical, chemical, petrochemical, and biotechnology sectors optimize the management of their laboratory assets.

"By establishing a full presence in Europe, existing and new customers will benefit from local support and solutions tailored to their individual needs," Thermo Fisher said in a statement.

The European Asset Management Services, based in the UK, will focus on companies there, in Germany, France, Spain, Italy, the Netherlands, and Scandinavia.

FDA Clears Cepheid's Meningitis Molecular Dx

The US Food and Drug Administration last week cleared for marketing a Cepheid test designed to help physicians detect viral meningitis within two and a half hours.

The test is Cepheid's third molecular diagnostic assay to receive 510(k) clearance since the beginning of 2006.

Cepheid's Xpert EV test is an automated diagnostic that uses reverse transcription PCR on cerebrospinal fluid samples to determine if a patient has enterovirus, which causes 90 percent of meningitis cases. The test is run on the company's GeneXpert diagnostic system, one of two RT-PCR platforms developed by Cepheid. The other is its SmartCycler.

According to a statement released by the FDA last week, in a multi-site study the test was 96-percent accurate in detecting viral meningitis, and 97-percent accurate in patients who did not have the enterovirus.

Bacterial meningitis is far more lethal than the viral form of the disease, and requires swift diagnosis. Because symptoms of the two diseases are so similar, doctors currently treat viral patients empirically with antibiotics for the bacterial form.

Being able to swiftly determine which pathogen a patient has should allow doctors to make more

accurate therapeutic decisions and avoid unnecessary antibiotic treatments, the FDA said.

Two Banks Upgrade ABI's Stock

Two investment banks last week upgraded their ratings on Applied Biosystems' stock.

Am Tech/JSA Research upgraded ABI's stock to "buy" from "neutral," while Matrix Research raised its rating for ABI to "buy" from "hold."

ABI's shares rose 4.1 percent over the past week to close at $29.73 on Tuesday.

Investment Bank Starts Coverage of Luminex

Investment bank Thomas Weisel Partners this week initiated coverage of Luminex with an "overweight" rating.

The rating follows an upgrade in December from investment firm Soleil Securities on Luminex's stock from "hold" to "buy."

Luminex's shares closed at $14.75 on Tuesday.

US Air Force Extends CombiMatrix Contract to Develop Influenza Dx

CombiMatrix last week said that the US Air Force has extended a contract to continue developing an influenza genotyping system.

Under the $890,000 deal, CBMX will develop a field-deployable system designed to identify all influenza strains, including H5N1 avian flu, and other respiratory pathogens.

The original program started in 2005 when the USAF's Institute of Occupational Health paid CBMX $340,000 for a one-year deal to begin developing an array to identify the infections.

Last year's agreement enabled the company to retain the right to sell the resulting assays to other customers.

CBMX CEO Amit Kumar said it is important to be able to track mutations in bird flu, and added that CBMX's system can "determine when any other strain of influenza A is gaining an upper hand in a population, and can identify other infectious diseases that cause flu-like symptoms.

The company said the first effort will be to update the array with new information from influenza A sequencing that is related to human disease.

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Exhibit 7

genomeweb

Gates Foundation Awards Md. Vaccine Center $5.6M for Pathogen Dx; Illumina, Ibis Tapped as Subcontractors

November 28, 2007

Gates Foundation Awards Md. Vaccine Center $5.6M for Pathogen Dx; Illumina, Ibis Tapped as Subcontractors

By a GenomeWeb staff reporter
NEW YORK (GenomeWeb News) The Center for Vaccine Development at the University of Maryland's School of Medicine said today that it has been awarded a $5.6 million grant from the Bill and Melinda Gates Foundation to develop a diagnostic for diarrheal disease.

Illumina and Ibis Biosciences will serve as subcontractors on the grant and will provide genomics and pathogenic identification services, the university said.

The center plans to develop a diagnostic that will be faster and more accurate than available models and will be aimed at identifying pathogens that cause diarrheal problems in developing countries.

This center will "detect all of the important organisms involved in diarrheal diseases in developing countries," the CVD said in a statement.

The research will be conducted on fecal samples obtained from Bangladesh, Mali, and Kenya through an agreement with researchers from the Center for Vaccine Development.

footer

ILLUM-3648

Exhibit 8

Journal of
Clinical Microbiology

Survey of Culture, GoldenGate Assay, Universal Biosensor Assay, and 16S rRNA Gene Sequencing as Alternative Methods of Bacterial Pathogen Detection

Brianna Lindsay, Mihai Pop, Martin Antonio, Alan W. Walker, Volker Mai, Dilruba Ahmed, Joseph Oundo, Boubou Tamboura, Sandra Panchalingam, Myron M. Levine, Karen Kotloff, Shan Li, Laurence S. Magder, Joseph N. Paulson, Bo Liu, Usman Ikumapayi, Chinelo Ebruke, Michel Dione, Mitchell Adeyemi, Richard Rance, Mark D. Stares, Maria Ukhanova, Bret Barnes, Ian Lewis, Firoz Ahmed, Meer Taifur Alam, Ruhul Amin, Sabbir Siddiqui, John B. Ochieng, Emmanuel Ouma, Jane Juma, Eunice Mailu, Richard Omore, Ciara E. O'Reilly, James Hannis, Sheri Manalili, Jonna DeLeon, Irene Yasuda, Lawrence Blyn, Raymond Ranken, Feng Li, Roberta Housley, David J. Ecker, M. Anowar Hossain, Robert F. Breiman, J. Glenn Morris, Timothy K. McDaniel, Julian Parkhill, Debasish Saha, Rangarajan Sampath, O. Colin Stine and James P. Nataro
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Survey of Culture, GoldenGate Assay, Universal Biosensor Assay, and 16S rRNA Gene Sequencing as Alternative Methods of Bacterial Pathogen Detection

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Cultivation-based assays combined with PCR or enzyme-linked immunosorbent assay (ELISA)-based methods for finding virulence factors are standard methods for detecting bacterial pathogens in stools; however, with emerging molecular technologies, new methods have become available. The aim of this study was to compare four distinct detection technologies for the identification of pathogens in stools from children under 5 years of age in The Gambia, Mali, Kenya, and Bangladesh. The children were identified, using currently accepted clinical protocols, as either controls or cases with moderate to severe diarrhea. A total of 3,610 stool samples were tested by established clinical culture techniques: 3,179 DNA samples by the Universal Biosensor assay (Ibis Biosciences, Inc.), 1,466 DNA samples by the GoldenGate assay (Illumina), and 1,006 DNA samples by sequencing of 16S rRNA genes. Each method detected different proportions of samples testing positive for each of seven enteric pathogens, enteroaggregative *Escherichia coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), *Shigella* spp., *Campylobacter jejuni*, *Salmonella enterica*, and *Aeromonas* spp. The comparisons among detection methods included the frequency of positive stool samples and kappa values for making pairwise comparisons. Overall, the standard culture methods detected *Shigella* spp., EPEC, ETEC, and EAEC in smaller proportions of the samples than either of the methods based on detection of the virulence genes from DNA in whole stools. The GoldenGate method revealed the greatest agreement with the other methods. The agreement among methods was higher in cases than in controls. The new molecular technologies have a high potential for highly sensitive identification of bacterial diarrheal pathogens.

he detection of pathogens in stools has been performed traditionally using techniques developed and applied in high-income countries. These methods, developed over the past century and rigorously tested and approved for use for clinical diagnostic purposes, include the standard clinical culture for identifying pathogens, sometimes after an enrichment step, on a selective or differential medium that inhibits the growth of many bacteria and is permissive to the pathogen of interest. After sufficient growth is attained, the colonies are tested by PCR for the selected genes or agglutinated with antisera specific for the pathogen. Alternatively, enzyme-linked immunosorbent assays (ELISAs) or PCR methods may be used directly on fecal samples. Despite the widespread use of these methods in high-income countries, their adoption for routine use in low-income countries has been very limited (1, 2). The availability and costs of technical expertise, supplies, equipment, and equipment maintenance are factors which have limited the adoption of diagnostic microbiology methods in these settings (3). Simple, inexpensive, and reliable point-of-care diagnostic tests that rapidly identify treatable etiologies of diarrhea are needed in low-income countries. We sought to compare the abilities of four different emerging technologies to identify the presence of enteric pathogens in stools from children in low-income countries.

The new molecular methods have great appeal as the technologies are increasingly robust and affordable and potentially more precise. Three alternative technologies are based upon detection of specific DNA sequences. One technology, the GoldenGate assay (Illumina, San Diego, CA), has been adapted for microbiological detection (4). The assay is based on the specific hybridization of primers to target DNA in the solution and the ligation of two primers to form a product that can be amplified with efficiency equal to that for the other ligated products. Amplified ligation products are hybridized to microbeads and detected in an optical microarray format in a Bead Express station (Illumina). The sec-

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ond technology is the Ibis Universal Biosensor assay (5). It employs a series of PCR primers to amplify selected targets; after amplification, the target DNAs are detected by electrospray ionization-mass spectrometry (ESI-MS) (Ibis, San Diego, CA). The third technology is high-throughput sequencing of fecal community DNA after amplification of 16S rRNA genes (6). Sequences are then processed to identify operational taxonomic units (OTUs). This method can distinguish between genera and some species of bacteria but cannot distinguish among pathotypes of a single species, e.g., enteroaggregative *Escherichia coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and *Shigella* spp.

These three new technologies were compared with conventional methods on stool samples collected from case and control children in The Gambia, Mali, Kenya, and Bangladesh during the Global Enterics Multicenter Study (GEMS) funded by the Bill and Melinda Gates Foundation (7, 8). Comparisons were done for seven detectable pathogens, EAEC, ETEC, EPEC, *Shigella* spp., *Campylobacter jejuni*, *Salmonella enterica*, and *Aeromonas* spp. Our analyses showed that no method is clearly superior, but the GoldenGate method agreed most frequently with the other methods.

MATERIALS AND METHODS

Stool samples and DNA isolation. Stool samples were collected as part of the GEMS. Children less than 5 years old seeking care for episodes of diarrhea (passage of ≥3 abnormally loose stools within the previous 24 h) at health care facilities serving field site populations were considered. Those who presented within 7 days of the onset of their illness and met the case definition of moderate to severe diarrhea were eligible for enrollment. The episodes meeting the inclusion criteria included exhibition of the most objective signs of dehydration, including sunken eyes, a loss of normal skin turgor, a decision to initiate intravenous hydration or hospitalize the child, or the presence of blood in the stool (dysentery). Within 2 weeks of enrollment of a case, 1 to 3 randomly selected control children without diarrhea, matched to the case by age, gender, and village, were enrolled from the community (8). Stool samples were cultured 7 h on average after defecation. The stool samples were tested for 15 potential diarrheagenic pathogens and their subtypes by culture and subsequent PCR and immunological methods (7). After homogenization of fecal samples by vortexing with 3-mm glass beads (Sigma Life Science, St. Louis, MO), bacterial cell walls were disrupted using a bead beater with 0.1-mm zirconium beads (catalog no. 110791012; BioSpec Products, Bartlesville, OK). The cell slurry was centrifuged, and the supernatant was removed and processed using the Qiagen (Hilden, Germany) QIAamp DNA stool extraction kit. The extracted DNA was precipitated, and the DNA was shipped to the University of Maryland (Baltimore, MD), following the appropriate in-country approvals. DNA was then resuspended, and aliquots were distributed to Ibis and Illumina. For the current study, we utilized specimens from The Gambia, Mali, Kenya, and Bangladesh, chosen based on geographic representation from Asia and sub-Saharan Africa and close collaboration with the sites' principal investigators.

Culture. The clinical pathogen identification methods were completed in each of the participating countries (The Gambia, Mali, Kenya, and Bangladesh) and were described in detail by Panchalingam et al. (7). Briefly, the stool specimens were collected in sterile containers and examined within 24 h of passage. The stool specimens were stored at 2 to 8°C while in transit to the laboratory. Conventional bacteriological, immunological, and molecular methods were used to identify bacterial pathogens. *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Aeromonas* spp., *C. jejuni*, *Campylobacter coli*, *Salmonella* spp., *Shigella* spp., and diarrheagenic *E. coli* were isolated from the appropriate selective media and identified by standard biochemical tests. The standard protocol was to test three putative lactose-positive and indole-positive *E. coli*-like colony morphologies selected from the MacConkey plate for diarrheagenic *E. coli* as previously described (7). The species and subtypes were confirmed by serotyping (for *Shigella* and *Salmonella* spp.) with commercially available antisera (Reagensia, Solna, Sweden, and Denka Seiken, Tokyo, Japan) and by PCR tests for both the heat-labile and heat-stable enterotoxin producers of ETEC, typical and atypical EPEC, and EAEC.

GoldenGate assay. Performed at Illumina, the GoldenGate assay consists of allele-specific oligonucleotides (ASOs) or locus-specific oligonucleotides (LSOs) that are hybridized to the target sequence and ligated together, and the ligated products are PCR amplified using primers attached to the 5′ ends of the ASOs or LSOs. The primers were designed using GoldenGate design software (see reference 9 for details in the GoldenGate assay workflow). In addition, a "Laguna" probe was designed to hybridize to the target DNA, specifically avoiding sequences from human and the various target organisms. The probe is attached to streptavidin and, when precipitated, increases the purity of the target DNA. Multiple probes sets were designed for each target organism.

Sixty-nine sets of probes were simultaneously hybridized to the target DNAs following the methods described in the GoldenGate Genotyping with VeraCode Technology instructions (4). After the beads were scored in the array, the background intensity was calculated for each sample by taking the 50th percentile for all probes present, because it is unlikely that more than half of the targets would be present in a sample. After background subtraction from all the probes, the mean intensities and standard deviations were calculated for all the probes. Greater than 3 standard deviations was the threshold for a probe to detect a target. To call a target present, at least half of its probes had to be detected.

Universal Biosensor assay. Performed at Ibis, this method uses an automated process for aliquoting extracted DNAs, amplifying DNA using PCR primers for the detection of microorganisms of interest, and then subjecting the amplified products to ESI-MS (5). Six pairs of primers targeting 16S and 23S rRNA genes were used to identify microbes broadly (Gram positive, Gram negative, aerobic, and anaerobic), followed by primer pairs to characterize 17 additional genes (*ctxA*, *ctxB*, *east*-1, *east*-2A, *estA1*, *eltA*, *eltB*, *stx1A*, *stx1B*, *aggR*, *aatA*, *eae*, *invA*, *ipaH*, *ipaA*, *ipaC*, and *ipaB*) used to identify the pathogen-specific markers. The mass spectrometer measures the amplicons of the PCR product to calculate a molecular weight, and the composition of the nucleotides can be deduced for each amplicon present. The Universal Biosensor assay utilizes a database of sequence base composition of known microorganisms to determine which microorganisms are present. The Universal Biosensor assays tested for broad groups of microorganisms, including *Campylobacter* spp., *Clostridium* spp., *E. coli*, *Escherichia* spp., nontyphoidal *Salmonella* spp., *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *V. cholerae*, *V. parahaemolyticus*, ETEC, EPEC, EAEC, and *Yersinia* spp. and the genes *ctxA*, *ctxB*, *eae*, *east*-1, *invA*, *ipaB*, *ipaC*, *ipaD*, and *ipaH*.

16S rRNA gene survey. The DNA from stool samples was amplified using "universal" primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 338R (5′-CATGCTGCCTCCCGTAGGAGT-3′). A single reverse primer and a set of 96 barcoded forward primers were used. The presence of amplified products was confirmed on agarose gels, and aliquots from each of the 96 samples were added in equimolar amounts to a final mix. This was sequenced using the FLX sequencing kit and the 454 FLX sequencing platform (454 Life Sciences, Branford, CT). Individual reads were filtered for quality using custom in-house scripts that perform the following checks suggested by Huse et al. (10): (i) sequences containing any ambiguous bases (N) were removed, (ii) sequences that were shorter than 75 cycles of the 454 instrument were removed (each cycle yields an average of 2.5 bp, depending on the sequence composition), and (iii) sequences for which a barcode could not be identified were removed (10). The remaining sequences were separated into sample-specific sets according to their barcodes, and the barcodes were removed. The conservative OTUs were constructed by pooling together the sequences from all samples and then were clustered using DNACLUST with the default parameters (98% identity clusters) (11). Pooling the samples ensures that the definition of an OTU is consistent across all the samples. To obtain taxo-

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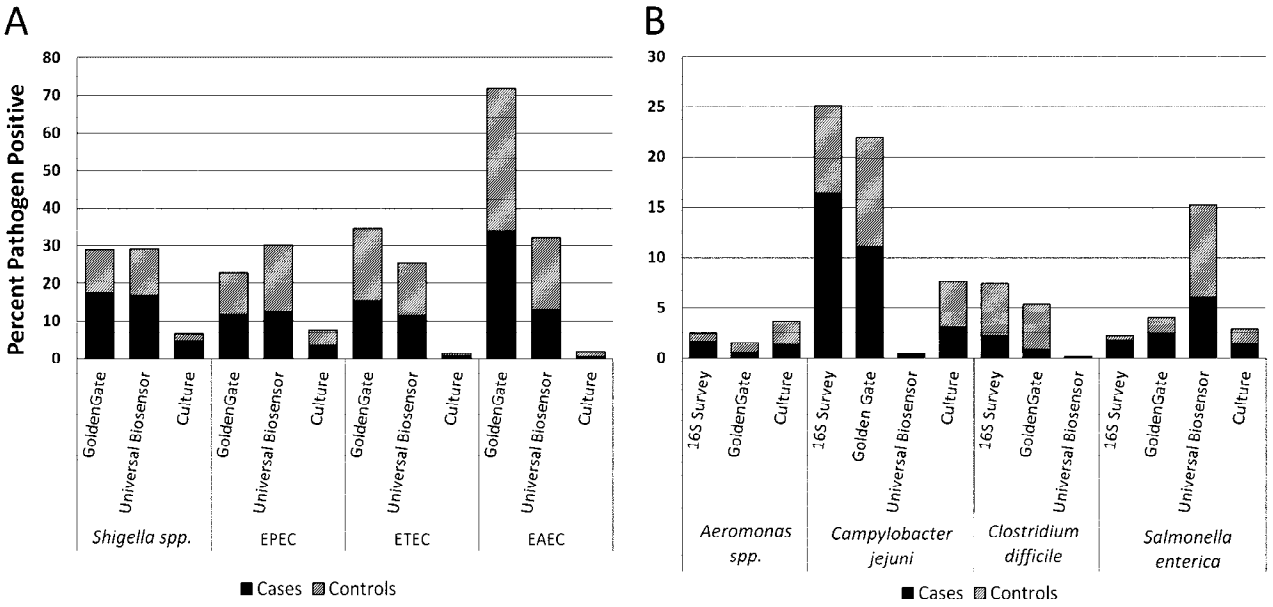

FIG 1 Histogram of the percentages of samples positive for specific pathogens by each method of detection. The pathogens were separated into two panels because the 16S rRNA gene survey is unable to identify the pathogens in panel A. (A) Percentage of samples that were positive for *Shigella* spp. and specific pathotypes of *E. coli* by the GoldenGate, Universal Biosensor, and culture methods. (B) Samples that were positive for specific species. The heights of each histogram bar was calculated by dividing the number of samples where the pathogen was detected by the total number of samples. The bars are colored according to the proportion of cases and the proportion of controls.

nomic identification, a representative sequence from each OTU was aligned to the Ribosomal Database Project (RDP) (release 10.4 [see http://rdp.cme.msu.edu]) using BLASTN with long word length (-W 100) in order to detect only nearly identical sequences. A reference database of 16S rRNA gene sequences from known enteric pathogens was manually curated by extracting full-length 16S rRNA gene sequences from isolated genomes in the RDP (on 1 April 2011) for *Clostridium difficile*, *V. cholerae*, *V. parahaemolyticus*, *S. enterica*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *C. jejuni*, *C. coli*, *Helicobacter pylori*, and *Yersinia enterocolitica*. All sequences from each OTU were searched against the full RDP and the enteric pathogen subset using BLAST (best hit with similarity, ≥97%).

Statistical analysis. The percentages of samples positive for diarrheal pathogens in each method were determined by dividing the number of samples identified as positive by the total number of samples tested. Cohen's kappa was used to describe the agreement between two detection methods. Cohen's kappa is advantageous because it does not require the specification of a gold standard but simply quantifies the agreement between binary outcomes of tests (positive or negative), taking into account agreement occurring by chance (12). The kappa term ranges from −1 to 1 and can be negative if the agreement is less than what would be expected by chance. The following labels were assigned to the corresponding ranges of kappa strength: poor agreement, <0; slight, 0.0 to 0.20; fair, 0.21 to 0.40; moderate, 0.41 to 0.60; substantial, 0.61 to 0.80; and almost perfect, 0.81 to 1.00 (12, 13). All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

Pathogen detection in stool samples. The frequency of detection of each pathogen varied from one method to the next (Fig. 1A and B). The comparisons were based on the percent frequency of detection in order to normalize the methods because different numbers of specimens were tested. We tested 3,610 stool samples with the culture method, 3,179 DNA samples with the Universal Biosensor method, 1,466 DNA samples with the GoldenGate method, and 1,006 DNA samples with the 16S rRNA gene survey method (Table 1). Although attempts were made to identify *V. cholerae* and *V. parahaemolyticus* on each sample, no method detected more than 10 specimens with these pathogens, so no comparisons were made. Identification of EHEC was attempted with only the Universal Biosensor method, so these results are not presented.

The pathotypes of *E. coli* and *Shigella* spp. were differentiated

TABLE 1 Demographic characteristics of enrolled children and detection methods by case and control status

Characteristics and detection methods	Cases (n = 1,500)		Controls (n = 2,110)		Total (n = 3,610)
	No.	%	No.	%	
Age group (mo)					
0 to <12	645	45.9	759	54.1	1,404
12 to <24	497	39.9	750	60.1	1,247
24 to 59	358	37.3	601	62.7	959
Site					
The Gambia	418	44.0	532	56.0	950
Mali	139	48.8	146	51.2	285
Kenya	694	44.2	875	55.8	1,569
Bangladesh	249	30.9	557	69.1	806
Male	834	41.3	1,187	58.7	2,021
Detection method					
Culture	1,500	41.6	2,110	58.5	3,610
16S rRNA gene survey	513	51.0	493	49.0	1,006
GoldenGate assay	678	46.3	788	53.8	1,466
Universal Biosensor assay	1,327	41.7	1,852	58.3	3,179

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on the basis of virulence genes specific to each subtype. As the 16S rRNA gene survey method does not detect virulence genes, it was not compared to the other methods for these pathogens. As seen in Fig. 1A, the culture-based method detected *Shigella* spp., EPEC, ETEC, and EAEC in a smaller proportion of the samples than either of the methods (GoldenGate or Universal Biosensor) based on detection of the virulence genes from DNA from whole stools. For *Shigella* spp., EPEC, and ETEC, the proportions of samples in which the virulence genes were detected were very similar for the Universal Biosensor and GoldenGate methods, while for EAEC, the GoldenGate method detected the virulence genes in a substantially higher proportion of samples.

Two pathogens, *C. jejuni* and *S. enterica*, could be detected by all of the four methods (Fig. 1B). *Aeromonas* spp. were detected by the culture based method, the 16S rRNA gene survey, and the GoldenGate method, and *C. difficile* was detected by the 16S rRNA gene survey, the GoldenGate method, and the Universal Biosensor method. The Universal Biosensor method rarely detected *C. jejuni* and *C. difficile*, but it identified *S. enterica* in the highest proportion of cases. As with the pathotypes, the culture-based method consistently detected pathogens in fewer stool samples than did the GoldenGate method. The 16S rRNA gene survey method detected *C. jejuni*, *C. difficile*, and *S. enterica* in proportions similar to those detected by the GoldenGate method.

Shigella spp. and EPEC were detected relatively more consistently among the methods. Figure 2A and B shows Venn diagrams that highlight the numbers of *Shigella* spp. and EPEC identified by each method and the overlap of the methods. Generally, as the number of detection methods that positively identified the pathogen increased, so did the proportion of cases within that group.

Comparison between methods. The agreement between methods was determined by comparing the positive and negative results for each sample successfully tested by each possible pair of methods. The best agreement exceeding the threshold (0.41) for moderate agreement was observed in four instances; the Universal Biosensor and GoldenGate methods agreed on EPEC, ETEC, and *Shigella* spp., and the GoldenGate and 16S rRNA gene survey methods agreed on *C. jejuni*. Fair agreement (kappa values, 0.21 to 0.40) was observed between the GoldenGate and culture methods for five pathogens (EPEC, *Shigella* spp., *C. jejuni*, *Aeromonas* spp., and *S. enterica*), and between the Universal Biosensor and culture methods for two pathogens (EPEC and *Shigella* spp.). We found that the agreement between methods was higher in the cases than in the controls in 21 of the 27 possible comparisons (Fig. 3).

To determine whether there were any site-specific biases, kappa values were computed for the data from each individual site (Table 2). After a comparison across all four sites, the GoldenGate method appears to be the one that contributes to relatively high kappa values most frequently. The GoldenGate and Universal Biosensor methods detect pathogenic ETEC, EPEC, EAEC, and *Shigella* spp. at similar rates and at greater-than-conventional culture rates. The GoldenGate and 16S rRNA gene survey methods detected *Aeromonas* spp., *C. jejuni*, *Salmonella enterica*, and *Clostridium* spp. at similar rates, greater than the culture-based rates.

16S rRNA gene survey results. The survey detected a total of 97,666 nonunique OTUs. Of these, only 25,834 that were detected in more than 5 samples or had more than 20 sequences in at least 1 sample were included in further analyses. The number of OTUs per sample averaged 465.27 and ranged from 3 to 1,439. For the pathogens *C. jejuni*, *C. difficile*, *S. enterica*, and *Aeromonas* spp., representative 16S rRNA gene sequences were selected from the Ribosomal Database Project, and OTUs that varied by less than 2% from these sequences were assigned to that species. This was not done for OTUs matching *E. coli*, as *E. coli* strains can be either commensal or pathogenic. Seventy OTUs were designated *C. jejuni*, 8 *C. difficile*, 3 *S. enterica*, and 3 *Aeromonas* spp. *C. jejuni* was detected most often, being present in 25% of the total number of samples, with *C. difficile*, *S. enterica*, and *Aeromonas* spp. being present in 7%, 2%, and 2%, respectively.

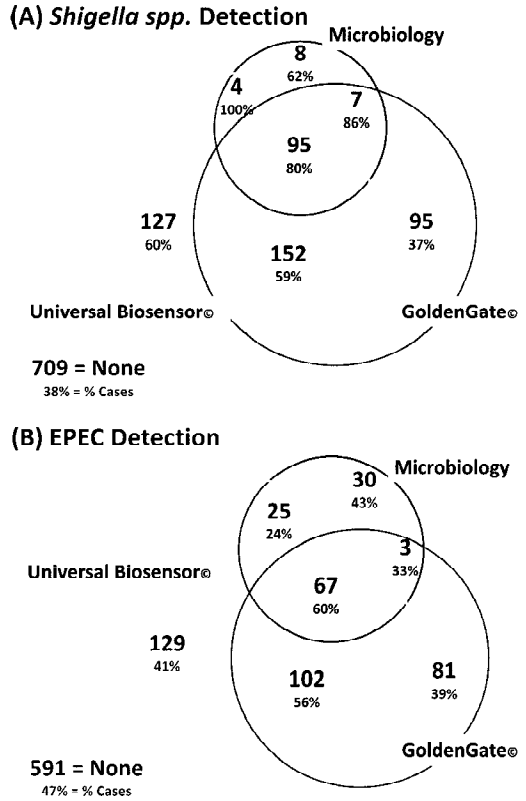

FIG 2 Venn diagram depicting identification overlap between methods. Each case and control sample was tested by all three methods. The top number in each sector represents the number of positive cases and controls identified by each method, and the lower percentage is the number of cases identified divided by the total number of samples (cases and controls) positive by each method. The size of the circle and overlap between circles are proportional to the categorical n values. (A) *Shigella* spp. (n = 1,197). In 709 samples, no *Shigella* sp. was detected. (B) EPEC (n = 1,028). In 591 samples, no method detected EPEC.

DISCUSSION

Culture-based methods are commonly used for detecting enteric pathogens, and in our hands, the standard tests detected many pathogens. It is notable, though, that there were numerous samples where the alternative molecular techniques detected the apparent presence of a pathogen despite the absence of that pathogen according to the standard clinical method. However, that same statement could be made for every other method as well; each method failed to detect a specific pathogen in a sample where one or more of the other methods did detect the same pathogen. *A priori* reasons abound for false positives and false negatives for

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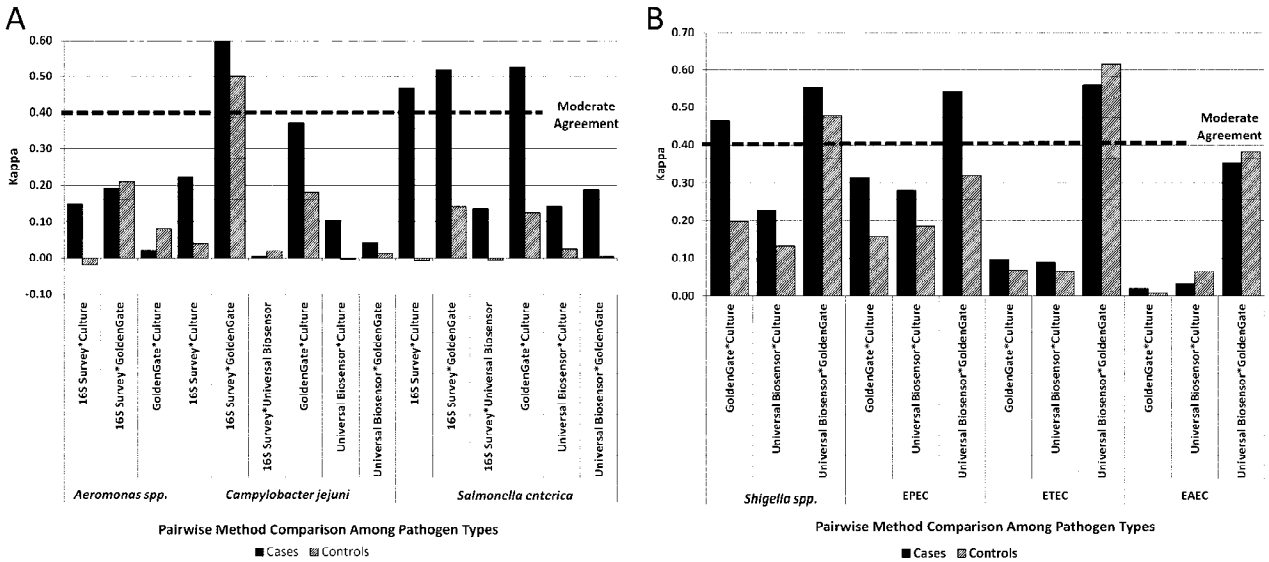

FIG 3 Agreement between methods among cases and controls for detection of pathogens. The kappa values between pairs of methods for individual pathogens are plotted. A moderate agreement between methods is marked by the dashed line (kappa > 0.41). (A) Kappa values for *C. jejuni*, *S. enterica*, and *Aeromonas* spp.; (B) kappa values between methods for pathotypes of *E. coli* and *Shigella* spp.

each of the methods; the potential sources include contamination, PCR failure, limits to detection, genetic variation, and complexity of the composition of the stools (14). Nevertheless, the different methods of detection often detect the same pathogen in the same stool sample, indicating that the methods have some agreement. We interpret these data to indicate that, at this time, there is no gold standard for detecting pathogens. The lack of a gold standard limits the analyses.

When the culture methods identified the target organisms, we were able to confirm that the microbes were truly present. However, a negative result may have been either true, because the organism was not present, or false, because (i) the organism did not grow as a result of inadvertent inhibition by the selective medium used, (ii) the specimen transport conditions compromised the organism, or (iii) laboratory inefficiencies were present. Published results and our own unpublished results revealed that testing a greater number of *E. coli* isolates (e.g., testing 5 colonies instead of 3 picked from a MacConkey plate) will increase the rates of recovery, thereby confirming the possibility of some false negatives (15). Compared to those of other methods, the culture-based results showed fair agreement with the GoldenGate method on *Aeromonas* spp., *C. jejuni*, and *S. enterica* and with both the Universal Biosensor and GoldenGate methods for EPEC and *Shigella* spp.

The Universal Biosensor and GoldenGate methods are the two most similar methods. Both methods detected EAEC, EPEC, ETEC, and *Shigella* spp. at much higher rates than did the culture-based method and had the highest rates of agreement for these pathogens. Each method starts with the specific hybridization of two primers to DNA and detects the same virulence genes, although with distinct primers, but they have limits of detection based on the ability of the primers to hybridize. Any sample with the specific DNA of interest below the detection limit will produce a false negative that may be overcome in the culture-based method, which has the potential to enrich and grow a bacterium from a single CFU. False positives will occur if the virulence genes, usually found on mobile elements, are found in bacteria other than the expected pathogen.

The sequence survey method is based on amplifying the 16S rRNA gene of bacteria using universal primers and then counting the presence of the different 16S rRNA gene sequences. In order to be counted, the bacteria must constitute $1/n$ or more (where n is the number of sequences counted) of the community. Since n is on average 3,900, the bacteria in question must be ~0.03% of the sample, and those near the cutoff level are also subject to sampling error (for an example, see Fig. 1B in reference 16). The method cannot distinguish among the pathotypes of *E. coli* and *Shigella* spp. However, the method did distinguish among *Aeromonas* spp., *C. jejuni*, *S. enterica*, and *C. difficile* as expected, and for the latter three, the method detected these pathogens at levels that were most similar to those of the GoldenGate method. The two methods revealed moderate agreement for *C. jejuni*. The Universal Biosensor method for detecting *C. jejuni* and *C. difficile* is based on the detection of 16S rRNA genes, and the underestimation of these pathogens is likely the result of the complexity of the stool samples such that even in samples from cases, the sequence-based method yielded extremely large numbers of distinct 16S rRNA gene sequences. The amplification of the whole sample for the sequencing approach raises the question of whether an alternative approach, like complete shotgun sequencing using next-generation sequencing, would detect taxa similar to those detected by the other methods.

The agreement between methods was consistently greater in cases than in controls. Almost certainly this observation can be attributed to the higher concentration of a pathogen in the cases than in the controls and to the fact that the higher the concentration the more likely a pathogen is to be detected. Quantification of the level of pathogens in the stool may improve the ability to accurately diagnose the cause of the diarrhea (17). This may have particular importance as the increased levels of detection (seen in

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TABLE 2 Kappa agreement values stratified by country and case/control status

Organism[a]	Kappa agreement value[b] for organisms detected in: The Gambia	Mali	Kenya	Bangladesh	Cases	Controls	Overall kappa value
Aeromonas							
16S survey*Micro				0.1290	0.1806	−0.0234	0.1014
16S survey*Illumina	−0.0056	−0.0196	**0.4439**	−0.0231	0.1814	0.1859	0.1827
Illumina*Micro				0.0735	0.0216	0.0816	0.0587
Campylobacter							
16S survey*Micro	0.0843	0.0060	0.1399	**0.4482**	0.2229	0.0356	0.1538
16S survey*Illumina	**0.5812**	**0.5722**	**0.5315**	**0.5338**	**0.5920**	**0.4846**	**0.5574**
16S survey*Ibis	0.0189	−0.0096	0.0229	0.0000	0.0048	0.0191	0.0106
Illumina*Micro	0.0864	0.0356	0.3363	0.5493	0.3715	0.1799	0.2711
Ibis*Micro	−0.0059	−0.0063	0.0581	0.0354	0.1023	−0.0032	0.0421
Ibis*Illumina	0.0083	−0.0115	0.0517	0.0652	0.0402	0.0125	0.0271
Salmonella							
16S survey*Micro	0.1500	0.1931	0.2207	−0.0278	0.2245	−0.0177	0.1603
16S survey*Illumina	0.0279	**0.5588**	**0.5007**	−0.0386	0.3103	0.0775	0.2379
16S survey*Ibis	0.0338	0.1563	0.0951	0.0446	0.1449	−0.0041	0.0778
Illumina*Micro	0.1577	0.3932	0.5903	0.5376	**0.5246**	0.1242	0.3691
Ibis*Micro	0.0344	0.0625	0.0957	0.0128	0.1419	0.0252	0.0725
Ibis*Illumina	−0.0062	**0.4182**	0.1589	−0.0183	0.1870	0.0038	0.0855
Shigella spp.							
16S survey*Micro	0.1080	0.0280	−0.0108	0.0921	0.0057	0.0002	0.0311
16S survey*Illumina	0.0361	0.0499	0.0383	0.1167	0.0004	−0.019	0.0303
16S survey*Ibis	0.3372	0.2857	0.2192	0.077	0.2723	0.1345	0.2487
Illumina*Micro	0.2519	0.1492	0.3458	**0.5672**	**0.4646**	0.1969	0.3835
Ibis*Micro	0.1634	0.0922	0.1469	**0.4129**	0.2280	0.1325	0.2027
Ibis*Illumina	**0.5119**	**0.4904**	**0.5024**	**0.6112**	**0.5548**	**0.4790**	**0.5388**
EPEC							
Illumina*Micro	0.0620	0.3155	**0.4783**	0.1970	0.3156	0.1573	0.2341
Ibis*Micro	0.1858	0.2128	0.2164	0.3357	0.2778	0.1856	0.2247
Ibis*Illumina	0.3692	**0.6856**	**0.4991**	0.2276	**0.5438**	0.3206	**0.4278**
ETEC							
Illumina*Micro	0.0830	0.1223	0.0520	0.0729	0.0964	0.0686	0.0811
Ibis*Micro	0.0547	0.0732	0.0796	0.1173	0.0882	0.0637	0.0753
Ibis*Illumina	**0.5642**	**0.5884**	**0.6175**	**0.5696**	**0.5614**	**0.6165**	**0.5909**
EAEC							
Illumina*Micro	0.0013	0.1280	0.0245	0.0216	0.0193	0.0076	0.0129
Ibis*Micro	0.0565	0.0258	0.0439	0.0726	0.0302	0.0653	0.0514
Ibis*Illumina	0.3064	0.3284	**0.4279**	**0.4353**	0.3523	0.3820	0.3689

[a] Micro, microbiology; 16S survey, 16S rRNA gene survey; Illumina, Illumina GoldenGate assay; Ibis, Ibis Universal Biosensor assay. *, pairs of methods were compared.
[b] Agreement: fair, 0.21 to 0.40 (underlined); moderate, >0.40 (bold). Cells without data indicate a zero cell count.

the Golden Gate and Universal Biosensor methods) will mean that multiple pathogens will be detected in an increasing number of stool samples. The proportion of hospitalized patients in Kolkata, India, with multiple pathogens using cultures was approximately 30%, and when multiple pathogens were present, there were often strong associations between them (18, 19). With the GEMS revealing many pathogens in control stool samples, quantitation of pathogens may be an important diagnostic criterion.

In conclusion, our results demonstrate that traditional culture-based methods underestimate the numbers of stool samples in which specific enteric pathogens can be detected compared with those by the alternative molecular methods. Not surprisingly, the alternative methods based on detection of specific unique genes produced the most similar results. Methods based on 16S rRNA gene sequences had distinct limitations. However, in the absence of a gold standard, replication and consistency between methods become important criteria. The GoldenGate method revealed the greatest agreement with the other methods. However, improvements in terms of targeted genes and the ability to quantify the number of pathogens present would enhance the usefulness of the method used for diagnosis.

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Exhibit 9

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EraGen Partners with Illumina to Develop Molecular Dx Assays

November 18, 2009

EraGen Partners with Illumina to Develop Molecular Dx Assays

By a GenomeWeb staff reporter

NEW YORK (GenomeWeb News) – EraGen Biosciences said today that it has formed a strategic partnership with Illumina to develop high-throughput, multiplexed clinical assays.

Under the agreement, EraGen, based in Madison, Wis., has non-exclusively licensed the use of Illumina's BeadXpress platform to develop and commercialize molecular-based, high-throughput, clinical assays using EraGen's MultiCode-PLx technology.

Meantime, Illumina has non-exclusively licensed EraGen's MultiCode-PLx technology for the life sciences, research, and clinical markets.

Financial terms of the agreement were not disclosed.

"Our newly formed relationship with Illumina further validates our MultiCode technology's versatile application in the development and commercialization of molecular-based assays for the clinical market," Irene Hrusovsky, president and CEO of EraGen, said in a statement.

EraGen's MultiCode technology detects RNA and DNA targets by combining multiplexed PCR with its ISO bases, which are structurally similar to standard nucleobases but have altered base-pairing specificity.

EraGen said that the pact is part of its strategy to continue expanding its commercial efforts in the molecular diagnostics market.

Related Stories

- Esophageal Cancer Study Reveals Mutation Transitions with Diagnostic Potential
  June 23, 2014 / GenomeWeb Daily News

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- Trovagene, Illumina Reach Technology Assessment Deal
  June 25, 2013 / GenomeWeb Daily News
- CGC Genetics Rolls out BeadXpress-based Test for Skeletal Dysplasia
  July 17, 2012 / BioArray News
- Luminex Looks to Target Mid- to Low-Volume MDx Labs with GenturaDx Platform
  July 12, 2012 / PCR Insider
- AmberGen Secures $600K to Develop Allergy Test on Illumina's BeadXpress Platform
  May 15, 2012 / BioArray News

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Exhibit 10

Complete NGS Target Enrichment in Less Than a Day
Agilent Technologies

At Goldman Sachs Confab, Illumina Predicts Future Growth in AgBio, Diagnostic Sectors

June 17, 2008
By Justin Petrone

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Around 30 percent of Illumina's revenues currently come from sales to commercial customers, but the company expects that portion to shift in five years to 40 percent of its revenue, due in part to growth in the agricultural biotechnology and molecular diagnostics segments.

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March 8, 2012 / BioArray News

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OGT Debuts Cyto CGH and SNP Array for Hematological Cancers; Plans More Oncology Chips
March 6, 2012 / BioArray News

Cleveland Clinic to Evaluate IntegraGen's Autism SNP Panel in 600-Patient Prospective Study
March 6, 2012 / BioArray News

As Marketing Push Drives Q4 Revenue Spike, CombiMatrix Plans Expanded Test Menu, Sales Force
March 6, 2012 / BioArray News

In this issue of BioArray News

1000 Genomes Project Could Fuel 'New Round of Chip Development,' Experts Say
1000 Genomes Project Could Fuel 'New Round of Chip Development,' Experts Say
At Goldman Sachs Confab, Illumina Predicts Future Growth in AgBio, Diagnostic Sectors
Almac's New Discovery Group Likely to Work with Sister Array-Based Dx Firm

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Narrowing the Focus
Scientists and policy-makers don't always see eye-to-eye.
March 12, 2012

Hollywood's Help
Celebrities are lending their fame to science, one blogger says.
March 12, 2012

The Microbiome's Role
The microbiome may be a driver of evolution.
March 12, 2012

Physicians, Patients Talk Genetic/MDx Tests
The LA Times reports on a new study that examines genetic and molecular diagnostic test spending in the US.
March 12, 2012

Papers of Note
People on the Move

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Exhibit 11

Amplify Single Cell DNA Samples, WGA
GenomePlex® Whole Genome Amplification
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SIGMA Where bio begins

Matt Dublin on
New algorithms

Illumina Aims to Have FDA-Cleared Dx, CLIA Lab In '09; CEO Says Arrays Have Dx Future

November 18, 2008
By Justin Petrone

The firm's first assay will run on its digital microbead-based BeadXpress reader, but the CLIA lab will offer both sequencing and array-based services, a sign that Illumina believes these two platforms may play a larger role in its diagnostics offering in the future.

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Q&A: Oxford's Dagan Wells on the Adoption of Arrays for Preimplantation Genetic Screening
May 10, 2011 / BioArray News

Agendia, AstraZeneca, NKI Partner to ID Colorectal Cancer Subtypes
May 10, 2011 / GenomeWeb Daily News

FDA Clears Affymetrix Gene Expression Reagents for IVD Use
May 10, 2011 / BioArray News

1. **GenomeWeb Daily News**
UPDATE: Report Suggests Big Payoff from Human Genome Project

2. **In Sequence**
Complete Genomics to Design New Generation of Sequencers; Reports Backlog of 2,100 Genomes

3. **GenomeWeb Daily News**
Fluidigm's Q1 Revenues Climb 30 Percent

4. **GenomeWeb Daily News**
CardioDx Raises $60M in Private Financing

5. **BioArray News**
Affymetrix to Launch Next-Gen Cytogenetics Array in Q3, Plans FDA Submission

In this issue of BioArray News

Illumina Aims to Have FDA-Cleared Dx, CLIA Lab In '09; CEO Says Arrays Have Dx Future

Illumina, OGT Betting Their New Chips Will Tap 'Huge' Cyto-Testing Market

Some Panomics Customers 'Surprised,' 'Concerned' About Affy's Impending Buy

Microarray-Related NIH Grants in Fiscal Year 2008, Sep. 16 — Nov. 3, Part II of II

Young Investigator Profile

Blog

Papers of Note

Influence of Mammalian TE Distribution on Gene Transcription
Zhang et al., PLoS Computational Biology
A trio of researchers at the British Columbia Cancer Agency and the University of British Columbia show that in both human and mouse, the "distributions of transposable elements reveal hazardous zones in mammalian introns." The researchers also report "several factors that likely contribute to whether a particular TE can influence gene transcription," they write, adding that "TEs in extremely close proximity — less than 20 bp — to exons show a strong bias to be near splice-donor sites."

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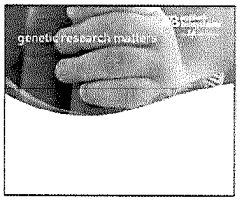

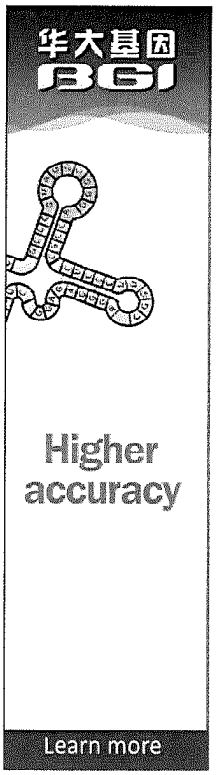

Tech Guide Archives

Real-Time PCR Technical Guide, Volume One

'Functional Complotype' and Susceptibility to Alternative Pathway-Driven Disease
Heurich, Martinez-Barricarte et al., PNAS
A team led by investigators at Cardiff University shows that "combining disease 'risk' variants in add-back assays yielded six-fold higher hemolytic activity compared with 'protective' variants" in their investigations of age-related macular degeneration. The team says its results "introduce the concept of a functional complotype — combination of polymorphisms — defining complement activity in an individual, thereby influencing susceptibility to AP [alternative pathway]-driven disease."

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- No, I've always only submitted however many proposals my research agenda has afforded.

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Science

A study published online in *PLoS ONE* this week underscores the genetic variation that can exist amongst individuals sharing the same self-reported ancestry — findings that the authors say have implications for understanding and applying genetic information in a personalized medicine context.

Business

PerkinElmer reported after the close of the market on Thursday a 14 percent jump in first quarter revenues year over year, as well as the acquisition of Geospiza. The deal supports the recent launch of its next-generation sequencing service and fits its shift toward a business model with greater emphasis on recurring revenues.

Funding

The European Cooperation in Science and Technology (COST) organization will receive €30 million ($44.5 million) in new funding from the European Commission. COST's Biomedicine and Molecular Biosciences programs support networks in all areas of genomics, proteomics, and metabolomics research.

Genome Technology Magazine

In this month's Q&A, *Genome Technology* speaks with the Whitehead Institute's Yaniv Erlich. Erlich recently published a study showing that blood-based DNA samples from discordant monozygotic twins might not always show distinct genotypes, and *GT* spoke with him to find out more.

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Illumina Unveils Strategy to Enter Molecular Diagnostics Market

January 21, 2009

Illumina Unveils Strategy to Enter Molecular Diagnostics Market

By Turna Ray

Illumina plans to enter the molecular diagnostics space by forging partnerships with customers, opening a new CLIA lab, and launching a research project to study cancer genomes, CEO Jay Flatley said during a recent presentation to investors.

Speaking at the JPMorgan Healthcare Conference in San Francisco last week, Flatley said the company will invest to improve existing sequencing technologies and develop new ones to enable it to play in the molecular diagnostics space, which he estimates to be worth $3 billion.

"We are really excited about what's happening in the sequencing market," Flatley said during his presentation. "We think over the next few years, it's going to be the most exciting segment in life sciences tools, so we continue to make major investments in this space."

As part of what Flatley called Illumina's "platform partnering" program, the company plans to work with customers to develop diagnostic applications using its existing BeadXpress genotyping platform and the sequencing technology it acquired last summer when it bought Avantome.

With the BeadXpress platform, Illumina has already begun partnerships to develop genotyping assays for blood typing, pharmacogenomics, and prenatal testing, Flatley said. Illumina hopes to garner clearance from the US Food and Drug Administration for the BeadXpress platform in the second half of the year.

Illumina has yet to commercialize the Avantome technology. Flatley did not update investors on the types of sequencing-based diagnostic tests Illumina plans to develop with the Avantome platform, or the partners Illumina is working with on this effort.

Additionally, Illumina plans to open its own CLIA-certified diagnostic laboratory, which will allow the company to introduce tests and testing services while waiting for the US Food and Drug Administration to clear test kits being developed under its partnering program.

He said the CLIA lab will also enable Illumina to offer services based on proprietary content from

its licensing and discovery programs, and to offer sequencing services for "traditional [genetic] targets" such as carrier testing for Rett syndrome; drug-resistance testing for HIV and Mycobacterium; mutation detection in the genes P53, KRAS, BRAF, EGFR; and HLA testing.

The company plans to apply for CLIA-certification during the first half of this year and hopes to start generating revenue from diagnostic services in the second half of 2009. An Illumina spokeswoman told *PGx Reporter* sister publication *In Sequence* this week that the company's primary interest for the CLIA lab is to provide sequencing services on its Genome Analyzer.

The last piece of Illumina's plan to enter the diagnostics space involves an internal discovery-research project to study ovarian and gastric cancer. To that end, the company plans this year to sequence approximately 50 cancer genomes and their controls, and to conduct whole-trascriptome and methylation-profiling analyses of these samples, Flatley said.

After validating the results in a larger number of samples, Illumina plans to implement diagnostic tests using these results on its array platform. Specifically, for ovarian cancer, Illumina aims to "identify very early markers for diagnosis" and to "begin to look at the genetics of therapy resistance, in particular resistance to platinum therapy," according to Flatley.

He said Illumina can embark on a project of this scale because "we can sequence so rapidly, and at such great cost points."

The decreasing cost of sequencing technologies has particularly impacted the nascent consumer genomics industry. Both 23andMe and Navigenics have said they plan to offer their customers whole-genome sequencing as the costs of this technology decrease.

Flatley told the JPMorgan conference that the molecular diagnostics market will grow from $3 billion to $5 billion by 2011, while the sequencing market will grow from $1.1 billion to $1.5 billion during that period. Illumina is betting that its sequencing know-how will enable it to win a slice of the broader molecular diagnostics space.

In this regard, Flatley said Illumina plans to launch several new products in the near term, including a sequencing add-on module, called Harmonia. The module works in concert with Illumina's iScan genotyping platform, set for launch in the second half of this year, and will be marketed to the company's genotyping customers who want to try sequencing technology.

Last week, as reported in *In Sequence*, Illumina announced it had made an $18 million investment into UK-based startup Oxford Nanopore Technologies. Oxford's nanopore sequencing technology "holds tremendous promise to be one of the first technologies to reach the sub-$1,000 genome and become the cheapest and fastest way to sequence DNA," Flatley said during his presentation (see *In Sequence* 1/13/2009)

— *Julia Karow, editor of* In Sequence, *contributed to this article.*

Related Stories

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  November 12, 2013 / GenomeWeb Daily News
- Empire Genomics Clinical Lab Gets CLIA Certification, New York Approval
  November 12, 2013 / GenomeWeb Daily News

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  November 11, 2013 / Clinical Sequencing News
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  November 8, 2013 / Clinical Sequencing News
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Exhibit 13

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Illumina: Revolutionizing Healthcare through Personalized Medicine and Genomics

Date:

Wednesday, September 30, 2009 - 6:00pm - 9:00pm

Pricing:

Event Overview: With a current market capitalization of around $4B, Illumina is one of the handful of players working towards making personalized medicine a reality. The vision of personalized medicine—"the right drug for the right patient at the right time" will offer an entirely new approach to medicine, including more accurate assessments of disease risk, better predictions of response to treatment, and safe, more effective treatments. This research will lead to better health care for all patients and long-term savings in the cost of health care.

John Stuelpnagel (co-founder and former CEO) and Jay Flatley (current President & CEO) will share their experiences in conceiving of and executing Illumina's formation and subsequent transformation to a world-class player revolutionizing healthcare.

Illumina was founded in April 1998 by John Stuelpnagel, David Walt, Larry Bock, Anthony Czarnik and Mark Chee and completed its IPO in July, 2000. Illumina currently has about 1500 employees. From FY 2005 to 2008, Illumina's revenue grew approximately ten-fold to about $570 million through strategic acquisitions of organizations such as Solexa and Avantome, in-licensing of novel technologies, and by developing and launching innovative products and services.

Illumina develops, manufactures and markets integrated systems and services for the large scale analysis of genetic variation and biological function. Illumina enables researchers to correlate genetic variation and biological function to advance the field of personalized medicine e.g. drug discovery and clinical research, allow diseases to be detected earlier and permit better choices of drugs for individual patients. Illumina recently launched personalized genome sequencing services.

Speakers

Jay Flatley, President & CEO, Illumina, Inc.
John Stuelpnagel, Co-founder & former CEO, Illumina, Inc.

Program Details

09/30/2009 06:00 PM Registration & Networking
09/30/2009 06:45 PM Indian Dinner & Networking
09/30/2009 07:15 PM Soapbox Pitch
09/30/2009 07:20 PM Speakers
09/30/2009 08:30 PM Question & Answer Session
09/30/2009 09:00 PM Event Ends

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Exhibit 14

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Monday,

Illumina Starts Validating Ovarian, Gastric Cancer Genes from Discovery Project; Adds Colorectal Cancer

January 18, 2011

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By Julia Karow

This article was originally published January 13.

As part of its Cancer Discovery Initiative, Illumina is validating genes discovered by sequencing ovarian and gastric cancer samples in larger sample sets and expects to have results for ovarian cancer in the latter half of the year, according to a company executive. It has also embarked on a third cancer type, colorectal cancer.

The aim of the initiative, which Illumina launched two years ago (*IS 1/20/2009*), is to use sequencing to discover biomarkers for early disease detection and to predict response to therapy or relapse. Eventually, the firm wants to develop new diagnostics on the BeadXpress platform. According to its website, Illumina is partnering with "leading cancer research institutions" for the project.

The company chose to tackle ovarian and gastric cancer first, where survival rates are poor if the disease is detected late. Also, for ovarian cancer, most diagnoses are currently made at a late stage. The aim is to discover biomarkers for early detection and to predict resistance to platinum therapy. Last fall, Illumina reported that it had completed sequencing the genomes, methylomes, and transcriptomes of 25 tumor/normal ovarian cancer samples (*IS 11/2/2011*), and had found about 40 "significant genes," half of which had not previously been implicated in the disease. It had also completed sequencing 25 tumor/normal gastric cancer samples.

At the JP Morgan Healthcare Conference in San Francisco last week, Illumina CEO Jay Flatley said that the company is currently validating the ovarian cancer genes — along with some additional related genes — by targeted sequencing in 300 samples. The project is expected to be completed mid-year, and in the second half of the year, "we'll determine what's come out of this cancer discovery activity," Flatley said.

For gastric cancer, he said, company researchers have discovered three "very important genes," and are beginning to validate those in a set of 250 samples.

The company has also selected colorectal cancer as a third cancer type to investigate. Half of colorectal cancers that are treated with surgery alone relapse, according to the firm's website, and therapies selected by stage of diagnosis are not well associated with the likelihood of response. The goal, thus, is to discover biomarkers for disease prognosis and relapse. "Sequencing on those cancer samples is just now beginning," Flatley said. He did not mention the number of samples to be analyzed.

In this issue of In Sequence

Illumina's Low-Cost MiSeq Promises to Speed up Next-Gen Sequencing

Two Sequencing-Based Prenatal Tests Make Strides Toward the Clinic

Illumina Passes Terabase Milestone for HiSeq; Customers to Reach 600 Gb per Run this Spring

Acquisition of Epicentre Gives Illumina Exclusive Access to Nextera Sample-Prep Method

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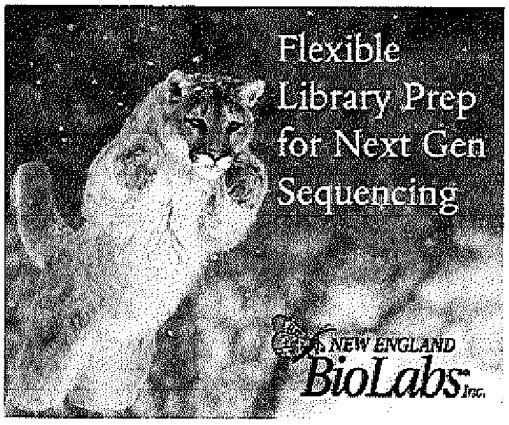

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People on the Move

David Botstein, **Eric Lander**, and **Craig Venter** will share the 2012 Dan David Prize, and will split the $1 million prize the award carries.
The three winners have been recognized for their contributions to the Human Genome Project and an array of genomics research efforts, from developing microarrays and DNA sequencing technologies to computational genomics and SNP mapping, and launching labs, companies, and institutes that have driven cutting-edge genomics.
Botstein currently is a director of the **Lewis-Sigler Institute for Integrative Genomics** at **Princeton University**. Venter is founder, chairman, and president of the **J. Craig Venter Institute**, as well as the **Institute for Genomic Research**, and **Celera Genomics**. Lander is founding director of the **Broad Institute of Harvard and MIT** and director of its

ILLUM-0608

According to the website, if validation pans out, Illumina plans to offer diagnostic services for ovarian cancer in its CLIA lab. Ultimately, it wants to run prospective trials on these markers and gain regulatory clearance for diagnostics developed on its BeadXpress platform, which received 510(k) clearance from the US Food and Drug Administration last year.

Have topics you'd like to see covered in *In Sequence*? Email the editor at jkarow [at] genomeweb [.] com.

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Clinical Genome Sequencing Requires Better Standards for Accuracy, Completeness, NHGRI Team Says
August 24, 2011 / Clinical Sequencing News

Illumina, Helicos Platforms Offer Distinct Advantages for Ancient DNA Sequencing, Danish Group Says
August 23, 2011 / In Sequence

Illumina and University of Oxford to Sequence 500 Human Genomes to Inform Diagnosis, Treatment
August 3, 2011 / Clinical Sequencing News

Illumina Books Orders for More than 135 MiSeqs, Starts Early-Access Shipments
August 2, 2011 / In Sequence

New Products
July 19, 2011 / In Sequence

Genome Biology Program.

Jorge Garces has joined **GenMark Diagnostics** as senior vice president of R&D, the company said recently. Garces most recently served as CEO and president of **Enigma Diagnostics**' US business. Prior to that, he was VP of R&D and site operations manager at **Hologic**. He has also held positions at **Third Wave Technologies**, **Genzyme Genetics**, and **Athena Diagnostics**.

Combimatrix this week said it has named two San Diego area executives, **Richard Ding** and **Joseph Limber**, to seats on its board of directors, and that former President and CEO **Amit Kumar**, as well as **Rigdon Currie** and **John Abeles**, will not be candidates for reelection at its next stockholders meeting.
Ding is CEO of **BioTheranostics**, a molecular diagnostics test developer and lab, and formerly was VP of strategy and business development at **BioMerieux**. Limber is president and CEO of **Prometheus Laboratories**, a pharmaceutical and diagnostics company focused on personalized medicine. He also previously was president and CEO of **Aclara BioSciences**, subsequently named **Monogram Biosciences**.

Upcoming Events

Science	Business	Funding	Genome Technology Magazine
Investigators from the Wellcome Trust Sanger Institute, the University of Cambridge, and elsewhere used exome sequencing to reveal the compound inheritance patterns behind an autosomal recessive condition called thrombocytopenia with absent radii, or TAR, syndrome. By underscoring the potential importance of non-coding, regulatory regions in the genome, the work may also inform studies of other conditions.	Affymetrix said that it has released eBioscience from certain obligations under the terms of their proposed merger as the Santa Clara, Calif.-based array firm continues in its efforts to salvage the $330 million deal. Affy said in a Form 10-K filed with the SEC that it is in discussions with financing sources and eBioscience about restructuring a financing agreement that could support the deal, but no agreement has been reached.	Research councils from 22 participating countries are providing roughly $13.5 million in funding to support the BIOMARKAPD program, a multinational collaboration aimed at improving and harmonizing methods for using molecular biomarkers for neurodegenerative disorders, such as Alzheimer's and Parkinson's disease. The consortium, led by the Karolinska Institute, will also support projects to identify new markers.	Using a MeDIP-seq approach to study an unsequenced species, the University of Birmingham's Kevin Chipman and his colleagues identified a potential link between environmental pollutants, epigenetic changes, and cancer in a fish called the common dab. In addition to finding epigenetic changes in the tumors themselves, Chipman and his team saw changes to the methylation profiles of DNA in surrounding tissue as well, which suggest a few ways that tumor-associated changes could occur.

ILLUM-0609

Exhibit 15

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genomeweb

Illumina Sees Clinical Applications for MiSeq; Plans FDA Submission in 2012

August 03, 2011

Illumina Sees Clinical Applications for MiSeq; Plans FDA Submission in 2012

Citing an example of clinical applications for the MiSeq, a company representative said that one customer plans to use the instrument for MRSA testing in a hospital.

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- IOM Workshop Discusses Interim Strategies to Handle Unresolved Challenges of Clinical Sequencing
  July 27, 2011 / Clinical Sequencing News
- Sequenom Signs Supply Deal for T21 Test with Illumina; Firms to Collaborate on Regulatory Approval
  July 12, 2011 / Clinical Sequencing News
- Illumina to Apply Rare-Cell Capture to Ovarian Cancer Program; Expects Clinical Apps for MiSeq
  May 4, 2011 / Clinical Sequencing News
- Illumina Starts Shipping 600 Gb HiSeq Reagent Kits; Takes First Orders for MiSeq
  May 3, 2011 / In Sequence
- Following Diagnostic Sequencing Success, MCW Creates Comprehensive Framework to Guide Future Cases
  March 29, 2011 / Clinical Sequencing News

ILLUM-0660

Exhibit 16

YAHOO! FINANCE

Illumina Names Dr. Daniel S. Grosu Chief Medical Officer

As New Medical Point Person for Regulators, Grosu to Provide Medical Perspective to Project Teams, Work Closely with Illumina Clinical Services Lab

Business Wire - Mon, Oct 31, 2011 6:00 AM EDT

SAN DIEGO--(BUSINESS WIRE)-- Illumina, Inc. (NASDAQ:ILMN - News) today announced that it has appointed Dr. Daniel S. Grosu, M.D., as Vice President and Chief Medical Officer. In this newly created position, Dr. Grosu will play a key role in building Illumina's diagnostic capabilities through collaborations with regulatory agencies, internal product development teams, and Illumina's CLIA-certified clinical laboratory.

"Dr. Grosu brings the experience, credentials, and intellectual rigor required to move Illumina's cutting-edge technology into the clinic," said Illumina's Gregory Heath, Senior Vice President and General Manager, Diagnostics. "As Chief Medical Officer, he will assist in aggressively driving our business towards this enormous new market opportunity."

"During the next few years, we will see tremendous advances in medicine driven by technological breakthroughs in genomics and related systems biology areas," said Dr. Grosu. "These developments will bring us fully into the long-awaited era of personalized medicine, and Illumina is ideally positioned to be both a key enabler and a key player in this historic process. I am delighted to join Illumina at such an exciting time for our industry."

Prior to joining Illumina, Dr. Grosu was Group Director, Clinical Science, Clinical and Medical Affairs at Ortho-Clinical Diagnostics. He was also previously the Director and Global Clinical Leader, Global Clinical Development, Diagnostic Imaging at Bayer Healthcare Pharmaceuticals. Prior to that, Dr. Grosu was at Siemens Medical Solutions. He holds bachelor's and master's degrees from Wheaton College in Wheaton, Ill., a medical degree from Missouri's Saint Louis University, and a master's degree in business administration from the University of Oxford.

About Dr. Grosu's Compensation

Illumina will grant an option to purchase 60,000 shares of Illumina's common stock and 6,700 restricted stock units (RSUs) to Dr. Grosu as part of his inducement to join the company. An RSU represents a conditional right to receive one share of our common stock at a specified future date. The options and RSUs were approved by Illumina's compensation committee pursuant to NASDAQ Marketplace Rule 5635(c)(4) and will be granted under Illumina's New Hire Stock and Incentive Plan, which was approved by Illumina's Board of Directors on January 29, 2008. The option will have the following terms: an exercise price equal to the closing fair market value of Illumina's common stock on the grant date, a ten-year term, and vesting over four years with 25 percent of the option vesting one year from the grant date and 1/48th of the option vesting monthly thereafter. The RSUs will vest over four years with 25 percent of the RSUs vesting on each of the first four anniversaries of the grant date. The grant date for the option and RSUs to Dr. Grosu will be his start date with Illumina, which is expected to be October 31, 2011.

About Illumina

Illumina (http://www.illumina.com) is a leading developer, manufacturer, and marketer of life science tools and integrated systems for the analysis of genetic variation and function. We provide innovative sequencing and array-based solutions for genotyping, copy number variation analysis, methylation studies, gene expression profiling, and low-multiplex analysis of DNA, RNA, and protein. We also provide tools and services that are fueling advances in consumer genomics and diagnostics. Our technology and products accelerate genetic analysis research and its application, paving the way for

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molecular medicine and ultimately transforming healthcare.

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Exhibit 17

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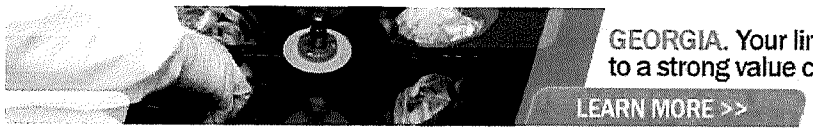

IVDTInsight

A blog from the editors of IVD Technology

Siemens, Illumina Partner on Infectious Disease Testing Using Next-Generation Sequencing

Posted: November 2, 2011

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Siemens Healthcare Diagnostics and Illumina have entered into a partnership aimed at setting new standards in the use of next-generation sequencing for the rapid, accurate identification of patients' infectious disease states and potential treatment paths. Through this agreement, the companies plan to make existing Siemens molecular HIV tests compatible with the recently launched Illumina MiSeq next-generation sequencing platform, with the ultimate goal of introducing breakthrough sequencing-based infectious disease assays for the clinical diagnostics market.

"Next-generation sequencing is a transformational technology that we believe will significantly impact clinical diagnostics over the next five years," said Michael Reitermann, CEO, Siemens Healthcare Diagnostics. "Our partnership with Illumina brings together two innovation leaders to set a new standard of care in the next wave of clinical diagnostics and personalized medicine."

Next-generation genome sequencing (DNA sequencing) is the determination of the precise sequence of nucleotides in a sample of DNA. These data points help provide physicians with deeper insights into patients' genetic characteristics, including suitability to drug regimens based on the genetic profile of an infectious disease; source of infection and insights into treatment directions; and personalized medicine status – both current disease state and susceptibility to future disease. According to a recent report in Genetic Engineering & Biotechnology News, Scientia Advisors estimates the next-generation sequencing market could reach $1.5 billion in sales by 2014, with a broader picture putting that figure closer to $3.6 billion.

Ten years ago, the Trugene HIV-1 Genotyping Assay, the first DNA sequencing-based test for HIV to be cleared by FDA, was launched to a worldwide market. This laid the foundation for Siemens to become a leader in infectious disease testing solutions that employ DNA sequencing technology. Since then, Trugene has become one of the market's leading DNA sequencing tests for infectious disease testing. By making its Trugene test compatible with Illumina's MiSeq analyzer, Siemens expects to be well positioned to help even more clinical laboratories leverage next-generation sequencing for their infectious disease testing with the fastest turnaround time and highest accuracy possible.

"Partnerships with global leaders like Siemens are a key element of our strategy to drive widespread adoption of MiSeq in the clinical setting," said Jay Flatley, CEO of Illumina. "Siemens has a long track record of success in developing regulated products for infectious disease testing, and we are excited to join them in taking next-generation sequencing to this important market."

-Richard Park

Ivdt insight blog Tags: Business News, Collaboration, Infectious Diseases, Siemens

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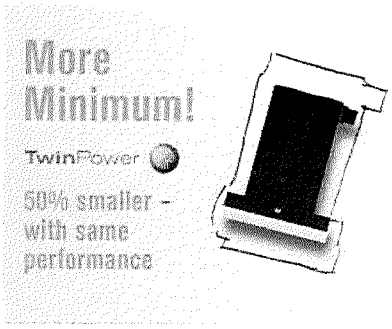

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Exhibit 18

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Q&A: Matt Posard on Illumina's Clinically Focused Business Unit

March 14, 2012

Q&A: Matt Posard on Illumina's Clinically Focused Business Unit

Name: Matt Posard
Title: Senior Vice President and General Manager, Translational and Consumer Genomics, Illumina
Experience: Vice President of Global Sales, Illumina, 2007-2012;
Vice President of Worldwide Marketing, Illumina, 2006-2007;
Regional Sales Director, Biosite, 2003-2006
Education: University of California, San Diego, BA in Quantitative Economics and Decision Making

In January, Illumina launched its Translational and Consumer Genomics unit, which will focus on services, the consumer market, CLIA certification and regulation, and business-to-business, with a particular focus on the clinical sequencing market (*CSN 1/11/2012*).

Illumina has said that clinical and commercial customers are increasingly making up a larger portion of its customer base, and that sequencing will become a core technology in molecular diagnostics (*CSN 2/8/2012*).

Recently, *Clinical Sequencing News* visited Illumina's headquarters in San Diego and spoke with Matt Posard, who is heading up the new unit, on its focus and goals, as well as the direction he think the clinical sequencing market is headed, and the remaining challenges for implementing sequencing in the clinic.

What is the goal of the Translational and Consumer Genomics business unit?

The mission really is to enable genomics-based healthcare. The way we intend to do that is not just look at what we sell as an instrument and a set of consumables, but [also] the report that a physician is going to look at. So our primary customer, if you will, is going to be clinical geneticists as well as pathologists because it's those groups that will ultimately sign off on the report.

The product is essentially that report, and we view our responsibility as making sure we provide the complete solution, so that the clinical geneticist and pathologist can sign off on that report

confidently and in as short a period of time as possible.

How are you developing the report?

There really will be three different categories of what will be ordered by clinicians. We think there will be fixed panels, we think there will be exomes, and we think there will be genomes. I think market adoption will essentially be in that order. There will be groups that will be interested in each of those three categories, but what we're hearing from the medical pathologists is that the largest demand — at least for the next year or two — is going to be in fixed panels. So, Illumina will be developing those panels in collaboration with clinical and academic centers. You'll be hearing more about that in months to come.

Through those panels we also intend to partner with teams that have already incorporated clinical reporting infrastructures for genomics. So, rather than have a black box reporting tool, we're going to adapt to the tools that are already out there.

The [TruSeq Amplicon – Cancer Panel] that we just [launched] is a research [use] only product. ... But generally speaking, to perform these panels as [laboratory-developed tests], customers have to custom-design the content. We don't plan on selling a fully-kitted product for translational use. That's really intended for research use.

What will the clinical products look like then?

Generally, the clinical products are going to be narrower in content than the research products. And the reason is that each institution has its own criteria on what they're going to report clinically. You have some institutions that are only going to report what's in the guidelines — the 23 genes in cystic fibrosis, for example. You'll have others that will only report what's reimbursed. You'll have others that will be a little bit more liberal in the content they provide their physicians, but it has to be confirmed pathogenic genes.

Then, at the other end of the spectrum you have these academic centers that will go all the way to the point where there's some publication-based association with the disease, in which case they'll say [the variant is] likely pathogenic. Each institution obviously has its own criteria and physician base that will help determine that. Our approach is to offer the genes of interest for each individual consumer.

So, each institution or customer would essentially design its own targeted gene panel?

Right, but there is a lot of support that's necessary that we think differentiates this business unit from other options on the market as well as from the research user. We will do all the optimization work for customers for these various panels up front. We'll do all the design work to optimize A to Z. And then customers can order whichever of the letters from A to Z for their specific panel.

Do you anticipate that these customers would be doing the sequencing in house, and then you'd be providing the interpretation? Or would you also be doing the sequencing?

We see the market as being bifurcated into two groups. In the first group are the people who want to bring the technology in house and do their own testing.

The second area of the market [will be users] who, for various reasons, just want to outsource

their samples. They'll either outsource them to reference labs, [to] which we are marketing to offer our products, or they're going to send them to Illumina. And our primary offering that we intend to continue to evolve out of CLIA is whole-genome sequencing. For whole genome what we'll be offering is today's rigorous QC that allows us to run it under CLIA and report the results, but we'll evolve it to include technical as well as clinical annotation services, so full interpretation of what those genome results are.

Where we see this evolving over the next several months is [to] something that takes the onus off the end user to interpret that genome. We'll do the bulk of that interpretation for the end user.

How are you developing the clinical interpretation pipeline? Is this something you're doing in house or are you collaborating with outside groups?

This requires an external evolution, which is happening very quickly, on biological annotation and clinical annotation. So all the public databases of the world essentially have individuals that can populate various variants that they find and show biological and clinical correlation to various diseases and responses to therapies. Then, from an in-house point of view, we have to automate that. We have to keep it updated and pull that information on a timely basis and scale it.

That's something we're taking on at Illumina — developing it internally and taking on solutions from other companies that have developed these systems. [We want something] that makes sense for us so that we can scale when the market gets to the size we expect it to be pretty shortly, which is definitely going to be thousands and thousands of clinical genomes.

How do you see the market for clinical sequencing growing?

Everybody has their own crystal ball, but ... while the number of years that they're looking out may vary — some people would say maybe a few years, others would say five years — everybody expects genomic-influenced healthcare to be based on whole genomes. And we know some very large institutions and some very large entities that are already formulating plans to sequence babies at birth. We're also talking to oncologists and cancer centers that are putting plans in place to ensure that every cancer patient is sequenced during the course of their therapy.

I believe that clinical sequencing for all cancer patients will be seen as standard of care. I think for my children and their generation, particularly, for them and for their kids, clinical sequencing and whole-genome [sequencing] are going to become standard of care.

How have the physicians and pathologists that you've worked with responded? Are they receptive of the technology?

It's really dangerous to generalize, but I would say like all large groups, you have early adopters and you have late adopters. Based on feedback from those we interface with regularly as well as from the American Molecular Pathology group, next-generation sequencing is definitely grabbing the majority of mindshare among that group at levels they've never seen before. So, whether an early adopter or a late adopter, I think everybody's convinced it's coming and [is] extremely interested to see how it actually evolves. [Next-gen sequencing] has had incredible success in the research community, but how it actually makes that leap to the clinical community [is a concern].

So that's a long answer. The short answer is that everybody is prepared and excited about it

coming, but wants to make sure that it's reimbursed, [and that] the quality standards are consistent with a clinical as opposed to a research laboratory.

[Another concern] is, frankly, what I would say is the rate of change. What's made next-gen sequencing so popular in the research community has been in large part its rate of change, but change in a clinical community, if not managed, is very intimidating and generally seen as a negative. So stability and manageability, if you will, of this amazing technology is going to be really critical.

How important are collaborations in bringing sequencing-based diagnostic assays to the market?

Clinical collaborators are very important. They are the subject matter experts. Illumina may be the technology and the instrument expert, but translating that technology into something that actually impacts patient care positively is only something you can find from a clinical collaborator.

The technology also tends to be received faster and have more credibility by customers if they know that the product was developed in collaboration with a medical institution that has a lot of respect.

Where are you in discussion with the US Food and Drug Administration on obtaining 510(k) clearance for the MiSeq, and how has your experience with clinical sequencing informed your discussions?

We've submitted a pre-[investigational device exemption] for the MiSeq and are working with the FDA toward getting that into a clinical trial and ultimately 510(k) cleared. Our diagnostic business unit is in routine discussions with the FDA to ensure that we get the proper labels for the various products that we've been talking about.

And I think that, to their credit, the FDA has been amazingly involved and supportive of next-gen sequencing. They obviously want to make sure that the supply companies get it right. And it's going to shatter some old paradigms and I think create some new standards by which these technologies are evaluated.

For the MiSeq to get 510(k) cleared it will be done with a specific assay. We haven't publicly announced that yet, but there will be a specific assay that goes with that and then all the following assays on top of that, which will be panels.

What are the remaining major challenges to adopting next-gen in the clinic?

For any new diagnostic or technology to be accepted it really has to address the following: it must have compelling clinical utility; clinical and economic outcomes have to be established; and reimbursement has to be established and routine.

A lot of these technologies, including whole-genome sequencing, have been demonstrated to be reimbursed but it's early days yet to determine how long it will take to be standard of care for a reimbursed product or a reimbursed diagnostic nationwide or routinely.

Coupled with that, it has to be something that fits within a clinical laboratory. So whether someone wants to do the testing themselves or outsource it, there has to be ease in ordering that product or diagnostic, relative speed in getting results back, and interpretation that results in

clinically relevant decisions. All of that has to be established in a protocol that is reproducible for other users as well as scalable.

Do you think that whole-genome sequencing will always be physician-mediated, or will it evolve to a point where a consumer can just go and get his or her genome sequenced?

I think it depends on ... what we're calling the consumer. If it's someone that's struggling with an undiagnosed disease, it's always going to go through a physician, without question. If it's somebody that's healthy but just being proactive about understanding genomics or their own DNA, which I would say is more a curiosity angle and more educationally driven, then there are certainly products on the market today on the genotyping side that allow those people to explore.

And for somebody to do that on their own genome, while they couldn't get a CLIA-based genome without going through a physician, I think in the future there will be opportunities for people to explore their own DNA and their entire genome without it having clinical consequences for that patient today because they're healthy.

The risk or the responsibility that's on Illumina and the other providers is [that], when somebody is exploring their own genome, they will find markers that have risk predisposition for different diseases. Making sure those results are provided responsibly and, in some cases, with professional counseling or support to help that individual through that information is the responsibility of the provider of those products.

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Exhibit 19

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Illumina in Discussions with 100 Potential Clinical Customers for MiSeq, Plans Cancer Diagnostics

April 25, 2012

By Monica Heger

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Over half of the orders taken for Illumina's MiSeq instrument are for clinical customers and the company is in active discussions with more than a hundred potential customers looking to use the instrument for diagnostics, CEO Jay Flatley said this week during a conference call to discuss the company's first-quarter earnings.

"The clinical market is emerging very rapidly and will be a key story for 2012," he said. "We believe clinical sequencing is nearing an inflection point."

While Flatley did not discuss the number of MiSeqs sold in the quarter, he said that the instrument is well suited for clinical applications, such targeted cancer panels, and over half of MiSeq orders are coming from clinical customers.

Nevertheless, he said that commercial diagnostic customers such as Sequenom and Genomic Health are using the company's HiSeq for clinical purposes and that going forward those customers would be significant drivers of HiSeq consumable revenue.

Of Illumina's current commercial clinical customers, "Sequenom is leading the pack," Flatley said.

Sequenom launched its sequencing-based MaterniT21 prenatal test last year. The company said last week that it had sold more than 4,900 MaterniT21 Plus tests during the first quarter of 2012 and increased its full-year sales goal to 40,000 from a previous target of 25,000 (*CSN 4/18/2012*).

Sequenom also said last week that it had extended its supply agreement with Illumina from three years to five years, ending in July 2016.

Clinical Products and Services

Flatley said that Illumina is tackling the clinical market on a number of fronts.

The company's Translational and Consumer Genomics business unit, created in January (*CSN 1/11/12*), is focused on "the top CLIA labs" both inside and outside of the US, he said. This group focuses on instrument and reagent sales to diagnostic companies like Sequenom and Genomic Health, as well as the company's partnership with Siemens to develop an HIV assay on the MiSeq (*CSN 11/9/2011*).

"[In] that segment, we're very well set up," Flatley said.

One area that is not so far along is developing *in vitro* diagnostic products based on the company's own proprietary content, Flatley said.

As he explained previously, the company plans to develop products that it takes through FDA clearance within its molecular diagnostics unit, headed up by Greg Heath (*CSN 2/8/2012*).

The products would be based on biomarkers that it has discovered through its ovarian, gastric, and colon cancer sequencing initiative (*IS 1/18/2011*).

In this issue of Clinical Sequencing News

Illumina in Discussions with 100 Potential Clinical Customers for MiSeq, Plans Cancer Diagnostics

Life Tech to 'Capitalize' on Existing Instrument Base to Expand MDx Business

Q&A: Illumina's Tina Hambuch on Clinical Whole-Genome Sequencing and CLIA Validation for NGS

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Papers of Note

Whole-genome haplotype reconstruction using proximity-ligation and shotgun sequencing
Selvaraj, Dixon, *et al. Nature Biotechnology*
Meanwhile, in *Nature Biotechnology*, a team from the University of California, San Diego, and the Ludwig Institute for Cancer Research publish details on a new approach for whole-genome haplotype reconstruction called HaploSeq. Using proximity ligation and sequencing, the investigators showed that alleles on homologous chromosomes occupy distinct territories, and they were able to preferentially recover physically linked DNA variants on a homolog. Computational analysis of such datasets allows for accurate reconstruction of chromosome-spanning haplotypes for around 95 percent of alleles in hybrid mouse cells with 30x sequencing coverage, the researchers say. To resolve haplotypes for a human genome, they combined HaploSeq with local conditional phasing to obtain haplotypes for about 81 percent of alleles with approximately 98 percent accuracy from just 17x sequencing.

Demographic divergence history of pied flycatcher and collared flycatcher inferred from whole-genome

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He did not elaborate on the types of products it plans to develop from its cancer sequencing efforts, but said that he would provide more information in three to six months.

The aim of this effort would be to sell "more broadly to oncologists and physicians," Flatley said — a goal that would require the firm to "supplement our distribution," either by "building it ourselves or potentially through an acquisition."

He added that the company is interested in transactions with diagnostic companies, such as a merger, acquisition, or partnership, when the time is right to help accelerate its "penetration into the [diagnostic] market, either with products, distribution, reimbursement, or all of the above."

Flatley's comments came a week after Illumina successfully fought off a hostile takeover bid from Roche, which had highlighted the clinical promise of the company's sequencing technology as a key selling point.

Illumina has said that it intends to submit the MiSeq to the US Food and Drug Administration for 510(k) clearance, and Flatley reiterated this week that the company is still on track to do that by the end of the year.

So far, in conjunction with Siemens, Illumina has submitted a pre-IDE application for the HIV assay the companies are co-developing. Additionally, he said Illumina "is working on some internal assays" for the MiSeq that it plans to submit to the FDA this year as well.

Front and Back Ends

Aside from diagnostic assays, Flatley indicated that Illumina would be developing a number of other clinically focused products both on its own and together with partners in the areas of sample prep, analysis, and interpretation.

It is developing a PCR-free sample prep protocol that it has already begun to roll out in its services business, as well as sample prep for low-input formalin-fixed paraffin-embedded samples. It plans to launch both the PCR-free sample prep and FFPE sample prep as kits, likely next year.

Additionally, Flatley said that its recently developed software aligner Isaac, will increase genome analysis four- to six-fold and reduce compute requirements from a Linux cluster to a single server.

Illumina has also "nearly completed" an annotation project that aggregates data from multiple publicly available databases, to offer "simple push-button analysis for clinical interpretation," he said.

Combining the sample-prep and analysis improvements with sequencing on the HiSeq 2500 "yields a full workflow allowing clinical genomes to be prepped, sequenced, and interpreted in less than three days," he added.

Also on the interpretation front, Illumina this week launched MyGenome, an application for Apple's iPad that enables users to view a demonstration human genome, learn about possible health implications, and view reports on different disease-associated genetic variants.

Illumina plans in the future to release a version that links to its website, "allowing customers to download and view their own genome once they have been sequenced via Illumina's physician-mediated individual sequencing service," Flatley said.

The company plans to use the app to deliver genetic data to ordering physicians, who will use it to visualize the genome and interrogate it for issues such as Mendelian disorders and pharmacogenomic drug response, before providing direct access to the consumer.

Financials

While Illumina's overall revenue fell 3 percent to $273 million in the first quarter of 2012 from $283 million in the first quarter of 2011, sequencing revenues grew by an undisclosed amount, driven by record orders for the MiSeq and a 20 percent increase in sequencing consumable revenues, Flatley said.

Product revenues, including instruments and consumables, were $255.6 million, down 4

re-sequencing data
Nadachowska-Brzyska, Burri, *et al. PLOS Genetics*
Swedish researchers used a combination of computational approaches to assess past divergence patterns between the pied flycatcher and the collared flycatcher — work they present in *PLOS Genetics*. The team considered more than a dozen different demographic models in an effort to understand the bird species' split, incorporating whole-genome re-sequencing data for 20 birds. Results of the analysis pointed to relatively recent divergence between the pied flycatcher and collared flycatcher, supporting the notion that avian speciation may occur more quickly than previously suspected. The work also picked up on population declines in bird populations following this split, study authors say, along with gene flow into collared flycatcher populations from the sister species.

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Are you surprised that the results of some 30 percent of registered clinical trials are never published?

- Yes. Such findings, even negative ones, could help patients.
- Yes. Clinical trials are supposed to benefit society.
- No. Negative results are rarely published.
- No. I'm surprised it's not higher.
- I'm of two minds.

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percent from $266.7 million in the first quarter of 2011, while revenue from the company's sequencing services business increased 8 percent to $17.1 million from $15.8 million last year.

Research and development expenses for the first quarter of 2012 were $48.8 million compared to $50.2 million in the first quarter of 2011. Selling, general and administrative expenses for the first quarter of 2012 were $68 million compared to $65.7 million for the first quarter of 2011.

Additionally, Illumina incurred $8 million in expenses associated with Roche's unsolicited acquisition bid, and $2.6 million in restructuring charges.

It ended the quarter with $1.3 billion in cash, cash equivalents, and short-term investments.

Illumina's board authorized the company to repurchase up to $250 million of outstanding common shares.

Monica Heger tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Monica Heger or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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Illumina Acquires Moleculo, Pre-announces Q4 Revenue Growth of 8 Percent
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Macrogen Expands Sequencing Fleet in Preparation for Clinical Sequencing Service Launch
March 28, 2012 / Clinical Sequencing News

Illumina Sees Growing Commercial Customer Base; Doubled Clinical HiSeq Placements in 2011
February 8, 2012 / Clinical Sequencing News

Science	Business	Funding	GenomeWebinars
Members of the International Genomics Alzheimer's Project consortium brought together data for tens of thousands of individuals with or without late-onset Alzheimer's disease, and through GWAS and meta-analyses of that data tracked down 19 risk loci for the neurodegenerative condition, including 11 not found previously. The new candidate genes were detected in amyloid and tau protein-related pathways and pathways involved in immune response, inflammation, cellular migration and transport processes.	Illumina signed a definitive agreement to acquire clinical software firm NextBio, which provides platforms to aggregate and analyze large amounts of phenotypic and genomic data for research and clinical applications. Illumina plans to combine its BaseSpace cloud computing environment for next-generation sequencing data with NextBio's platform for integrating patient data. Illumina will integrate NextBio into its Enterprise Informatics business and will retain NextBio Co-founder Ilya Kupershmidt and CTO Satnam Alag.	The Carlos Slim Foundation has pledged $74 million to fund the second phase of a genomics-focused collaboration between the Broad Institute, Mexico's National Institute of Genomic Medicine, and the Carlos Slim Health Institute. In this second phase of the collaboration, researchers plan to develop diagnostic tools for breast cancer and diabetes, complete their genetic analyses of these diseases, develop therapeutic roadmaps to guide the development of new treatments, and launch "a full-scale effort" to target the MCKD1 gene.	Is Next Generation Sequencing Accelerating Your Cancer Drug Development and Clinical Trial Programs? Sponsor: Asuragen This webinar was recorded Oct. 3. GenomeWeb and Asuragen invite you to view a webinar discussing the use of next-generation sequencing to illuminate druggable targets in oncology while addressing the limitations in DNA quality and yield from formalin-fixed specimens. Register here to view the playback or download the recording.

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Exhibit 20

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Illumina Sees Diagnostics as Largest Growth Opportunity; Dx Strategy to Focus on Cancer

June 20, 2012

Illumina Sees Diagnostics as Largest Growth Opportunity; Dx Strategy to Focus on Cancer

By Julia Karow

Illumina sees the clinical market as its largest opportunity for growth and will focus its diagnostic strategy on cancer, a company representative said last week.

"What we expect to be the largest market, and the most important market for the company over the next decade, is the emerging clinical and translational market," said Christian Henry, Illumina's senior vice president and general manager of genomic solutions, during a presentation at the William Blair Growth Stock Conference, which was webcast. "The diagnostics opportunity is by far the most significant opportunity."

While genetic diagnostics is about a $6 billion market today that is "focused heavily" in the area of infectious disease, he said, it could grow to $23 billion by 2020, driven by infectious disease, cancer, and reproductive medicine, with contributions from pharmacogenomics.

Illumina's goal will be to develop a diagnostic strategy to take advantage of this market opportunity, Henry said. "The focus for us will be primarily in cancer, because we think it's the most complicated [area] and will use the most sequencing, so our core competencies are uniquely suited to going into that market."

Henry also made a projection for how Illumina could gain more than $600 million in additional annual revenues from clinical or diagnostic sequencing once the '$1,000 genome' is available, which he said will likely happen over the next few years.

About $350 million in new revenues could come from newborn screening by whole-genome sequencing, assuming that 1 percent of all newborns in the US and other industrialized countries — about 34.5 million in total — would undergo such a test.

Another $250 million in annual revenues could come from cancer diagnostics if 1 percent of all new cancer patients in the world — a total of about 12.7 million in 2008, according to the World

Cancer Research Fund International — would have their tumor as well as a normal control sequenced. It is likely that each tumor would be sequenced multiple times during the course of therapy, he added. "Achieving 1 percent penetration in this market we expect will be very straightforward, and we think we'll be able to achieve much higher levels of penetration over the next decade," Henry said.

Finally, $25 million in annual revenues could derive from the clinical trial market if 1 percent of all patients entering clinical trials in the US each year would undergo whole-genome sequencing.

These projections are aimed to "impress upon you how significant these markets are going to become, and we are at the very earliest stages of taking advantage of this opportunity," Henry concluded.

He pointed out that the company's HiSeq 2000 sequencer is already being used by several companies, such as Sequenom, in prenatal diagnostics to test for Down syndrome and other chromosomal abnormalities. "This is a really significant opportunity for those companies and for Illumina," he said, adding "that market could be well over $1 billion and move very fast."

Illumina is already using the HiSeq 2500, which has not been commercially released yet, in its service laboratory to generate fast clinical results, for example to "help a clinician with a specific cancer case with a five-day sample-to-answer turnaround," he said. "The implications of this are very significant because it allows us to start pushing the system deeper and deeper into clinical applications, because if you are a patient, you don't want to wait from 10 days to a month for the answers."

Besides rapid turnaround, having a simple workflow, accurate data, and being able to work with small amounts of input DNA will be important for clinical sequencing applications, Henry said, noting that Illumina's TruSeq PCR-free protocol allows users to perform whole-genome sequencing with as little as 500 nanograms of DNA.

"One of our competitive advantages is that we have the fastest workflow, and we have the simplest workflow in the industry," Henry claimed. "That allows researchers of various skill levels to get engaged with the technology, which becomes more and more important as we start to move into the clinical markets."

Julia Karow tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Julia Karow or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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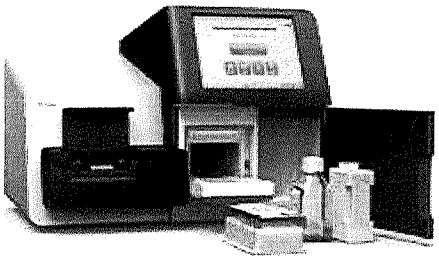
Illumina Alliance Introduces Clinical Next Generation Sequencing Tools to Pathologists

12 Sep 2012

Illumina, Inc. and Partners HealthCare have announced the formation of a strategic alliance to offer medical geneticists and pathologists, infrastructure and networking tools to support the interpretation and reporting process for genetic sequencing data.



The tools will integrate the functionality of Illumina's MiSeq®

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sequencing system and Partners GeneInsight Suite®, an IT platform that streamlines the analysis, interpretation, and reporting of complex genetic test results. The GeneInsight Suite is registered with the FDA as a Class I exempt medical device.

Through the alliance, Illumina and Partners HealthCare are leveraging their respective areas of expertise to jointly enable a comprehensive sequencing and clinically relevant reporting solution for the Illumina family of next-generation based content sets. The new tools will link to Illumina's BaseSpace cloud computing platform and the MyGenome app for iPad®, as well as to a clinical lab's local laboratory information system (LIS). The solution creates a seamless workflow, starting on the MiSeq and delivering results directly into the GeneInsight tool, allowing laboratory personnel to interpret data accurately and with confidence.

"Illumina and Partners HealthCare share a vision of better patient care through genomics," said Matt Posard, Senior Vice President and General Manager of Illumina's Translational and Consumer Genomics business. "This alliance highlights our commitment to a collaborative model that will establish the new standard in NGS clinical-based applications and enable a truly integrated, high-quality solution."

Illumina and Partners HealthCare will initially release the combined MiSeq-GeneInsight solution to a limited number of pilot customers in the clinical testing space. These pilot sites will include a select group of reference laboratories within leading academic institutions, molecular and genetic pathology laboratories looking to bring next-generation-based genetic testing in-house, and commercial reference laboratories. Additionally, Illumina will use the GeneInsight Lab® application in its CLIA-certified sequencing laboratory to support clinical interpretation and reporting.

GeneInsight software has supported the interpretation and reporting workflow for more than 24,000 complex genetic tests across multiple diagnostic reference laboratories, including Partners HealthCare Laboratory for Molecular Medicine, where the software has supported complex genetic tests since 2005.

"We expect the collaboration between our organizations to yield significant benefits to our patients, and to patients worldwide," said Anne Klibanski, M.D., Chief Academic Officer, Partners HealthCare. "We're excited about this extraordinary opportunity to work together to improve patient care and equally pleased that Illumina is committed to developing solutions that continue to advance our understanding of human genetics and disease."

Illumina have also just announced the launch of TruSight™ content sets for targeted sequencing, designed for use in next-generation sequencing. The combination of the content sets and new interpretation and reporting tools is a major step forward in realizing the benefits of next generation sequencing. Illumina remains on track to submit the MiSeq system to the U.S. Food and Drug Administration (FDA) for 510(k) clearance before year end.

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Sonia Nicholas
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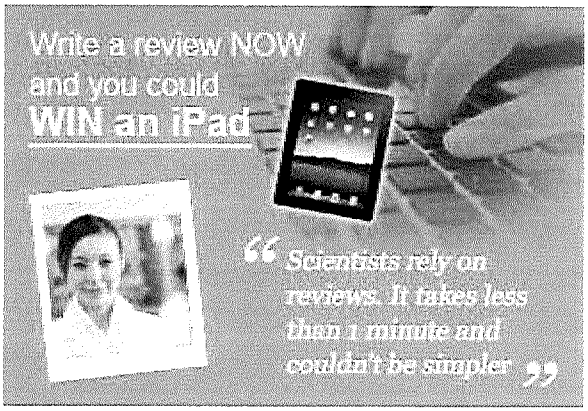

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Exhibit 22

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Tuesday, November 12, 2013

Illumina Solidifies Clinical Strategy with TruSight Products, Collaboration with Partners HealthCare

September 12, 2012

By Monica Heger

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Illumina this week made further inroads into the clinical market with the launch of disease-specific targeted sequencing products, diagnostic whole-genome sequencing on the HiSeq 2500, and a new collaboration with Partners HealthCare for clinical interpretation of sequencing data.

The company's disease-specific content sets, dubbed TruSight, are specific for autism, cardiomyopathy, inherited cancer, inherited diseases, and exome sequencing.

These products are the latest step in Illumina's ongoing strategy to capture a share of the nascent clinical sequencing market and shed some light on the direction that its Translational and Consumer Genomics business unit is taking.

Earlier this year, Matt Posard, general manager of the Translational and Consumer Genomics business unit at Illumina, told *Clinical Sequencing News* that the group was focused on enabling genomics-based healthcare and would seek to develop a physician's report as well as disease-focused panels that could be customized by clinical labs that wanted to launch them as laboratory-developed tests (*CSN 3/14/2012*).

The new products are "the realization of that plan," Posard said this week.

Illumina's other clinically focused unit, its Diagnostics business, has also been moving forward as it seeks to increase hires within its clinical and medical affairs group (*CSN 9/5/2012*).

The TruSight products are designed to be run on the MiSeq and to be interpreted using Partners Healthcare's GeneInsight Suite, a genome interpretation software package that is already registered with the US Food and Drug Administration as a Class I exempt medical device, Posard said.

"We identified very early on that an individual customer's ability to analyze and interpret sequencing data was a bottleneck in adoption with clinical research customers," Posard said. To address that bottleneck, Illumina considered various options, including building an interpretation tool itself, buying one, or partnering.

Posard said that after evaluating the landscape, the GeneInsight Suite by Partners HealthCare quickly "rose to the top." The software has been in the field for over seven years and a number of clinical labs already use it. The tool can also scale to evaluate both targeted panels and whole genomes.

It is compliant with CLIA and HIPAA and the fact that it is registered with the FDA could potentially be helpful as Illumina looks to bring its MiSeq system through FDA 510(k) clearance along with one or several of the TruSight assays, Posard said

"The company's intention is to take any or all of these products through the FDA based on clinical trial design and market interest in this research phase of the product's usage," he said.

Posard said that the company would be looking to get market feedback about the products before deciding which or when to take them through the FDA.

In this issue of Clinical Sequencing News

Illumina Solidifies Clinical Strategy with TruSight Products, Collaboration with Partners HealthCare

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Because the TruSight assays are being sold as research-use-only products, Posard said that labs interested in using them for diagnostic purposes would have to validate them in a CLIA-approved setting.

Additionally, he said, the TruSight products have been designed so that "individual customers can augment the design, or content of the oligos, and create additional content if they wish, and therefore create a customized product that would satisfy the [laboratory-developed test] requirement" under CLIA.

The TruSight products are based on the Nextera "tagmentation" chemistry. Tim McDaniel, Illumina's director of scientific research, told *CSN* that the company chose to use the Nextera chemistry versus the TruSeq chemistry because it was much simpler and fast — requiring 90 minutes for library preparation. By comparison, the TruSeq kits require eight hours for library prep.

Illumina partnered with experts in the field to develop each of the TruSight assays.

The TruSight Autism assay was developed with Jonathan Pezsner at the Kennedy Krieger Institute and targets around 100 genes. TruSight Cancer targets germline mutations in around 94 genes associated with inherited forms of cancer and was developed with Nazneen Rahman at the Institute of Cancer Research in London. The assay includes the BRCA 1 and 2 genes.

TruSight Cardiomyopathy focuses on 46 genes and was developed with Heidi Rehm's group at Partners HealthCare's Laboratory for Molecular Medicine. Rehm's team had already been developing a cardiomyopathy test that originally used both next-gen and Sanger sequencing (*CSN 2/22/2012*).

The TruSight Inherited Disease assay was developed with Stephen Kingsmore at Children's Mercy Hospital. His team had been working on its own inherited disease panel — comprising the same 552 genes that are now in the TruSight assay — originally as part of the National Center for Genome Resources, but moved the test to Children's Mercy Hospital for the clinical launch.

Last year, Kingsmore's team began a validation study of the assay in a clinical trial of 1,000 known patient samples using the HiSeq 2000 and Agilent's SureSelect enrichment kit (*CSN 8/9/2011*)

And finally, Illumina's TruSight Exome assay targets 2,761 genes — all the genes that are known to be associated with clinical ailments or conditions as reported by the Human Genome Mutation Database.

Additionally, Illumina has expanded its diagnostic sequencing services and now offers whole-genome sequencing on the HiSeq 2500 in its CLIA-certified laboratory. The service will require a physician's prescription and will initially be available on a limited basis. Illumina will evaluate cases submitted for sequencing on the 2500 to prioritize which patients will most benefit from a faster turnaround.

Posard said that Illumina does not intend to offer its TruSight assays as a diagnostic service, because that would compete directly with its customers.

Monica Heger tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Monica Heger or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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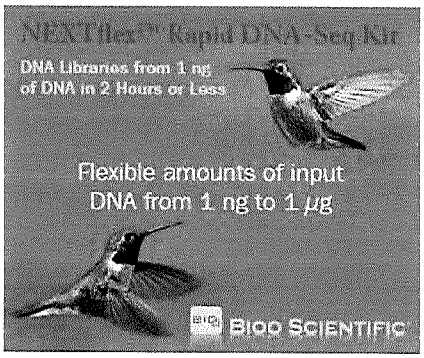

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April 25, 2012 / Clinical Sequencing News

Science

Members of the International Genomics Alzheimer's Project consortium brought together data for tens of thousands of individuals with or without late-onset Alzheimer's disease, and through GWAS and meta-analyses of that data tracked down 19 risk loci for the neurodegenerative condition, including 11 not found previously. The new candidate genes were detected in amyloid and tau protein-related pathways and pathways involved in immune response, inflammation, cellular migration and transport processes.

Business

Illumina signed a definitive agreement to acquire clinical software firm NextBio, which provides platforms to aggregate and analyze large amounts of phenotypic and genomic data for research and clinical applications. Illumina plans to combine its BaseSpace cloud computing environment for next-generation sequencing data with NextBio's platform for integrating patient data. Illumina will integrate NextBio into its Enterprise Informatics business and will retain NextBio Co-founder Ilya Kupershmidt and CTO Satnam Alag.

Funding

The Carlos Slim Foundation has pledged $74 million to fund the second phase of a genomics-focused collaboration between the Broad Institute, Mexico's National Institute of Genomic Medicine, and the Carlos Slim Health Institute. In this second phase of the collaboration, researchers plan to develop diagnostic tools for breast cancer and diabetes, complete their genetic analyses of these diseases, develop therapeutic roadmaps to guide the development of new treatments, and launch "a full-scale effort" to target the MCKD1 gene.

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Exhibit 23

Press Release: 19th September 2012 - Illumina Acquires BlueGnome

Acquisition Supports Illumina Goals in Diagnostics, Enhances Position in Reproductive Health and Cancer SAN DIEGO—(BUSINESS WIRE)—September 19, 2012—Illumina, Inc. (NASDAQ:ILMN) today announced that it has acquired Cambridge, U.K.-based BlueGnome Ltd. for an undisclosed sum. BlueGnome, now a wholly owned subsidiary of Illumina, is a leading provider of solutions for the screening of genetic abnormalities associated with developmental delay, cancer, and infertility.

"The BlueGnome acquisition supports Illumina's goal to be the leader in genomic-based diagnostics and enhances the company's ability to establish integrated solutions in reproductive health and cancer," said Jay Flatley, President and CEO of Illumina. "BlueGnome is the leader in the rapidly growing IVF market and is well-known for their software and comprehensive assay workflows."

"By joining forces with Illumina, we will be able to leverage the industry's leading microarray and sequencing platforms for our next generation products," said Nick Haan, President and CEO of BlueGnome. "The throughput and data quality of Illumina's sequencers enable us to consider revolutionary new approaches to genetic testing."

In collaboration with some of the world's leading in vitro fertilization (IVF) centers, BlueGnome developed 24sure™, a preimplantation genetic screening (PGS) test for counting the chromosomes in a single human cell – a test that holds enormous promise for increasing IVF success rates. Clinical data has shown that PGS greatly improves IVF success, increasing pregnancy rates for women and reducing

miscarriages.

BlueGnome's offerings also include CytoChip, a powerful test for the investigation of genetic abnormalities mainly associated with developmental delay or with complex leukemias. CytoChip is used by more than 200 labs across 40 countries worldwide as a first-line cytogenetic test, replacing traditional G-band karyotyping.

The company's founders, Nick Haan and Graham Snudden, will continue to lead BlueGnome which will report to Greg Heath, SVP and General Manager of Illumina's Diagnostics business. In addition to its headquarters in Cambridge, England, BlueGnome has offices in Fairfax, Virginia and Singapore.

Added Greg Heath, "Our technologies are rapidly moving towards clinical applications that can make dramatic and immediate impacts on people's lives, and the BlueGnome acquisition is an excellent fit with Illumina's overall genomic solutions and goals. We are very excited to welcome this talented team to Illumina."

About Illumina

Illumina (www.illumina.com) is a leading developer, manufacturer, and marketer of life science tools and integrated systems for the analysis of genetic variation and function. We provide innovative sequencing and array-based solutions for genotyping, copy number variation analysis, methylation studies, gene expression profiling, and low-multiplex analysis of DNA, RNA, and protein. We also provide tools and services that are fueling advances in consumer genomics and diagnostics. Our technology and products accelerate genetic analysis research and its application, paving the way for molecular medicine and ultimately transforming healthcare.

Forward-Looking Statements

This release may contain forward looking statements that involve risks and uncertainties. Important factors that could cause actual results to differ materially from those in any forward-looking statements are detailed in our filings with the Securities and Exchange Commission, including our most recent filings on Forms

10-K and 10-Q, or in information disclosed in public conference calls, the date and time of which are released beforehand. We do not intend to update any forward-looking statements after the date of this release.

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Exhibit 24

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New Players, Clinical Applications, and the Lure of Commercialization among 2012 Bioinformatics Trends

January 04, 2013

By Uduak Grace Thomas

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Increasing activity from sequencing vendors and other new players, as well as a flurry of activity around clinical data analysis software and the commercialization of a number of open source tools were the key trends in the bioinformatics community in 2012.

Much of this activity spilled over from 2011, which was marked by an influx of newcomers into the next-generation sequence data analysis space and continued efforts to develop software tools for clinical data analysis and personalized medicine (*BI* 12/22/2011).

Sequencing vendors continued to encroach on the bioinformatics arena in 2012. The year had barely begun when Life Technologies subsidiary Ion Torrent unveiled plans to launch Ion Reporter, a commercial cloud-based variant analysis software package that it launched in November (*BI* 1/13/2012 and *BI* 11/9/2012).

Illumina took a more aggressive stance with the launch of BaseSpace Apps — a dedicated application store for BaseSpace, the genomics cloud computing platform that the company launched in 2011 (*BI* 4/27/2012).

Illumina secured the buy-in of bioinformatics companies like Golden Helix, Biomatters, Ingenuity, and newly minted firms like Station X and Spiral Genetics, all of whom signed on to develop apps for the store, which is slated for launch later this month. While these developers are willing to give the app-based model a try, several made it clear that they would keep a close eye on adoption rates when the store came online (*BI* 8/24/2012 and *BI* 11/2/2012).

Illumina has done its best to sweeten the deal for app developers by allowing them to set the price of their apps themselves. It has put in place a pricing structure for the core BaseSpace platform that offers users a terabyte of free space and relatively low-cost options for those that require additional storage or processing space (*BI* 7/27/2012).

Illumina is not the only company that finds the app-based approach promising. That list includes 23andMe, which released an application programming interface that will allow authorized third-party developers to build a broad range of apps for the web and mobile devices (*BI* 9/21/2012); and Portable Genomics, which develops applications for browsing personal genomic information on mobile devices such as iPhones and iPads (*BI* 2/17/2012).

Besides BaseSpace, Illumina also formed partnerships with companies like Diagnomics, Ingenuity, and Knome, who were all selected to provide analysis services for customers of the Illumina Genome Network, which links researchers conducting whole human genome sequencing projects with institutes that offer Illumina sequencing (*BI* 4/13/2012).

Other sequencing vendors also sought partnerships in the bioinformatics market this year. Accelrys and Oxford Nanopore Technologies continued to work on "nanopore-specific" workflows for the Pipeline Pilot workflow software that will enable higher-level data analysis for Oxford Nanopore's sequencers (*BI* 2/24/2012). Also, Complete Genomics signed a deal with Ingenuity that allows the sequencing services provider to offer access to the Ingenuity Variant Analysis software (*BI* 7/27/2012).

In this issue of BioInform

New Players, Clinical Applications, and the Lure of Commercialization among 2012 Bioinformatics Trends

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Sequencing vendors weren't the only nontraditional players looking to grab a share of the bioinformatics market in 2012. Other new entrants into the genomics analysis space include Oracle, which released Omics Databank, a platform that provides tools to pull in data from -omics experiments and link this information with clinical data from electronic medical records (*BI* 1/30/2012).

Also, Qiagen and SAP announced a partnership that will focus on developing genomic sequence data alignment tools as well as variant identification software (*BI* 7/6/2012).

More broadly, telecommunications giant BT dipped its toe into the life science informatics market with partnerships with Accelrys and BioTeam that led to the launch of the cloud-based BT for Life Science R&D service and a Siri-based task management application (*BI* 4/27/2012).

The Commercial Alternative

2012 also saw some well-known open source tools go the commercial route.

In August, the Broad Institute signed an agreement with St. Louis-based cloud computing firm Appistry to distribute and support a commercial version of the Genome Analysis Toolkit. The Broad said at the time that the arrangement would allow it to provide better support for commercial users of its software and provide the resources needed to further develop the widely used toolkit (*BI* 8/3/2012 and *BI* 10/19/2012).

Then in December, Biobase signed a deal with University of Southern California and the Children's Hospital of Philadelphia that allows it to commercialize Annotate Variation, or ANNOVAR, an open source software tool for annotating genomic variants (*BI* 12/14/2012).

Also this year, University of Washington partnered with Insilicos, a Seattle-based biomedical company, to market a cloud-based version of the university's Rosetta protein and molecular modeling software (*BI* 9/7/2012).

Finally, the Joint BioEnergy Institute tapped startup TeselaGen Biotechnology to launch a cloud-based commercial version of the j5 software package, its automated DNA cloning tool (*BI* 10/26/2012).

Meanwhile, the Los Alamos National Laboratory is seeking industry partners to commercialize Sequedex, a software program developed at the lab for classifying short DNA sequence reads according to phylogeny and function (*BI* 10/19/2012).

In a related vein, several new startups spawned from academia moved into the limelight this year. A few examples include GenoSpace — started by researchers from the Dana-Farber Cancer Institute — which launched a cloud-based platform intended to provide access to a variety of 'omic and phenotype data (*BI* 6/29/2012).

And from the University of California, Santa Cruz, came companies like Maverix Biomics, which offers a cloud-based platform for next-generation sequence data management, exploration, and visualization (*BI* 11/9/2012); and Five3 Genomics, which began offering cloud-based cancer data processing and interpretation services using tools developed at UCSC (*BI* 10/26/2012).

Clinical Activity

With the rise of clinical applications for next-generation sequencing technology, there was a spike in informatics activity focused on this market.

Informatics efforts in this area included academic efforts such as Children's Hospital Boston's CLARITY (Children's Leadership Award for the Reliable Interpretation and appropriate Transmission of Your genomic information) contest, which aimed to "advance standards for genomic analysis and interpretation and the reporting of clear, actionable results to clinicians and patients." (*BI* 1/30/2012 and *BI* 8/24/2012).

The results of CLARITY indicated that the community largely agrees on the best informatics approaches for clinical genomic data analysis and interpretation, although there's still room for improvement (*BI* 11/16/2012).

Meanwhile, PerkinElmer said that it was developing a custom bioinformatics platform to support a next-generation sequencing-based cancer genotyping assay that is under development at the Massachusetts General Hospital (*BI* 8/31/2012); and GenoLogics made changes to its laboratory informatics management system that will enable clinical

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laboratories to adhere to regulatory requirements (*BI* 10/12/2012). In addition, vendors such as GenomeQuest took steps to ensure that their software toes current federal regulatory lines (*BI* 8/10/2012).

Also, IBM and the Memorial Sloan-Kettering Cancer Center began prepping IBM's Watson artificial intelligence technology to help with cancer diagnosis and therapy selection (*BI* 4/6/2012). IBM also began testing a prototype of an informatics platform that uses a combination of clinical knowledge with patients' genomic information and electronic medical records to make treatment decisions (*BI* 3/16/2012).

NextBio, meantime, launched a clinical version of its life sciences platform in April (*BI* 4/27/2012) and then a few months later announced a partnership with the Cancer Care Institute that aimed to demonstrate NextBio Clinical's ability to integrate and interpret genomic and clinical data from oncology patients in a medical setting (*BI* 6/29/2012).

In September, semantics-based software developer IO Informatics announced that it was working with the Center of Excellence for the Prevention of Organ Failure to build a web-based application that will be able to assess patients' disease risk and make treatment recommendations (*BI* 9/14/2012). IO later launched a new business arm dubbed IO Healthcare that will focus on helping customers in the personalized medicine market use clinical information to enhance their research and development activities (*BI* 12/07/2012).

Other efforts focused on improving clinicians' treatment decisions include a partnership between bioinformatics firm Annai Systems and IT consultancy ThoughtWorks to develop a software system that extracts pertinent information from individuals' molecular data and integrates it with information from the scientific literature and public databases (*BI* 11/30/2012); and one between MolecularHealth and SAP that will integrate MolecularHealth's oncology treatment decision support solution with the SAP Hana platform (*BI* 11/16/2012).

In November, Ingenuity said that it was working with two collaborators, including the Laboratory Corporation of America, to develop a new informatics solution that will address challenges associated with interpreting and analyzing sequence variants in the clinical setting (*BI* 11/9/2012).

Other activity in the clinical arena during 2012 included a grant that Integromics received from the European Regional Development Fund and the Spanish government to develop informatics solutions for analyzing next-generation sequence data in clinical laboratories (*BI* 2/10/2012). In addition, newbie N-of-One released its first product — an oncology informatics platform — and simultaneously announced a partnership with Foundation Medicine (*BI* 1/20/2012).

Buyers and Sellers

Acquisitions for the year included Accelrys' $35 million purchase of VelQuest — a developer of software for federally regulated lab environments — an acquisition that extended the company's product portfolio beyond research and development into late-stage product development and quality control (*BI* 1/6/2012).

Then in March, Certara bought Simcyp, a UK-based modeling and simulation software company, for $32 million in a bid to offer pharmaceutical customers a single informatics platform for drug discovery through preclinical and clinical development and clinical trials (*BI* 3/2/2012).

Meanwhile, Simulations Plus purchased software intellectual property from Enslein Research, a computational metabolism and toxicity prediction systems firm (*BI* 3/9/2012); and StarLIMS technologies, a subsidiary of Abbott Laboratories, purchased several distributors of its laboratory information management systems in an effort to improve services for its European customers (*BI* 3/9/2012).

In September, CLC Bio threw its hat into the drug discovery software ring with its acquisition of Molegro, a developer of molecular modeling software (*BI* 9/7/2012).

Finally, Life Technologies bought Compendia Bioscience, an Ann Arbor, Mich.-based cancer bioinformatics company, for an undisclosed sum (*BI* 10/12/2012).

Cloud Competition

While cloud computing continued to gain ground in the bioinformatics market during 2012, Amazon saw its position in the sector challenged by Google. In June, privately

held Numerate said that it had integrated its computational drug design platform with Google Compute Engine — a new service launched by the search provider that bears more than a passing resemblance to Amazon's Elastic Compute Cloud (*BI* 6/29/2012).

Google also worked with a bioinformatics team at the Institute for Systems Biology to evaluate Google Compute Engine's ability to handle life science computing requirements.

A side-by-side comparison of the two platforms revealed some similarities and differences in terms of price, capabilities, and associated services and so those who have used both systems say the right cloud platform will vary from case to case. Google representatives would not comment on whether they intend to aggressively pursue life science clients (*BI* 7/20/2012).

Amazon responded quickly with the launch of Amazon Glacier, a new low-cost cloud storage solution for archiving rarely used data in August. At the time of the launch, an Amazon representative told *BioInform* that the company believed that the life sciences would be "a very big market" for Glacier.

The company said it expected to bag customers from several market segments including genomics and biomedicine. However, the new resource met with mixed reviews from the bioinformatics community, with some suggesting that Glacier would be "extremely useful" while others believed that life scientists would pass on the new infrastructure (*BI* 8/31/2012).

Also posing something of threat to Amazon's dominance is Microsoft with its Windows Azure platform. Microsoft was able to woo customers such as TeraDiscoveries, which inked a deal that allows the company to run its Inverse Design computational modeling software on Azure (*BI* 4/27/2012).

Microsoft also partnered with UK-based drug discovery services firm Molplex, which is using its cloud infrastructure to build and support its computational compound screening business (*BI* 12/14/2012).

Uduak Grace Thomas is the editor of GenomeWeb's *BioInform*. She covers bioinformatics, computational biology, and life science informatics. E-mail Uduak Grace Thomas or follow her GenomeWeb Twitter account at @BioInformGW.

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Science	Business	Funding	GenomeWebinars
Members of the International Genomics Alzheimer's Project consortium brought together data for tens of thousands of individuals with or without late-onset Alzheimer's disease, and through GWAS and meta-analyses of that data tracked down 19 risk loci for the neurodegenerative condition, including 11 not found previously. The new candidate genes were detected in amyloid and tau protein-related pathways and pathways involved in immune response, inflammation, cellular migration and transport processes.	Illumina signed a definitive agreement to acquire clinical software firm NextBio, which provides platforms to aggregate and analyze large amounts of phenotypic and genomic data for research and clinical applications. Illumina plans to combine its BaseSpace cloud computing environment for next-generation sequencing data with NextBio's platform for integrating patient data. Illumina will integrate NextBio into its Enterprise Informatics business and will retain NextBio Co-founder Ilya Kupershmidt and CTO Satnam Alag.	The Carlos Slim Foundation has pledged $74 million to fund the second phase of a genomics-focused collaboration between the Broad Institute, Mexico's National Institute of Genomic Medicine, and the Carlos Slim Health Institute. In this second phase of the collaboration, researchers plan to develop diagnostic tools for breast cancer and diabetes, complete their genetic analyses of these diseases, develop therapeutic roadmaps to guide the development of new treatments, and launch "a full-scale effort" to target the MCKD1 gene.	Is Next Generation Sequencing Accelerating Your Cancer Drug Development and Clinical Trial Programs? Sponsor: Asuragen This webinar was recorded Oct. 3. GenomeWeb and Asuragen invite you to view a webinar discussing the use of next-generation sequencing to illuminate druggable targets in oncology while addressing the limitations in DNA quality and yield from formalin-fixed specimens. Register here to view the playback or download the recording.

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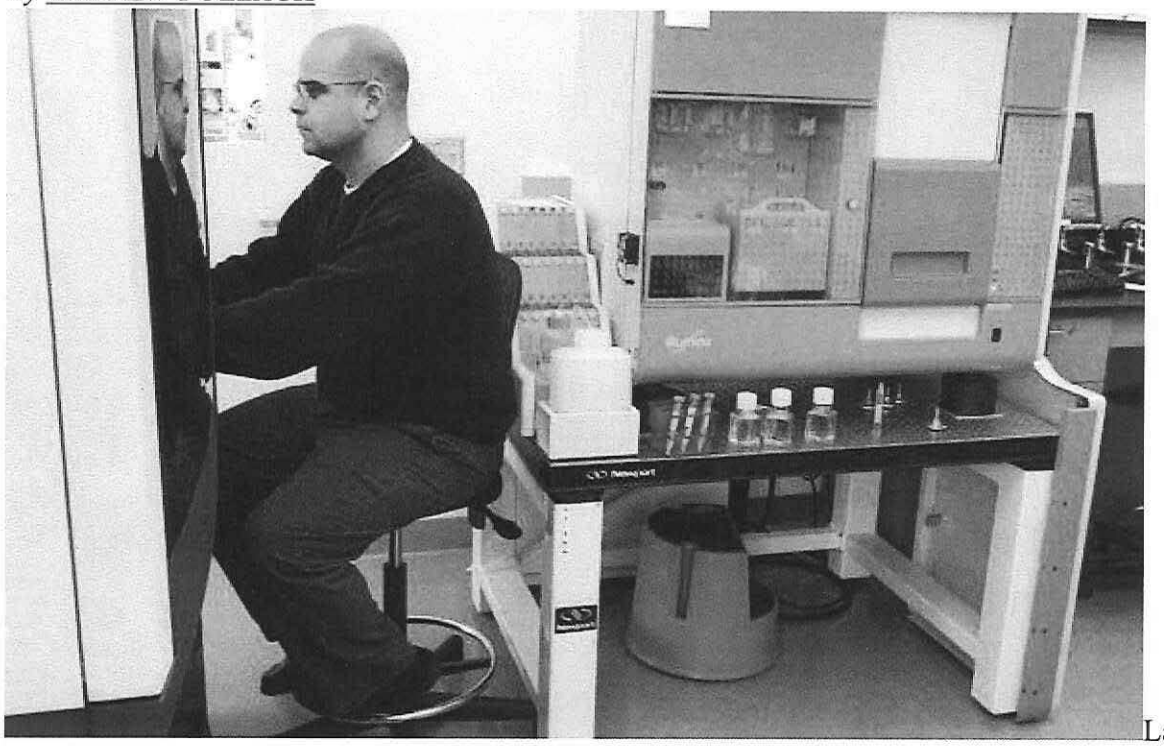
Illumina Buys Maker of Down Syndrome Test

By ANDREW POLLACK



Larry Downing/ReutersIllumina's technology provides genetic analysis at a laboratory in Rockville, Md.

SAN FRANCISCO — Illumina, the leading manufacturer of DNA sequencing machines, said on Monday that it would buy the privately held Verinata Health for at least $350 million in cash to expand its push into the diagnostics business.

Verinata, based in Redwood City, Calif., sells a test that uses a blood sample from a pregnant woman to determine whether her baby will have Down syndrome or some other chromosomal abnormalities.

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Such tests, which have been available for only about a year, have been rapidly catching on as an alternative, in some situations, to invasive tests like amniocentesis that carry a slight risk of inducing a miscarriage.

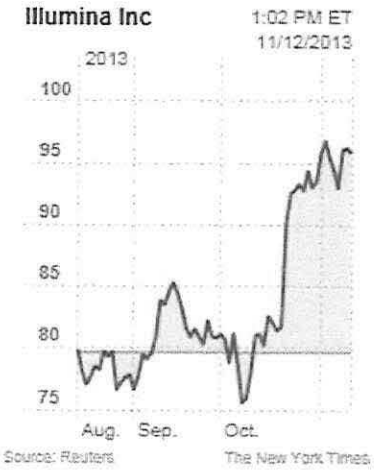
Illumina's stock fell almost 8 percent in early trading on Monday, though that was probably more because of reports that Illumina itself would not be acquired by Roche Holding, the Swiss pharmaceutical and diagnostics company. Illumina shares closed at $50.88, down 7 percent.

Roche's chairman, Franz B. Humer, was quoted on Sunday by a Swiss newspaper, Sonntags Zeitung, as saying a deal was off because Illumina wanted too high a price.

In April, Roche had dropped a hostile bid for Illumina, valued at $51 a share, or about $6.7 billion.

But a different Swiss newspaper had reported in December that Roche was trying to buy Illumina again, this time for $66 a share. Neither Illumina nor Roche commented publicly on that report.

Both Roche's interest in Illumina and Illumina's acquisition of Verinata suggest that DNA sequencing, which until now has mainly been used for research studies like the Human Genome Project, is moving toward being used for medical diagnosis.



Illumina wants to be more than a seller of sequencing machines. It already offers a service sequencing the genomes of people to help diagnose rare diseases or figure out the best treatment for a cancer. In September, it bought BlueGnome, a British company that uses sequencing to screen for various genetic abnormalities.

"The agreement with Verinata demonstrates Illumina's commitment to developing innovative diagnostic solutions and providing our partners with the most advanced technologies for improved patient care," Jay T. Flatley, chief executive of Illumina, said in a statement.

Verinata's test, called Verifi, uses sequencing to analyze fragments of fetal DNA that can be found in a pregnant women's blood. That allows for detection of Down syndrome, in which a person has three copies of chromosome 21 instead of the usual two.

Such noninvasive tests for Down syndrome appear to be catching on rapidly. Verinata, however, is believed to substantially lag the market leader, Sequenom, in market share.

Sequenom, a publicly traded company, introduced the first noninvasive Down syndrome test in October 2011.

It said on Sunday that it had performed 60,000 of its MaterniT21 Plus tests in 2012, and by the end of the year was operating at an annualized run rate of 120,000 tests.

Others selling or developing such tests include Ariosa Diagnostics and Natera. The companies are involved in various patent lawsuits against one another. They are also broadening their tests to detect chromosomal abnormalities beyond Down syndrome, including those linked to abnormalities in the sex chromosomes.

Some of these other companies use Illumina sequencers to perform their tests. It is possible they may now become more reluctant to rely on machines made by a company that is a competitor.

Illumina said there were about 500,000 high-risk pregnancies a year in the United States that would be candidates for a noninvasive prenatal test. It said the potential market for such tests would be more than $600 million in 2013.

Verinata said on its Web site that it would continue to operate as a subsidiary of Illumina. Beyond the initial payment of $350 million, Verinata shareholders will be eligible to receive up to an additional $100 million in milestone payments through 2015.

Illumina said the deal would dilute its earnings per share by 20 cents in 2013 but add to them in 2014.

Bank of America Merrill Lynch and Covington & Burling advised Illumina on the deal.

Illumina made its announcement on the eve of the J. P. Morgan Healthcare Conference in San Francisco, an annual Wall Street and medical industry gathering at which numerous companies make announcements.

A version of this article appears in print on 01/08/2013, on page B3 of the NewYork edition with the headline: In Diagnostics Push, Illumina to Buy Maker of Down Syndrome Test.

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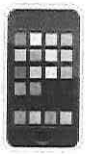

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Exhibit 26

Illumina CEO Jay Flatley on Diagnostics, the $1K Genome & China

Luke Timmerman 1/15/13 Follow @ldtimmerman

Illumina is the dominant player in the high-speed gene sequencing business, and has been for a number of years. That powerful position in a field that's vital to the future of healthcare has made it the object of intense scrutiny, and in some cases, scorn, from customers, competitors, and potential acquirers.

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Last year, the big event came when the San Diego-based instrument maker (NASDAQ: **ILMN**) fought off a $6.7 billion **hostile takeover bid from Switzerland-based Roche**, saying in essence that it could be a lot more valuable on its own. Many of its actions since could be interpreted as the moves of a hunter, not a company that sees itself as prey.

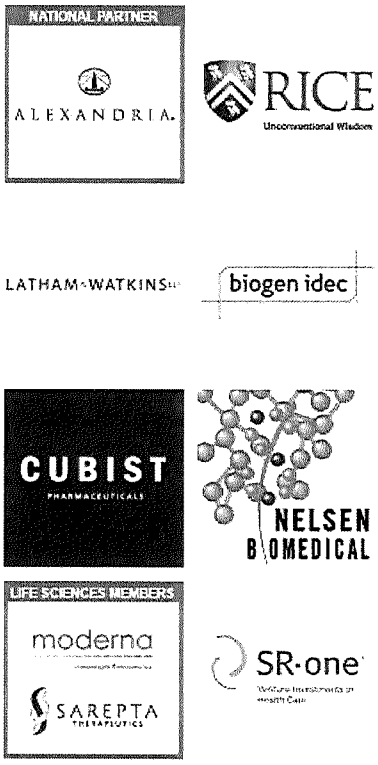
After the dust settled last spring in the Roche takeover battle, Illumina bought a couple of diagnostics companies, **BlueGnome** and **Verinata Health**, to follow through on its stated plan to morph into a more diversified maker of research tools and genomic diagnostic tests. The company has been racing to fend off rivals in the sequencing business like Carlsbad, CA-based **Life Technologies** (NASDAQ: **LIFE**), and smaller players such as **U.K.-based Oxford Nanopore** that pose technological threats to its platform for DNA sequencing. Illumina has ruffled more than a few feathers in the industry with some aggressive moves, including **an unsuccessful bid to stop BGI-Shenzhen from acquiring** Mountain View, CA-based Complete Genomics (NASDAQ: **GNOM**).

I met with Illumina CEO Jay Flatley to discuss all of these issues and more during a wide-ranging interview last Tuesday (January 8th) at the JP Morgan Healthcare Conference in San Francisco. Here are excerpts of the conversation, edited for length and clarity.

Jay Flatley, CEO of Illumina

Xconomy: You have been one of the busiest newsmakers in the industry lately. You bought Verinata Health, then another company, **Moleculo**, a spinout from Stanford University. You pre-announced **fourth quarter revenues of $309 million** that

UNDERWRITERS AND PARTNERS



Xconomy Events Across the Network

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were higher than consensus expectations on Wall Street. You reportedly said no to another recent acquisition inquiry from Roche. What else is up your sleeve for this year?

Jay Flatley: We have a lot going on, we're busy fellows. We have a pretty rich pipeline of opportunities that come our way now, because of the size we are and the presence we have in the market. We get to look at lots of different companies. It's driven in part by the challenges in the life sciences venture capital industry. Some are getting out of the business. Sometimes small companies get to the first step in development, and they are looking for strategic partners, or for somebody to buy them out. So we get to look at a lot of things. We don't do that many. We do a handful of tuck-ins per year, and occasionally a big one like Verinata.

X: How has your strategy around acquisitions changed after the whole Roche thing ended last spring? Do you see yourselves increasingly as the acquirer, rather than the acquired?

JF: Our M&A strategy changed a bit a few years ago, and Roche didn't particularly influence any change. We used to be more opportunistic—if something came to us and looked interesting, we'd look at it. Now we're much more proactive. We have a full-time staff that does nothing but this, scouring the States and the world for good licensing opportunities, or good companies that we think we ought to own.

X: What kinds of things are you most interested in now? Diagnostic companies, or new technologies to build up the platform?

JF: It spans a wide range. We're clearly looking at diagnostics, we've been very public about that the past couple of years. We're looking for something to really enhance our penetration in diagnostics, more rapidly than we could organically. Verinata certainly does that. We've looked across the entire space. There frankly aren't that many high-quality assets that can move the needle for us, and we think Verinata can.

We're always looking for good technology pieces, and Moleculo is a great example. It's a company that's young and small, but they have a great technology that will help inch our product line forward in some interesting ways. We are always looking for interesting assay methods out there. Software is an area we have been looking.

X: Do you worry about how getting aggressive in M&A might backfire? There has been some commentary made about you guys moving to compete against Sequenom (NASDAQ: **SQNM**) in the prenatal genetic testing market. These guys, I believe, are your No. 1 customer.

JF: They are a very important customer to us. The goal here certainly isn't to compete directly with Sequenom. One of the things we've tried to make very clear—and we talked to them before we announced this—is that our goal is to make the whole field expand and continue to have them be a strong customer for us. There are couple components to this. One is that we think Verinata has the foundational IP in the field, and we think the field is being held back a little bit by IP overhang. There may be a way now that we can work that out. I'd like to see if that's possible.

We clearly have a partnering strategy to take this technology to the market. We'd love to partner this (Verinata Health's prenatal genetic test). Part of what we've done here is in recognition of the fact that in five years, this is going to be an IVD (in vitro diagnostic) market. People will want an FDA-approved test they can run in lots of labs. While the technology was split up, with assays being in other companies, and us having the platform, there was no really easy way to get an application through the

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FDA. We'd love to work with all these companies. We want to help Sequenom, help Ariosa (Diagnostics) and further our business as well.

X: What other kinds of diagnostics are you looking to tuck in?

JF: We've been very active in the cancer field, but these are not things we think are going to be acquisitions. They are tools we put out on the market. For example, we put out a somatic cancer panel. It's to help accelerate CLIA (centralized clinical labs). They can add additional content on top of this. But it's to get these labs to begin to do cancer vs. normal tissue screening. It's not that likely we're going to have a material acquisition in cancer anytime soon. But we're continuing to watch the field. We have a lot of customers. It's a very big market with lots of indications. I don't think there's any risk of us competing with our customers in cancer.

X: What about technology acquisitions? Moleculo is one of those. They'll enable you to do more long-read DNA sequence lengths, right? Others, like PacBio, have tried to push ahead on that front to gain an advantage. What's your rationale for that acquisition?

JF: The great thing about the Moleculo technology is that in order to get long reads, you don't have to sacrifice throughput or cost. That's the problem with the other systems. You sacrifice accuracy, with, say, [Oxford] Nanopore's technology or **PacBio**. Here we get the accuracy of SBS (sequencing by synthesis) chemistry, plus the long reads. The incremental amount of (extra) sequencing you have to do is very small. It's about one extra lane on a HiSeq machine to get a full human genome.

It opens up about 10 percent of the next-generation sequencing market that we think really wants long reads. It's for areas like structural variations in cancer, or de novo sequencing—particularly in complex plant genomes. There are applications like meta-genomics, where you're sequencing a complex soup of things, when you're looking at a number of different organisms present, and you're trying to ask, is this organism present? Having a couple hundred base reads sometimes isn't enough to figure that out. Certainly there are some clinical applications, in being able to determine whether you're dealing with a mutation in a gene, or whether it's from the paternal or maternal strand, can make a big difference in the diagnosis.

Over time, this will be a standard part of what we do. There is some inherent improvement in accuracy when you move to long reads.

X: Are you really going to be able to get reads that go all the way up to 10,000 bases of DNA?

JF: Just the data they have already hits that. The chart I showed today, the maximum read length was 13,000, the average read length was 7-8,000 base range. We actually have an internal program where we can get up to 100,000 base reads. These are synthetic, so to be clear, these aren't actually using SBS chemistry to read 10,000 bases in a row. It's labeling the ends of short reads, and then reconstructing them afterwards. So we call it a synthetic long read. But the accuracy is astounding.

X: Why did you say no to Roche's overtures again?

JF: We said no to Roche at our annual meeting in April, and that's the last comment we made on Roche.

X: Yes, but they made some recent overture that was reported…

JF: There's been a bunch of stuff reported in the press, but we didn't comment on it.

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X: So why remain an independent Illumina? Why does that make sense for the company and its shareholders?

JF: We're certainly not wedded to that notion. We've said that openly. This was a matter of having a fair price, and a price that we think puts an end to the upside our shareholders enjoy. To truncate that value at some fixed number requires a number that's materially bigger than any number we've seen. When you look at $51, we were trading at $55 just a few weeks ago. A $51 per share offer just wasn't in the ballpark.

X: When you look at the markets you can enter, how big do you think the opportunity is, and what kind of share price does that justify?

JF: The markets for sequencing are going to be enormous. Many, many, many billions of dollars when you look out 5-8 years from now. The cancer market will be enormous. The newborn screening market is going to be enormous. If you look just at NIPT (non-invasive prenatal genetic testing) alone, today there's a $1 billion of value just in doing amniocentesis. This is going to be much bigger than that, because it will be done by more women in the high-risk group who avoid amnio because of the risk to the fetus (potential miscarriages). And it will expand into low-risk pregnancies, because this is a test you can do with virtually zero risk. That market alone has multiple billions of potential.

X: And you think Verinata has the IP advantage in a four-way dispute (with Sequenom, Ariosa Diagnostics, and Natera) the other entrants?

JF: We think so. Nobody's certain about that until you get to the end of the process, but there's a chance we can make something work here with the other players.

X: Why did you guys oppose the Complete Genomics merger with BGI-Shenzhen (for $118 million)?

JF: Oppose is maybe…we asked that CFIUS (Committee on Foreign Investment in the United States) get involved, and they did. We think BGI owning it has national security implications, and we thought it was bad that they'd get it. To our surprise, CFIUS let it go through. Yesterday, the Federal Trade Commission approved it too, so we withdrew our bid today (Tuesday Jan. 8).

X: Why is it bad for national security?

JF: Because we think there's risk they could build very large databases, and get access to the genomes of lots of Americans. They could bring them back to China. There are lots of nefarious ways you could use the information. There are theoretical bad things you could do with those kind of databases if they aren't regulated by the law of the United States. So we were concerned about BGI's affiliation with the Chinese government. We'll have to see how it plays out.

X: Isn't BGI one of Illumina's biggest customers? They have bought a ton of your HiSeqs.

JF: Yes, they are a very significant customer of ours. We want to maintain a great relationship with them. But we're not sure it's in the U.S. national interest to sell the formula for Coke. It's different when people just buy Coke.

X: Has there been tension in the relationship with BGI since you took this action?

JF: Until this sorted out, in terms of who was going to make the Complete Genomics acquisition, we haven't had a lot of interaction with them. But I'm certain now that it looks like we know how it's going to go, we'll get re-engaged with them and have open discussions about how we can move the relationship forward.

X: How do you think you're doing vis-à-vis the competition? It's an extraordinarily competitive field, with Life Technologies, Complete Genomics, PacBio, Oxford Nanopore and others.

JF: I think we're doing well. We just pre-announced $309 million in revenue in the fourth quarter, which was a record quarter for us. We think we're continuing to add significant market share against our competitors. We take them seriously and think they are strong competitors.

X: Do you think Illumina still has the edge, technology-wise?

JF: Yes. We have a very rich pipeline of new products. We're fortunate enough now to be big enough that we can invest in a broad way to improve things like sample prep, and bioinformatics. It's not just for the sequencers. It's enabled us to introduce new products like **Basespace**, which we think is a very important cloud-based add-on to our sequencing ecosystem.

X: I want to come back for a bit to the diagnostics world for a minute. I've heard some rumblings this week about people being unhappy with Illumina moving into this area, and trying to take over the world. I've heard about some moves to jack up prices of reagents for diagnostics company customers. They seem threatened. Are you threatening a lot of your customers, who are aspiring molecular diagnostics companies?

JF: Not at all. We do think in the diagnostics market, the requirements those companies appropriately place on us, in terms of having different products, better lot tracking, keeping longer inventories, giving them advance notice of changes—it all requires us to build a different infrastructure inside the company, a parallel infrastructure. That's expensive for us. We're putting all those capabilities and systems and duplicate part numbers in place. As a result of that, we think premium pricing is justified for diagnostic kits.

X: So there was a recent price increase for diagnostic customers, compared with standard academic research labs?

JF: Pricing for our RUO (research use only) kit is different than for diagnostic customers. They are separate market segments. The diagnostic group does their pricing based on whatever the cost is of the infrastructure.

X: But was there a price increase recently?

JF: Don't think of it like that. It's not like it was some price one day and it changed. It wasn't an increase. But we're starting to have new products we put in the market that have different capability. They have different packaging, different lot tracking, different shelf life, different notification and supply agreements. They are priced appropriately.

X: So these customers have a different set of needs, and they are paying more?

JF: Exactly. And if they want to keep using our RUO reagents, they can continue to do that. We aren't forcing them to take those new products.

X: What worries you the most when you look at the business landscape?

JF: In 2012, (federal budget) sequestration was clearly the biggest worry we had. It caused a lot of uncertainty in the business, and we didn't know how customers would respond to it. We've come out of 2012 with much less impact than we might have anticipated. That's probably less of a worry for us now. Now, it's probably just

tracking what the competition is doing, and making sure we are in the market with products that as are competitive as we can make them.

X: What's the biggest problem your customers are facing, that you need to solve?

JF: It probably relates to interpretation of genomes. We've had great work done on the core sequencing engine, and made a lot of progress on sample prep. On the front end couple pieces of software, we've made lots of progress there, in terms of reducing file sizes and aligning genomes and call variants. Now the problems are moving into things like what the genome means, and what the variants mean. A lot of academic world is focused there, and we're trying to help. Particularly around cancer.

X: Did you actually hit the $1,000 genome threshold by the end of 2012? I know that both you and Life Technologies, if memory serves, said at this meeting (JP Morgan Healthcare Conference) last year that you'd be able to sequence an entire human genome in one day for $1,000.

JF: That's not quite accurate. What happened exactly a year ago at this meeting is that two companies announced they would have ability to sequence a complete human genome in a day. One company said they could do it for $1,000, and that was Life Technologies. We never put any pricing out in the market, but we said we could do the sequencing in a day. In February, we presented the first data on that. In the second quarter, we deployed the technology in our services operation. In the third quarter, we put it in the hands of customers. In the fourth quarter, we shipped it in volume. We delivered exactly on the program we promised.

X: So what does it cost now to do a whole genome, just the sequencing, on a HiSeq?

JF: It varies, depending on the volume of the customer. The discounts can vary, depending on what their usage rates are. If you look at the range of numbers, if you look at instrument depreciation and reagent costs, it varies from a couple thousand dollars up to $5,000. It depends also on what mode you run the instrument in, too. It's a bit more expensive to run the instrument in rapid mode than in high-throughput mode.

X: How do you stack up on cost with Life Technologies at this point?

JF: We're very competitive. Right now at least, they're not doing whole human genomes.

X: With the SOLiD or the Ion Torrent?

JF: The SOLiD isn't much of a factor in the market anymore. With Ion Torrent, to our knowledge, they aren't at the output levels people will use for human genomes.

X: It's more about targeted, regional sequencing, right?

JF: Yes.

X: Personally, this had to be a very intense year for you. How do you feel about what you're doing. Triumphant? Vindicated? Do you feel good about what you're doing?

JF: I've never been more optimistic about the company and the markets we're in. I feel very good about how the technology stacks up right now. We've got a great management team, and a great overall team. It's a lot of fun to do what we do. It was an intense year from a workload perspective. Particularly in that Roche (hostile takeover bid) window, there were three or four weeks when we did not much else.

But after that was over, we spent our time doing the blocking and tackling it takes to run a business and produce more innovation.

Luke Timmerman is the National Biotech Editor of Xconomy. E-mail him at ltimmerman@xconomy.com Follow @ldtimmerman

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Illumina's Clinical Business Driven by HiSeq 2500 Upgrades, Partnerships, Acquisitions

July 24, 2013

Illumina's Clinical Business Driven by HiSeq 2500 Upgrades, Partnerships, Acquisitions

By Monica Heger

Clinical and commercial customers are increasingly driving sales of Illumina's next-generation sequencing instruments, with 45 percent of HiSeq 2500 orders coming from such customers in the second quarter, the company said this week during a conference call discussing its second quarter earnings.

The firm is also expanding its presence in the clinical market through various partnerships and acquisitions. It recently announced a partnership with Pathogenica to co-market its HAI BioDetection kit on the MiSeq, and this week it announced a deal with HLA typing firm HistoGenetics to incorporate the MiSeq instrument into its CLIA-certified HLA typing working (see story, this issue).

Illumina has also been making progress expanding its presence in the noninvasive prenatal testing field through its acquisition of Verinata Health. During the conference call, Illumina President and CEO Jay Flatley said that in the second quarter, reimbursement coverage for Verinata's Verifi prenatal test was "significantly enhanced," and there are now 170 million covered lives in the US, with an additional 25,000 covered lives in Israel through an agreement with Teva. Additionally, more than 60 million lives are contracted for the Verifi test.

Clinical and translational customers are also making up an increasing portion of Illumina's whole-genome sequencing services business. During the second quarter, the US Department of Veterans Affair awarded Personalis and Illumina a contract to sequence 1,000 genomes as part of the Million Veterans Program, and it also secured an order from Inova Translational Medicine Institute, a nonprofit healthcare institute, to sequence 1,000 genomes. Previously, Inova had contracted Complete Genomics for a sequencing project to study preterm birth that it said would serve as a pilot for evaluating how whole-genome sequencing could be implemented within a healthcare setting (*CSN 9/14/2011*).

In total, Illumina received record orders for 6,000 whole genomes in the second quarter, and shipped 2,500 genomes.

FDA Progress

During the call, Flatley expanded on the company's various strategies for gaining regulatory clearance for its products. Earlier this year, the company submitted its MiSeqDx system along with two cystic fibrosis assays to the US Food and Drug Administration for 510(k) clearance (*CSN 1/9/2013*).

The company recently applied Europe's CE mark to the system and the assays — a carrier screening assay and a diagnostic assay (*CSN 7/3/2013*).

Going forward, Flatley said that the company's strategy would be a combination of developing and bringing products through regulatory clearance on its own as well as forging partnerships with specific expertise in various clinical fields.

"What we will put through the FDA will be highly selective," Flatley said, including "applications where we think having an [*in vitro* diagnostic] kit makes sense to be able to sell very broadly to labs around the globe."

In some cases, Flatley said that intellectual property would prevent Illumina from bringing products through on its own, "unless we specifically partner with that entity to put the product through the FDA jointly."

However, in the case of cystic fibrosis, "we were able to do that on our own."

Flatley said that the company will continue to "partner very broadly across lots of areas of diagnostics," including the HLA typing market, infectious disease, and cancer.

"HLA is a very large market opportunity and we're engaged with multiple customers that want to use next-gen sequencing in that market. HistoGenetics was the first of potentially several that we might work with," he said.

Illumina is also looking to bring Verinata's Verifi test through the FDA to sell as an IVD, and Flatley said that during the second quarter the company began its discussions with the FDA on a trial design, and that it is "pleased with the progress."

Currently, all four US-based NIPT companies use Illumina's sequencing instruments for their tests, so there was some concern that Illumina's Verinata acquisition would put it in direct competition with its customers (*CSN 1/16/2013*). Flatley said that the company is "broadly engaged in discussion" with those customers, but did not elaborate, and maintained that a key goal of the Verinata acquisition was to "enable overall growth of the NIPT market."

"We believe an IVD test will significantly expand the global market and enable all [NIPT] test providers to be successful in this field," he said.

Clinical Reagents

Another important aspect of Illumina's clinical business is its consumables. Clinical customers have different needs than research customers when it comes to reagents, said Flatley. As such, the company has a line of reagents that it calls TCG reagents.

Diagnostic customers require "more in our reagent kits than we typically provide," he said, including "lot tracking, longer shelf life, [and] the ability for us to continue to manufacture what

would otherwise be obsolete products in the RUO space."

The TCG line of reagents includes these features, Flatley said, which increases the cost to Illumina to produce and hold those products. "As a result of that, anybody who is using the TCG reagents pays a premium price to access those reagent sets, and they get essentially premium capability and services associated with those kits," he said.

Financials

Illumina's second quarter revenues increased 23 percent year over year to $346.1 million from $280.6 million. Product revenues increased to $313.5 million from $258.8 million a year ago, while service and other revenue rose to $32.6 million from $21.8 million during the second quarter of 2012.

R&D spending was cut 5 percent to $67.6 million from $71.2 million during the quarter, while SG&A costs rose 29 percent to $88.7 million from $68.5 million.

Illumina finished the quarter with $783.6 million in cash and cash equivalents.

Monica Heger tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Monica Heger or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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Illumina 'Optimistic' About Speedy FDA Clearance of MiSeq; Pitches Companion Dx Program to Pharmas

October 02, 2013

Illumina 'Optimistic' About Speedy FDA Clearance of MiSeq; Pitches Companion Dx Program to Pharmas

By Julia Karow

After being inspected by the US Food and Drug Administration two weeks ago, Illumina hopes its MiSeq sequencer will be cleared by the agency within the next several months, according to a company official.

While Illumina is waiting for the FDA's decision, it has applied the CE mark for expanded uses of the MiSeqDx in Europe, where it launched a CE-marked MiSeqDx with two cystic fibrosis assays this past summer (*CSN 7/3/2013*).

Marking its transition from a pure research tools company to one with a growing diagnostics branch, the company has also begun to market its services for companion diagnostics development to potential pharma partners.

Late last year, Illumina submitted a diagnostic version of its MiSeq platform, called MiSeqDx, as well as two cystic fibrosis assays to the FDA for 510(k) clearance (*CSN 1/9/2013*).

The submission has three components: the MiSeq in conjunction with a cystic fibrosis genotyping test, where most of the sequence except for the variants of interest is masked; the MiSeq with a cystic fibrosis diagnostic assay, where the entire gene is analyzed; and an "open" MiSeq platform with chemistry and software but no assay, which will allow customers to develop their own IVD assays more easily.

According to Nitin Udar, who leads Illumina's diagnostic assay program, the company was inspected by the FDA the week before last and is now awaiting clearance from the agency, which he said concluded its inspection "with no observations."

"I cannot speak for the FDA, but we are very optimistic that it is going to happen very soon – hopefully this quarter, or next quarter," said Udar, who spoke at Hanson Wade's NGS for Cancer Drug Development conference in Boston last week.

ILLUM-1607

As the first company to submit a next-generation sequencing platform to the FDA, Illumina has been collaborating closely with the agency, he said, educating it about the technology and conducting several pre-IVD meetings.

In Europe, Illumina appears to be one step ahead already. After CE-marking the MiSeqDx for use with the cystic fibrosis tests this past summer, the company said last week that is has "declared conformity with the requirement of the [EU] IVD directive and has applied the CE mark to expand the use of the MiSeqDx system in clinical laboratories."

Clinical labs in Europe can now develop their own diagnostic tests on a CE-marked MiSeqDx, using the MiSeqDx Universal Kit, which includes reagents for library prep, index primers, and sequencing consumables to develop amplicon assays. Illumina said the new kit will start shipping in November in several European countries that require the CE mark.

At last week's conference, which was attended by several dozen scientists, many from pharmaceutical companies, Udar also touted a partnership program for companion diagnostics development that Illumina is now offering.

Under a partnership with a pharmaceutical company, Illumina would design and develop the assay and manufacture its components, following a strict product development process where every step is documented, he said. For running the assay as part of a clinical trial, the firm would partner with a clinical research organization, since its own CLIA labs currently do not offer this as a service. "Both Illumina, the CRO, and the pharma partner would have a steering committee that overlooks this entire operation," he said.

Once clinical utility for the assay has been established and its targets have been locked down, "then Illumina will develop an IVD assay and submit that to the FDA on its own," he said, in parallel with the drug submission by the pharma partner.

Utar said Illumina would make a strong clinical partner because it has access to and continues to improve new technology and software; has a "strong assay development team;" has two CLIA laboratories and a network of CLIA partners; offers a global presence and distribution channels; and now has experience with regulatory agencies and in developing IVD assays.

Next-gen sequencing would be suitable for developing companion diagnostics, he said, which require researchers to look at large targets at first, but then to lock down the assay to only a few variants that have an association with a drug. "You can do that easily [with an NGS panel] – there are several different ways you can mask the results," he said. It could be either achieved by analyzing only the data of interest and masking the remaining sequence, or by analyzing the entire sequence and only reporting the variants of interest. An alternative, he said, would be to re-design the assay.

Julia Karow tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Julia Karow or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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Organizational Changes at Illumina Aimed at Growth, New Market Entries

October 16, 2013

Organizational Changes at Illumina Aimed at Growth, New Market Entries

By a GenomeWeb staff reporter

NEW YORK (GenomeWeb News) – Illumina today announced sweeping changes to its organization in order to maintain growth in its existing markets and to move into new ones.

In a letter to employees, which was filed with the US Securities and Exchange Commission, Illumina President and CEO Jay Flatley laid out the changes, which include the creation of new business units, new roles for existing managers, and the separation of the president's position from Flatley's continued role as CEO.

"These changes will enable us to continue growth in our existing markets, take the next steps to move into new markets, and further catalyze the use of our technologies in the clinic," Flatley said in his letter to employees.

The changes take effect on Jan. 1, 2014.

Flatley currently serves as president and CEO of Illumina, but starting in the new year, a new president will be hired. The president will be charged with "envisioning, developing, and producing our products," and will focus on leading Illumina's business units and core functions. That person will report to Flatley.

In the sole role of CEO, Flatley said he will set and drive Illumina's strategic vision and manage the commercial and corporate functions.

The company also is reorganizing into five business units, each with product marketing, market development, technology development, and target informatics resources.

The Life Sciences unit will focus on the company's core platform technologies and the core markets they enable, including complex disease, agrigenomics, microbiology, and metagenomics. A new Custom Products Group is being created, which will concentrate on content development for custom products and infrastructure to develop and manufacture them.

ILLUM-1735

Kirk Malloy, currently VP of Customer Solutions, will lead the unit. Illumina's molecular biology and PCR business will be under Malloy's leadership, as well. Mark Lewis, the current SVP and general manager of this business is retiring, but will provide interim leadership for the customer solutions function as Illumina searches for a new leader for the group.

The Reproductive and Genetic Health business unit will be headed by Tristan Orpin as general manager. He currently is chief commercial officer. The unit will include the Verinata, BlueGnome, and cytogenetics portfolio "and will focus on building a comprehensive, integrated offering in Reproductive Health and patient-directed Genetic Health," Flatley said.

The Oncology business unit will provide tools to the oncology community, which Flatley said is one of the company's largest market opportunities. Rick Klausner, Illumina's chief medical officer, will be interim leader of the unit.

Nick Naclerio will head the Enterprise Informatics business unit, while continuing in his current role as SVP of corporate development. The unit will be involved in storing, analyzing, understanding, and sharing genomic and phenotypic information.

"By establishing a strong focus in enterprise informatics solutions, we will be able to build the foundation upon which institutions can deliver precision medicine using genomic information," Flatley said.

Lastly, Illumina is establishing the New and Emerging Markets business unit so that the company can rapidly "identify, catalyze, and develop new markets and solutions." Matt Posard, the current SVP and general manager of Illumina's Translational and Consumer Genomics business, will head this unit.

In addition to the new business units, Illumina announced changes to its structure and leadership of core functions across the company, centralizing certain functions which will report directly to the president.

Product Development is moving out of the current Genomic Solutions business unit "to become a centralized function that will span systems, assays, consumables, reagents, and software." This organization will be responsible for delivering technology innovation across the company. Omead Ostadan will remain SVP of Product Development.

Also, Greg Heath will move from his current post as SVP and general manager of Illumina's Diagnostic business to become SVP of IVD Development, where he will be in charge of taking the San Diego-based company's technology solutions through the regulatory process. IVD Development will include functions such as medical affairs, clinical trials, and regulatory affairs.

Operations, currently part of Genomic Solutions, will become a centralized function, as well, and be headed by Bob Ragusa, who is joining Illumina in December as SVP of Operations.

Lastly, Quality and Regulatory Affairs will become two separate functions within Illumina. Regulatory Affairs will be folded into IVD Development, and Quality will be a standalone, central function. Melina Cimler will be VP of Quality, which is gaining importance as the company moves further into the clinical markets, Flatley said.

He added that Illumina is consolidating its commercial organization and channel in order to address the increasingly blurred lines between research customers and diagnostic customers

that have resulted from rapid technology development. Christian Henry, who currently serves as SVP and general manager of the Genomic Solutions business, will become Illumina's chief commercial officer.

"For me, these changes represent the next step in our evolution, and they will accelerate our growth and expand our reach into new and exciting markets," Flatley said in his letter to employees. "When Illumina was founded, the idea of precision medicine was just a concept; no one knew how it would come to pass. Now, we are on the cusp of it, we are not just talking about it, it's happening."

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Illumina dazzles with Q3 diagnostics expansion and revenue growth

October 23, 2013 | By Mark Hollmer

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Illumina's ($ILMN) stock soared more than 10% this week in anticipation of stellar revenue results for the 2013 third quarter. The San Diego gene sequencer did not disappoint, hitting numbers after the market's close Oct. 22 that reflect optimism over healthy gains and new full-year guidance revised higher for the second time in three months.

Illumina now predicts 22% revenue growth for the year, and non-GAAP-earnings per fully diluted share of $1.75 to $1.77, driven in large part by an expanded focus on diagnostics. At the end of the second quarter, Illumina nudged up its full-year guidance to a 20% revenue hike. The company's stock price closed Oct. 22 at $90.05.

Revenue soared to $357 million during the quarter, 25% higher than the $286 million booked during the 2012 third quarter. Net income nudged up to $31 million in Q3, up moderately from $30 million in net income booked over the same period a year ago.

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Illumina said its costs hiked during the quarter, in part because of a one-time charge stemming from the discontinuation of a noncore product line. Research and development expenses also grew. At the same time, however, Illumina's increased focused on diagnostics has propelled revenue growth to new heights. Earlier this year, the company closed its $350 million acquisition of Verinata Health, maker of Verifi, a noninvasive prenatal test. Through Q3, Illumina expanded Verifi's reach even further, announcing during the quarter that the test can now be used on women who are pregnant with twins. Earlier this year, Illumina also inked a deal with Teva ($TEVA) to market Verifi in Israel. Further, Verinata Health won a clinical laboratory permit from the New York State

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ILLUM-1610

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Beyond Verifi, Illumina also inked a three-year deal with Natera to supply the HiSeq 2500 sequencing system and related products for noninvasive prenatal testing.

Adding to the gains: Illumina launched two cystic fibrosis assays tied to its MiSeqDx sequencing platform in Europe earlier this year, after obtaining a CE mark for both. The company said it has been able to expand its overall use in clinical laboratories.

Illumina continues to build its gene-sequencing business as part of its overall strategy and made gains in that area during Q3 as well. Among those milestones: a project with Genomics England and the University of Cambridge to sequence 10,000 whole genomes, and a partnership with the Global Genomics Group to investigate new biomarkers and biological pathways connected to cardiovascular diseases.

"These results highlight another quarter of solid operational execution and record financial performance," Illumina President and CEO Jay Flatley said in a statement. "We continue to be pleased with the robust global trends demonstrated by our business and believe we are positioned to continue to deliver strong growth."

Illumina is much improved compared to its Q1 loss, which was driven by its defeat in a patent lawsuit from Syntrix Biosystems. Illumina said it still plans to appeal if its motion to vacate the verdict is unsuccessful.

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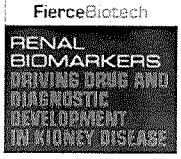

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Illumina Continues Push into Clinic with MiSeq Panels, HiSeq 2500, Reorganization

October 23, 2013

Illumina Continues Push into Clinic with MiSeq Panels, HiSeq 2500, Reorganization

By Monica Heger

Illumina is continuing its push into the clinical next-generation sequencing space. Customers are validating MiSeq panels for clinical use; HiSeq 2500 purchases are increasing from commercial, translational, and hospital customers; and the firm's recently announced reorganization is aiming to further drive the company into the clinic, President and CEO Jay Flatley said this week during a conference call discussing Illumina's third quarter earnings.

Additionally, the company is still negotiating with BGI on a clinical sequencing contract.

The HiSeq 2500 has been particularly popular in the clinical market. In the third quarter, 80 percent of HiSeq orders were for the 2500 version, and more than half of those were from commercial, translational, and hospital customers, Flatley said.

Additionally, more than 50 percent of MiSeq orders were from non-academic customers, "due to enthusiasm from pharmaceutical, translational, and clinical segments," Flatley said.

In particular, many of the MiSeq customers are interested in using it to run Illumina's TruSight panels, and to date around 200 customers have used those panels, with the tumor, inherited cancer, and exome among the most popular. Those customers are "validating the assays in their laboratory setting, a process that typically takes nine months," Flatley said.

He estimated that gene panels comprise around half of the clinical sequencing being done by its customers, with exome sequencing making up 25 percent to 30 percent and whole genomes around 5 percent to 10 percent.

For customers that are offering clinical sequencing services, Flatley estimated that reimbursement has been mixed.

"For complex panels, it's challenging," he said. Currently, only a small set of genes are routinely reimbursed, and that is typically done through stacked CPT codes, but "that is all changing," Flatley said.

With increased validation of panels, "we'll see increasing levels of reimbursement," he predicted.

Sequencing services and Verinata

Illumina saw a large boost in its services revenues, which were up 60 percent to $38 million. Much of that business was driven by a clinical sequencing project the firm is doing with the Global Genomics Group to provide whole-genome sequencing for the Genetic Loci and the Burden of Atherosclerotic Lesions, or GLOBAL, clinical trial.

This week, Illumina also announced the start of a three-year project with the University of Cambridge and Genomics England to sequence 10,000 whole genomes of children and young adults with rare genetic diseases, as part of the UK's efforts to sequence 100,000 genomes.

Verinata Health, which is also incorporated into the firm's services side, has "bolstered" Illumina's position in the noninvasive prenatal testing market, Flatley said, although he did not elaborate on Verinata's market position relative to the other US competitors Sequenom, Ariosa Diagnostics, and Natera. Illumina supplies all the US providers with sequencing equipment and reagents.

Reorganization will 'catalyze' move in the clinic

Last week, Illumina announced broad organizational changes regrouping the company into five business units, which Flatley said this week would not only help the company continue to grow in existing markets, but also "take the next steps into new markets and catalyze the use of our technologies in the clinic."

Two of the new business units — the Reproductive and Genetic Health business and the Oncology business — are directly focused on the clinical market. Illumina's BlueGnome and Verinata Health acquisitions will fall under the Reproductive and Genetic Health business, which will be led by Tristan Orpin.

"Reproductive health remains a key market, and our new organizational structure and leadership will enable us to create a strategic brand position by delivering a more integrative portfolio to our customers," Flatley said.

During the quarter, Illumina struck a three-year supply agreement with Natera, which will run its noninvasive prenatal test Panorama on Illumina's HiSeq 2500.

Illumina will take a slightly different strategy in the oncology market than it has taken so far in the reproductive health market with its acquisition of Verinata Health. Flatley said that within the oncology market, the firm does not plan to take a "proprietary position in particular tests in the oncology market that we would provide through our own laboratory operations."

Instead, Flatley said the Oncology business unit will focus on aspects of sample prep that need to be optimized specifically for the oncology market and on fine tuning software to better decipher cancer genomes.

"It's really a focus on the customer base for oncology, as well as improving the tool set, that this business unit will be focused on," he said.

Additionally, while Illumina's Chief Medical Officer Rick Klausner will serve as interim head of the unit, Flatley said that the firm will be looking to fill the position permanently with someone with

both expertise in cancer genomics, as well as someone with an operational background that can "work on large deal transactions" and "run groups of development teams inside the business unit."

Monica Heger tracks trends in next-generation sequencing for research and clinical applications for GenomeWeb's *In Sequence* and *Clinical Sequencing News*. E-mail Monica Heger or follow her GenomeWeb Twitter accounts at @InSequence and @ClinSeqNews.

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By Ivan Deryugin

Shares of Illumina (ILMN) have risen over 85% since our in-depth November 1, 2012 initiation of coverage, and for good reason: the company has, for several quarters in a row, proven that its core academic markets are steady in the face of continued headline risks, and that it is creating an increasingly diverse revenue base. The company's Q3 results, which included beats on revenue and EPS, as well as raised full-year guidance, highlight that even with its shares at an all-time high, Illumina should remain a facet of a balanced healthcare portfolio.

Broad-Based Growth

For Q3, total revenues grew by 25% to $356.8 million, beating consensus estimates by $13.58 million. EPS grew by 9.76% to $0.45, with costs being the primary driver of the divergence between sales and profits. Gross margins shrunk 30 basis points to 70.2% due to incremental dilution from Illumina's slate of recently acquired companies, as well as a lower mix of consumables (quarters with a higher proportion of system sales do shrink gross margins, but broaden Illumina's installed base, leading to higher consumables sales over time). **But surging R&D spending, which rose by 34.49% to over $61 million, caused the bulk of Illumina's tepid earnings growth.** That, combined with a rise in SG&A tied to expanded sales efforts (to further penetrate international markets, where Illumina has lagged Life Technologies) and other investments, pushed down operating margins to 32.1%, down 270 basis points from Q3 2012, but up 10 basis points sequentially. Naturally, CEO Jay Flatley opened the earnings call with discussion about the company's academic markets. Given the government shutdown, this is a point of concern for investors. However, as in previous quarters, Illumina demonstrated that its academic exposure continues to be an asset for the company, not a liability. Flatley noted that:

> "While the shutdown was very unfortunate for a few of our customers, we have not seen a material impact on spending patterns. Importantly, for most customers, external funding was flowing as expected during the shutdown. With the government now open and operating under a continuing resolution, we're seeing customary purchasing patterns. We expect this to continue going forward as customers and grant allocations maintain preference for sequencing over other technologies, as has been the case for the last few years."

Total sales in North America grew by 25% during the quarter, suggesting that the impact of the shutdown has indeed been muted. This performance places North America in the middle of the geographic pack; sales in Asia-Pacific rose by 22%, but sales in Europe rose by 27%, a figure made all the more impressive by the continent's continued macroeconomic uncertainty.

In addition, Illumina saw strong growth across product lines. System sales grew 21% to $100 million, and we note that Illumina's sales grow in part through a virtuous cycle: the larger its installed base of sequencing systems, the more consumables revenue the company can generate. Total consumables sales grew 22% to $216 million, representing well over 60% of total sales. Crucially, system sales were driven by solid demand for HiSeq systems, both through the completion of HiSeq 2500 upgrades, as well as organic orders, with the top-end HiSeq 2500 accounting for 80% of total orders. MiSeq shipments also saw year-over-year growth, but orders did fall sequentially due to an above average number of multi-unit orders in Q2 2013. Illumina's burgeoning service

segment, covering most of Illumina's recently acquired businesses, also saw solid growth, with total sales rising over 62% to $38.197 million (and up over 17% sequentially). Management noted several pockets of strength within the segment, including demand from agricultural and ordinary customers for genotyping services, as well as maintenance contracts on Illumina's growing installed base. In conjunction with its Q3 results, Illumina raised its guidance for 2013; the company now expects sales to rise by 22%, versus 20% previously, and EPS of $1.76 at the midpoint of its guidance range, versus a prior midpoint of $1.70.

The Road Ahead: Repositioning Illumina for a Genetic Future

But perhaps more important than the numbers themselves is the long-term transformation that has been taking place at Illumina, from a company focused on helping *research* the role of genes and genetics in healthcare, to one focused on helping customers utilize genes and genetics to *treat* patients. As recently as 2012, over 70% of the company's sales were generated from academic customers. In Q3 2013, the percentage of sales from the academic space dropped to 55% for the company as a whole, and just 50% in the HiSeq line. The MiSeq product line already generates a majority of its sales from non-academic customers, with CEO Jay Flately specifically noting that sales to the pharmaceutical industry are accounting for a growing portion of sequencing system sales. Illumina is repositioning itself as not simply a provider of sequencing systems, but as a provider of a genetics ecosystem, via a broad range of systems, as well as a growing ecosystem of applications hosted on BaseSpace. Illumina hosted a meeting of 120 developers at the 2013 ASHG (American Society of Human Genetics) annual meeting (running from October 22-26), and has launched a native API for developers to craft BaseSpace applications. Analysts on Illumina's earnings call pressed for information on BaseSpace, determined to find out if the business is profitable. Management said that BaseSpace revenue is largely immaterial at the moment, but we remind investors that this is not the point of BaseSpace, and if Illumina were focusing on it as a profit center, it would be cause for concern. In any business, focusing on how to generate them does not make great profits. Focusing on running a great business makes great profits. Apple chooses to virtually give away software to drive sales of its hardware, and we believe that in time, BaseSpace could play a similar role for Illumina. Management has made no secret of the fact that it sees BaseSpace as an important aspect of Illumina's business going forward, and for the last several quarters, every single earnings call has featured discussions of BaseSpace. Verinata is also continuing to grow, and on October 14, Illumina announced the release of new options for the verifi test that allow for the testing of fetal aneuploidy in twins. CEO Jay Flatley pledged to deliver more information about Verinata when the company delivers Q4 results in January.

Several days before its earnings release, Illumina disclosed that a meaningful reorganization has been taking place at the company, which we believe highlights its shift towards a future as a commercially focused healthcare company, with a decently sized academic business. First, Jay Flatley is stepping down as President of Illumina in order to spend more time on corporate strategy and vision; a new President will be named in due course. Secondly, Illumina will be reorganized into five separate divisions, each with a clear commercial focus.

- Life Sciences: This will become Illumina's main division, housing its "core platform technologies," as well as products that serve customers in agrogenomics, complex disease, and metagenomics.
- Reproductive & Genetic Health: Verinata, BlueGnome, and Illumina's cytogenetics business will be folded into this division, which will be headed by Illumina's current CCO.
- Oncology: Highlighting Illumina's growing sales to the pharmaceutical and biotechnology industries, this new division will be headed by Illumina's Chief Medical Officer to drive sales across the oncology market, ranging from both research of new drugs to customized treatments for cancer patients.
- Enterprise Informatics: This division will focus on products that offer the ability to store, analyze, and share genetic information; Illumina has said that it will target the growing precision medicine industry.
- New & Emerging Markets: Highlighting Illumina's penchant for tuck-in deals, this division will focus on new genetics markets, and will presumably be authorized to quickly acquire strategic start-ups. On its

earnings call, the company stated that it continues to expect a handful of tuck-in deals each year, and that it regularly looks for new opportunities across the sequencing and genetics industries for potential new deals.

A re-invigorated focus on these 5 markets will serve two purposes. The first is to accelerate Illumina's push into the commercial healthcare sector across a diverse slate of markets, and give these divisions increased autonomy to pursued deals and sales in their respective areas. The 2nd is that a decrease in academic sales (as a proportion of total sales) will serve to quell one of the few remaining points of potential criticism regarding and investment in Illumina. With nearly 19% of the company's float sold short, there is a subset of investors that continue to believe that Illumina's exposure to academic markets is a material risk for the company, and one that will cost it and its investors dearly. And while this may be a risk in the sense that *it could happen*, we believe that Illumina has done as much as any company can in proving the resilience of its academic customers. If a government shutdown and potential default, as well as ongoing stress in Europe cannot derail its academic markets, then what can? And as Illumina's exposure to the academic sector continues to fall, the importance of this issue will decline.

Competition: Managing Emerging Threats

For much of 2013, Illumina has seen a confluence of positive news regarding its competitors. Life Technologies has been sold to Thermo Fisher (TMO), a company that is not known for its furious pace of R&D innovation (the acquisition is on track to close in early 2014). As a frame of reference, Thermo Fisher spent just 3% of revenue on R&D in Q3 2013, versus 17.18% for Illumina. Pacific Biosciences of California (PACB) has been struggling to deploy its sequencing systems, and Oxford Nanopore has seen one delay after another. However, in recent weeks, there have been several developments involving Illumina's competitors. On September 25, Pacific Biosciences and Roche (RHHBY) inked a new agreement to develop and sell new sequencing systems. For Roche, this represents perhaps the last remaining opportunity to make a meaningful mark in genetic sequencing, other than conceding and attempting to buy Illumina once again. Given Roche's string of failures in sequencing (most recently, Roche scrapped a number of sequencing projects in April), it can be assumed that Roche recognizes that it needs to succeed with this collaboration. As part of the deal, Pacific Biosciences will receive $35 million upfront, up to $40 million in development milestones, and then royalties tied to sales of instruments, software, and consumables. Although Pacific Biosciences management team has said it has seen an increase in interest in its sequencing platforms after the Roche deal was announced, it remains to be seen if Roche can succeed where Pacific Biosciences has not; the company installed six 6 PacBio RS II systems, down from seven sequentially. We will be looking at news flow from Pacific Biosciences closely in the months to come to see if the deal with Roche leads to a change in its competitive position.

Oxford Nanopore has also seen progress, and is finally poised to release its MiniION platform to customers, albeit on a highly limited scale (as a reminder, Illumina owns 15% of Oxford Nanopore). Beginning in late November, researchers can request to receive a MiniION sequencer after paying a $1,000 deposit, which will be returned after Oxford Nanopore receives the MiniION. As with Pacific Biosciences, the impact of Oxford's move is unlikely to be material for Illumina. If anything, this may be a negative for Pacific Biosciences, given that Oxford's technology also utilizes the high read-length (but low throughput) technology of Pacific Biosciences. First, Illumina owns 15% of the company, allowing it to participate in at least some of Oxford's upside potential. Second, Illumina may move to acquire the reminder of Oxford should it see a need to do so. We note that as part of recent arbitration proceedings related to its 2009 collaboration agreement, Illumina has, until the end of 2016, the right to match any offer made for Oxford Nanopore's assets, including offers for its Nanopore sequencing technology (however, Oxford's CEO disputed comments that suggested that Illumina has the right to match offers made for shares of Oxford Nanopore). In addition, Illumina inked a licensing deal with the University of Alabama and the University of Washington this month related to Nanopore sequencing technology developed by scientists at the universities. The deal gives Illumina exclusive global rights to develop products based on this technology, and with 50% of Illumina's R&D spending devoted to its sequencing platforms, it is all but certain that Illumina is

examining ways to develop commercial-stage Nanopore sequencing technology of its own.

Conclusions

Even at all-time highs, we believe ILMN continues to offer long-term appeal for investors. The company is positioning itself for the next stage in its corporate life cycle - that of a commercial healthcare company. Illumina's reorganization should allow it to more rapidly pursue that vision, and its commitment to research & development and staying at the forefront of genetic sequencing has not wavered, as evidenced by an increase of over 30% in R&D spending. For the time being, Illumina's competitive position remains solid, as are its growth prospects in the remainder of 2013 and 2014. We expect the company's revenue diversification and sequencing innovation to continue in 2014, and believe that Illumina is far from reaching its full potential in the global genetics and sequencing markets.

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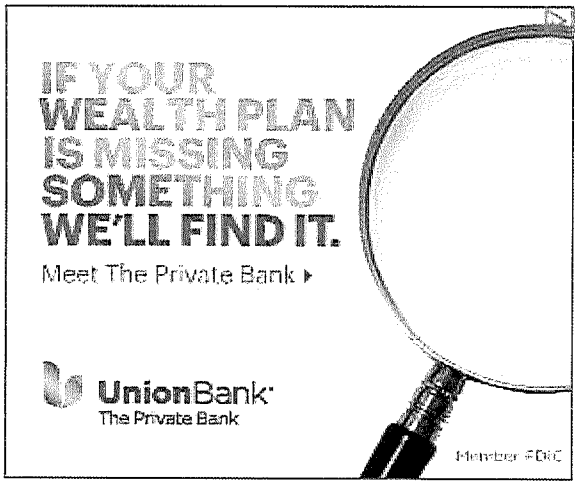

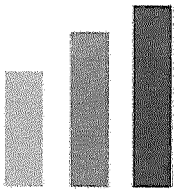

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RWK	-0.5%	3.6%	39.4%	33.2%
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PRFZ	-0.6%	5.0%	40.5%	32.1%
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MULTI ASSET & ALT COMPARISON

	TTM Total Return
Illumina, Inc. (**ILMN**)	0%

	TTM Total Return
Oppenheimer SteelPath MLP Alpha Fd	19.66%
Oppenheimer Capital Income Fund	6.37%
Oppenheimer Global Multi Strgy Fund	1.34%

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ILLUM-1606

Exhibit 33

Penn Medicine

Penn Medicine's
Center for Personalized Diagnostics

Precision Diagnostics for Personalized Medicine

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Center for Personalized Diagnostics

The Center for Personalized Diagnostics (CPD), a joint initiative between Penn Medicine's Department of Pathology and Laboratory Medicine and the Abramson Cancer Center, integrates Molecular Genetics, Pathology Informatics, and Genomic Pathology for individualized patient diagnoses and to elucidate cancer treatment options for physicians.

Video on The Future of Pathology at Penn

The Department of Pathology and Laboratory Medicine is at the leading edge of personalized medicine with its Center for Personalized Diagnostics and the Clinical Cell and Vaccine Production Facility

Watch Video

Massively parallel DNA sequencing has made it possible to identify a set of molecular changes and genomic signatures in the DNA of tumor cells that are distinct for tumor subtypes, that are not readily elucidated on the microscopic level—and that can reveal a genetic blueprint of the patient's tumor as discrete and singular as a fingerprint.

Because these mutation signatures can indicate whether and how a patient will respond to treatment, the CPD empowers physicians and patients to custom-tailor and individualize their treatment options accordingly.

Genomic Testing and Therapeutic Pathology

ILLUM-1838

The CAP/CLIA certified Center enables personalized therapies by offering genome-wide precision diagnostics to identify the specific tumor characteristics for each patient. In fact, of all the tumor patients tested to date, approximately 75% of them have received genomic testing results that altered prognosis or empowered the treating oncologists to alter the patients' treatment therapy or both.

The CPD will also serve as a cancer research center to detect clinical correlations for treatment and outcome, with the goal to identify prognostic markers for future diagnostic and treatment options.

The CPD is tightly integrated with the clinical mission at Penn medicine and works in close concert with oncologists and other physicians. In addition, the CPD leadership is part of the Abramson Cancer Center experimental therapeutics tumor board.

The instrumentation includes the Illumina HiSeq 2500, two Illumina MiSeq sequencers and a Personal Genome machine, all of which are instrumental in the detection and sequencing of genomic DNA and capable of detecting low-level mutation loads in tumor specimens.

The CPD was created to integrate genomic and pathologic information for clinical benefit on the basis of customized computational methods. These developments combine bio-informatics, genomic data and clinical knowledge in order to develop a comprehensive, personalized therapeutic approach for patients and their physicians.

This "next-generation" genomic sequencing technology provides the foundation for any form of truly personalized medicine by making individualized cancer mutation detection and predictive testing for cancer therapies possible.

Clinical Genetic Tests and Tumor Biomarkers

The current focus of the Center will be to report clinically useful mutations from specific targeted disease areas:

- **Hematologic malignancies** (including: leukemias)
- **Solid tumors** (including: brain, melanoma, and lung tumors)

In the near future, these tests will be expanded to include a wider range of malignancies so that all patients can benefit from the increased scrutiny of mutations present in tumor tissue.

The goal is to prospectively collect genomic mutation profiles and follow patients over time, so that the CPD can aid clinical researchers in the identification of new markers/mutation profiles to better predict the course of an individual patient's treatment response and suggest new targets for therapy. Additionally, the gene panels will be modified and expanded as new mutations and treatments are found, creating a dynamic process of mutation profiling with medically actionable targets.

We've Moved!

The CPD has found a new home in a state-of-the-art clinical genomics testing center at 3020 Market Street, Philadelphia, PA

Share this Page with a Friend:

Genomic Testing at Penn Medicine

- Highest Volume of Testing in the Region
- Fastest Turnaround Time (Average 10-14 Days)
- Actionable Findings with Therapeutic Significance (disease-associated genome mutations are found in 75% of tests performed)
- User-Friendly Reports Focus on Actionable Information
- Unique Custom Hematologic Malignancy Panel
- Custom Bioinformatics Allow Higher Sensitivity and Fewer False Negative Results
- Integrated Clinical and Translational Expertise of a Major Academic Medical Center

- Download CPD Brochure
- Download Test Requisition Form

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Exhibit 34

(/subsc

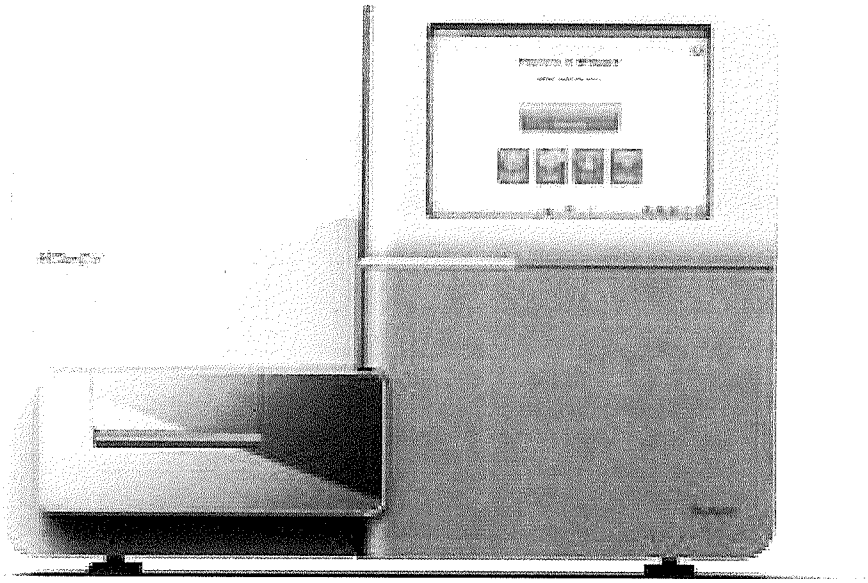
NEW HOMES U-T San Diego

BIOTECH

Illumina scores medical sequencing breakthrough

San Diego genome giant's gear first to be used for regular patient care

Bradley J. Fikes • U-T 08:05p.m. Nov 26, 2013



ILLUM-1590

(/photos/2013/nov/20/1158039/)

Gregory F. Heath, Ph.D., Illumina Senior Vice President & General Manager, Diagnostics. Courtesy photo

Increasingly powerful DNA sequencers have uncovered a mountain of data on how changes in the human genome affect health and disease. These "next-generation" instruments have greatly speeded up genome sequencing, while bringing down the price from hundreds of millions to as little as $5,000.

But few patients have benefited from this powerful technology, because the sequencers haven't been approved for clinical applications. They are used in research laboratories and only in rare circumstances have been used to guide treatment.

Thanks to a historic decision by the U.S. Food and Drug Administration, that knowledge will now be brought directly to patients as part of normal medical care. And the first company to do so will be Illumina, the San Diego-based DNA sequencing giant.

Illumina received FDA approval last week to sell its new next-generation sequencer for clinical use. The FDA also approved two tests for cystic fibrosis to be performed on the machine, and perhaps most significantly, a kit to help clinical laboratories develop their own tests for the sequencer. European regulators gave approval about two months ago for the instrument, called the MiSeqDx.

Using the kit, laboratories can develop innumerable tests, said Greg Heath, Illumina's senior vice president and general manager of diagnostics.

"The uses are limited only by their creativity," Heath said.

The approval's significance was highlighted by an article in the New England Journal of Medicine. The authors: Francis Collins, director of the National Institutes of Health, and Margaret Hamburg, FDA commissioner.

"Clinicians can selectively look for an almost unlimited number of genetic changes that may be of medical significance," Collins and Hamburg wrote. "Access to these data opens the door for the transformation of research, clinical care and patient engagement."

EXPANDING FIELD

Next-generation is a collective term for technologies that harness microelectronics to analyze genomes in a massively parallel fashion. The instruments use miniaturized devices to study the genome at many places at once. This not only speeds up analysis, it lowers prices, in part because the cost of the instruments can be amortized much more quickly.

Lower price means more patients can benefit. Increased speed means genome scanning can be performed in an increasingly routine fashion. The combination means that research knowledge can become part of daily patient care.

And for Illumina, more sales of sequencers means more revenue. As confidence in next-generation sequencing's future has grown, so has Illumina's market value, which now exceeds $12 billion.

Shares of Illumina closed Tuesday at $98.29. On Monday, shares briefly exceeded $100 apiece.

Illumina's market value was less than half that in January 2012, when Swiss drug giant Roche offered to buy Illumina for $44.50 per share, or about $5.7 billion. On Dec. 21, the day before rumors about the potential purchase surfaced, Illumina shares were trading for $27.17.

Illumina turned down that offer, and a sweetened offer of $51 per share, as far below the company's true value.

One of the few ways Illumina's research technology has reached the public is through the gene-testing company 23andMe, which uses Illumina sequencers. But that use was halted Monday when the FDA ordered the Mountain View company to stop selling its tests. The company said the tests are meant for guidance, not for medical diagnosis. But the FDA said 23andMe needs to prove clinical accuracy. Approval of Illumina's new clinical-grade sequencer offers another path to the market, with formal regulatory blessing.

The approval gives Illumina increased access to a vast and growing clinical diagnostics market, said John McCamant, editor of the Berkeley-based Medical Technology Stock Letter.

NATURAL COMPLEMENT

Clinical diagnostics are a natural complement to drugs, McCamant said. More accurate diagnostics is key to applying what's often called personalized medicine, in which treatments are matched with an individual's biological makeup.

Illumina's cystic fibrosis tests should pair nicely with a new drug being tested by Vertex Pharmaceuticals, McCamant said. There is no cure for cystic fibrosis, and no drug treats the underlying cause, mutations that cause thick mucus to build up in the lungs.

Illumina and its Carlsbad-based rival Life Technologies dominate next-generation sequencing, with a number of smaller competitors vying to leapfrog with other technologies.

Illumina, as its name implies, uses light-based methods to detect DNA sequences. An alternative that has received much attention in recent years directly detects sequences on a computer chip.

The technology was developed by Ion Torrent, now a division of Life Technologies. It is the basis of Life Tech's Personal Genome Machine and the newer Ion Proton next-generation sequencer.

However, the technology needs further development to compete with Illumina, according to a Nov. 11 analyst report from the research and investment banking company Leerink Swann. The Ion Proton device produces three times the error rate of the Personal Genome Machine, according to the report. Life Tech is working on Ion Proton improvements.

A smaller competitor, Oxford Nanopore, sequences DNA and proteins by sending them through an extremely small opening called a nanopore, and measuring changes in how electricity is transmitted as each letter, or base pair, is passed through. Illumina has long had a relationship with Oxford Nanopore, but on Nov. 15 Oxford said Illumina sold its 13.5 percent equity stake to other investors.

Other competitors include Complete Genomics, based in Mountain View; and Pacific Biosciences of Menlo Park.

GENE TO GENOME

Genetic diagnostics began when Linus Pauling discovered a mutation that causes sickle-cell anemia and in the process

described how to think of disease in a new way. Pauling co-wrote a paper on the discovery published in Science in 1949. Its title: "Sickle Cell Anemia, a Molecular Disease."

But the original technology was slow, requiring tedious work to find a disease-causing mutation in just one gene. Exploring the entire 3 billion letters of the human genome was unthinkable. And since genes make up only a small percentage of the genome, scientists focused on that portion, assuming most of the rest had no clinical significance.

Next-generation sequencing, pioneered in the late 1990s, changed that view. It was the product of competition between a government-led consortium and a private effort by Celera Genomics, headed by gene pioneer J. Craig Venter. The competition accelerated efforts on both sides, resulting in the draft of the first human genome, announced in June 2000.

Since then, the story of sequencing has been one of speed, lowered costs and discoveries in hitherto unknown areas. In recent years, the trend has surpassed Moore's Law, the famous statement of Intel founder Gordon Moore that predicted an exponential increase in the number of transistors on semiconductor chips, with a lowered cost.

This newfound ability to plumb the genome at will opens up possibilities that weren't practical before. Already, the genomes of numerous individuals have been sequenced to determine which variants are associated with physical traits. This is called a genome-wide association study. Its power is that GWAS can find variants in any part of the genome, not just in preselected genes.

Cancer will be one of the biggest applications of clinical next-generation sequencing, Heath said. Cancer cells are characterized by unstable genomes that mutate frequently. Some of these mutations fall into predictable patterns that "drive" cancer development. Next-generation sequencing allows frequent sequencing of a patient's cancer to track its progress.

Collins and Hamburg discussed this use in their article in the New England Journal of Medicine. "For instance, lung adenocarcinoma can now be divided into subtypes with unique genomic fingerprints associated with different outcomes and different responses to particular therapies," they wrote. "More broadly, recent work from the Cancer Genome Atlas demonstrates that the tissue of origin of a particular cancer may be much less relevant to prognosis and therapy than the array of causative mutations."

Moreover, next-generation sequencers routinely uncover previously unsuspected relationships between diseases and mutations in the great majority of the genome that has no genes. Much of these "non-coding" stretches of DNA have no identifiable function, but that proportion is less than originally thought.

Last year, a massive genome study called ENCODE used Illumina sequencers to determine that at least 20 percent of the human genome is functional. It set an upper limit of 80 percent of the genome that shows biochemical activity that may be functional. The actual number, which lies in between the two, will need more research to determine. That means more work for the researchers — and more business for Illumina.

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ILLUM-1593

Company wins grant for a better condom

(/news/2013/nov/26/gates-foundation-award-apex/)

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Exhibit 35

GEN
Genetic Engineering
& Biotechnology News

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GEN News Highlights : Jan 16, 2014

Illumina, Amgen to Develop CDx for Colorectal Cancer

Illumina is following up its big news earlier this week that its new HiSeq X10 system can make the $1,000 genome possible by announcing a partnership with Amgen to develop and commercialize a multigene, next-generation sequencing-based test as a companion diagnostic for Amgen's anticancer drug Vectibix (panitumumab). The firms plan to seek premarket approval of the test by the FDA and other regulatory agencies per the agreement.

Vectibix is a fully human anti-EGFR monoclonal antibody therapeutic designed to treat metastatic colorectal cancer. The aim of the collab, according to the companies, will be to validate a test platform that can identify the RAS mutation status of patients who would be good candidates for treatment with Vectibix. The test will be developed for use with Illumina's MiSeqDx™ instrument, and Illumina plans to commercialize the test with a focus on U.S. and EU markets pending regulatory approval in both locations.

Nick Naclerio, svp, corporate and venture development and general manager of Illumina's Enterprise Informatics, said in a statement that this agreement is part of the company's strategy to bring NGS to the clinical diagnostics field.

"With three FDA-cleared NGS products in our portfolio, we intend to complement internal development programs by taking products developed with external partners through the FDA submission process. Amgen is a key partner given their leadership in therapeutic development and strong track record in commercializing novel products," he added.

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Exhibit 36

510(k) Premarket Notification[6]

New Search Back To Search Results

Device Classification Name	Instrumentation For Clinical Multiplex Test Systems[20]
510(K) Number	K093128
Device Name	ILLUMINA BEADXPRESS SYSTEM
Applicant	ILLUMINA, INC. 5200 Illumina Way San Diego, CA 92122
Contact	Donald R Ellis
Regulation Number	862.2570[21]
Classification Product Code	NSU[22]
Date Received	10/02/2009
Decision Date	04/28/2010
Decision	Substantially Equivalent (SE)
Classification Advisory Committee	Clinical Chemistry
Review Advisory Committee	Hematology
Statement	Statement[23]
FDA Review	Decision Summary[24]
Type	Traditional
Clinical Trials	NCT00959504[25]
Reviewed By Third Party	No
Expedited Review	No
Combination Product	No

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CFR Title 21[14]|Radiation-Emitting Products[15]|X-Ray Assembler[16]|Medsun Reports[17]|CLIA[18]|TPLC[19]

New Search Back To Search Results

Device Classification Name	Test, Factor V Leiden Mutations, Genomic Dna Pcr[20]
510(K) Number	K093129
Device Name	ILLUMINA VERNCODE GENOTYPING TEST FOR FACOR V AND FACTOR II
Applicant	ILLUMINA, INC. 9865 Towne Centre Drive San Diego, CA 92121 1975
Contact	Donald R Ellis
Regulation Number	864.7280[21]
Classification Product Code	NPQ[22]
Subsequent Product Code	NPR[23]
Date Received	10/02/2009
Decision Date	04/28/2010
Decision	Substantially Equivalent - CLIA Submission (CS)
Classification Advisory Committee	Hematology
Review Advisory Committee	Hematology
Statement	Statement[24]
FDA Review	Decision Summary[25]
Type	Traditional
Clinical Trials	NCT00959504[26]
Reviewed By Third Party	No
Expedited Review	No
Combination Product	No

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Exhibit 37

Top News :

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Illumina Receives FDA 510(k) Clearance for Its BeadXpress® Multiplex Analysis System

Tuesday, May 04, 2010 6:57 AM

(Source: Business Wire)Illumina, Inc. (NASDAQ:ILMN) today announced that the U.S. Food and Drug Administration (FDA) has granted 510(k) market clearance for the company's BeadXpress system for multiplex genetic analysis. According to the FDA's indications of use, the BeadXpress system -- consisting of Illumina's BeadXpress Reader and VeraScan software -- is an in-vitro diagnostic device intended for the simultaneous detection of multiple analytes in a DNA sample utilizing VeraCode holographic microbead technology.

"This approval represents a significant and exciting transitional step for Illumina into the diagnostics field, where the potential is great for molecular medicine to make a real difference in the way disease is detected and ultimately prevented and treated," said Jay Flatley, president and CEO. "It demonstrates Illumina's ability to meet stringent regulatory requirements in designing and manufacturing an FDA-cleared in-vitro diagnostic device. This will serve as an important foundation for our future plans in the diagnostics area. Ultimately, our goal is to become a leader in translational medicine, focusing on complex diseases that benefit from high performance analysis, including genotyping, copy number, gene expression, methylation and protein analysis."

Illumina introduced the BeadXpress system in 2007 with Research Use Only kits for custom genotyping, gene expression, methylation and protein analysis. Since then it has been adopted by research, agricultural, industrial and pharmaceutical institutions worldwide. Utilizing uniquely inscribed digital microbeads, VeraCode technology provides high-quality data, broad multiplexing capability and assay flexibility. Illumina submitted the system for FDA review in September 2009.

"510(k) clearance opens up a wide range of new possibilities for our many clinical research and commercial partners, who can now pursue diagnostic development on our proven, high-performance BeadXpress platform," said Gregory Heath, Ph.D., senior vice president and general manager, Diagnostics. One of those partners is EraGen Biosciences, Inc., which concluded a licensing agreement with Illumina in 2009 to transfer their assays onto the BeadXpress System. "This clearance is a significant step forward in progressing our partnership in the clinical marketplace," said Irene Hrusovsky, M.D., president and CEO of EraGen Biosciences.

ILLUM-0658

For more information, please visit http://www.illumina.com.

About Illumina

Illumina (http://www.illumina.com) is the leading developer, manufacturer, and marketer of integrated systems for the analysis of genetic variation and biological function. Using our proprietary technologies, we provide a comprehensive line of products and services that currently serve the sequencing, genotyping, and gene expression markets, and we expect to enter the market for molecular diagnostics. Our customers include leading genomic research centers, pharmaceutical companies, academic institutions, clinical research organizations, and biotechnology companies. Our tools provide researchers around the world with the performance, throughput, cost effectiveness, and flexibility necessary to perform the billions of genetic tests needed to extract valuable medical information from advances in genomics and proteomics. We believe this information will enable researchers to correlate genetic variation and biological function, which will enhance drug discovery and clinical research, allow diseases to be detected earlier, and permit better choices of drugs for individual patients.

Forward-Looking Statements

This release contains forward-looking statements that involve risks and uncertainties. Important factors that could cause actual results to differ materially from those in any forward-looking statements include challenges inherent in new product development and manufacturing and the other factors detailed in our filings with the Securities and Exchange Commission, including our most recent filings on Forms 10-K and 10-Q, or in information disclosed in public conference calls, the date and time of which are released beforehand. We undertake no obligation, and do not intend, to update any forward-looking statements after the date of this release.

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The sector scan is based on 15-30 minutes delayed data. The Pattern scan is based on EOD data.

ILLUM-0659

Exhibit 38

BusinessWire
A Berkshire Hathaway Company

May 18, 2010 04:05 PM Eastern Daylight Time

Illumina Receives FDA 510(k) Clearance for VeraCode® Genotyping Test for Factor V (Leiden) and Factor II (Prothrombin)

SAN DIEGO--(BUSINESS WIRE)--Illumina, Inc. (NASDAQ:ILMN) today announced that the U.S. Food and Drug Administration (FDA) has granted 510(k) market clearance for the VeraCode® Genotyping Test for Factor V (Leiden) and Factor II (Prothrombin). The VeraCode Genotyping Test is comprised of the company's VeraCode digital microbead technology and proprietary assay chemistry. The BeadXpress® System, which received a separate 510(k) market clearance, is FDA-cleared for *in vitro* diagnostic use only with the VeraCode Genotyping Test for Factor V and Factor II.

About Illumina

Illumina (http://www.illumina.com) is the leading developer, manufacturer, and marketer of integrated systems for the analysis of genetic variation and biological function. Using our proprietary technologies, we provide a comprehensive line of products and services that currently serve the sequencing, genotyping, and gene expression markets, and we expect to enter the market for molecular diagnostics. Our customers include leading genomic research centers, pharmaceutical companies, academic institutions, clinical research organizations, and biotechnology companies. Our tools provide researchers around the world with the performance, throughput, cost effectiveness, and flexibility necessary to perform the billions of genetic tests needed to extract valuable medical information from advances in genomics and proteomics. We believe this information will enable researchers to correlate genetic variation and biological function, which will enhance drug discovery and clinical research, allow diseases to be detected earlier, and permit better choices of drugs for individual patients.

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ILLUM-0661

Exhibit 39

FDA NEWS RELEASE

For Immediate Release: Nov. 19, 2013
Media Inquiries: Susan Laine, 301-796-5349, susan.laine@fda.hhs.gov
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FDA allows marketing of four "next generation" gene sequencing devices
Two devices aid in screening and diagnosis of cystic fibrosis

Today the U.S. Food and Drug Administration allowed marketing of four diagnostic devices that can be used for high throughput gene sequencing, often referred to as "next generation sequencing" (NGS). These instruments, reagents, and test systems allow labs to sequence a patient's DNA (deoxyribonucleic acid).

The new technology also gives physicians the ability to take a broader look at their patients' genetic makeup and can help in diagnosing disease or identifying the cause of symptoms.

"NGS is changing the way we look at genomics," said Alberto Gutierrez, Ph.D., director of the Office of In Vitro Diagnostics and Radiological Health in FDA's Center for Devices and Radiological Health. "Before NGS, sequencing genes associated with a particular disease was a long and costly process. Today, we have the capability to read and interpret large segments of DNA very quickly in a single test and this information-rich technology is becoming more accessible for use by physicians in the care of their patients."

Two of the newly cleared devices are used to detect DNA changes in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which can result in cystic fibrosis (CF), an inherited chronic disease that affects the lungs, pancreas, liver, intestines, and other organs of those who inherit a faulty CFTR gene from both parents.

More than 10 million Americans are CF carriers and approximately 30,000 children and adults in the U.S. are affected with CF. Most children with CF are diagnosed by age 2 and the average life span for people with CF who live to adulthood is approximately 37 years.

The cleared devices include:

- The Illumina MiSeqDx Cystic Fibrosis 139-Variant Assay, which checks specific points in the patient's CFTR gene sequence to detect known variants in the gene. Information about which DNA changes are associated with symptoms of cystic fibrosis is found in the Clinical and Functional TRanslation of CFTR database (CFTR2).
- The Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay, which sequences a large portion of the CFTR gene to detect any difference in the CFTR gene compared to a reference CFTR gene.

Data submitted by Illumina for their cystic fibrosis tests included comparisons of the sequence results to Human Genome Build 19, a reference representation of the human genome. In addition, Illumina evaluated the performance of its instrument and reagent systems against a publically available quality-weighted human reference genome that was created through collaboration between the FDA and the National Institutes of Standards and Technology (NIST).

FDA authorized sequencing devices provide labs with quality and performance information

The FDA also granted de novo petitions for the Illumina MiSeqDx instrument platform and the Illumina Universal Kit reagents, two devices that make up the first FDA-regulated test system that allows laboratories to develop and validate sequencing of any part of a patient's genome. The Universal Kit reagents isolate and create copies of genes of interest obtained from patient blood samples, and the MiSeqDx platform analyzes the genes. The software compares the patient's genomic sequence to a reference sequence and reports back any differences between the patient and the reference.

"The FDA's review of the MiSeqDx and sequencer and Universal Kit reagents provides clinical laboratories with information about the expected performance of the device and the quality of the results," said Dr. Gutierrez. "This information was not previously available for next generation sequencers, and, with this platform, labs can develop tests for clinical use with greater confidence because they use FDA authorized devices."

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The FDA reviewed the Illumina MiSeqDx instrument platform and the Illumina Universal Kit reagents through its de novo classification process, a regulatory pathway for some novel low-to-moderate risk medical devices that are not substantially equivalent to an already legally marketed device.

For the de novo petitions, the FDA based its decision on the demonstrated performance of the MiSeqDx instrument and Universal Kit reagent systems across numerous genomic segments spanning 19 human chromosomes.

Illumina MiSeqDx instrument platform, Universal Kit reagents, MiSeqDx Cystic Fibrosis 139-Variant Assay, and MiSeqDx Cystic Fibrosis Clinical Sequencing Assay are manufactured by Illumina, Inc. in San Diego, Calif.

Relevant Web Links:

FDA: Medical Devices

NIH: What is Cystic Fibrosis?

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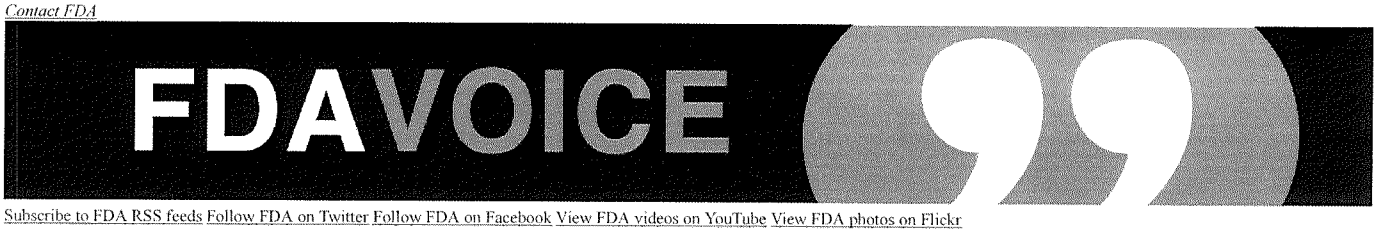

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Reviews

Microarray-Based Genomic DNA Profiling Technologies in Clinical Molecular Diagnostics

Yiping Shen[1,2,3] and Bai-Lin Wu[1,3,4*]

BACKGROUND: Microarray-based genomic DNA profiling (MGDP) technologies are rapidly moving from translational research to clinical diagnostics and have revolutionized medical practices. Such technologies have shown great advantages in detecting genomic imbalances associated with genomic disorders and single-gene diseases.

CONTENT: We discuss the development and applications of the major array platforms that are being used in both academic and commercial laboratories. Although no standardized platform is expected to emerge soon, comprehensive oligonucleotide microarray platforms—both comparative genomic hybridization arrays and genotyping hybrid arrays—are rapidly becoming the methods of choice for their demonstrated analytical validity in detecting genomic imbalances, for their flexibility in incorporating customized designs and updates, and for the advantage of being easily manufactured. Copy number variants (CNVs), the form of genomic deletions/duplications detected through MGDP, are a common etiology for a variety of clinical phenotypes. The widespread distribution of CNVs poses great challenges in interpretation. A broad survey of CNVs in the healthy population, combined with the data accumulated from the patient population in clinical laboratories, will provide a better understanding of the nature of CNVs and enhance the power of identifying genetic risk factors for medical conditions.

SUMMARY: MGDP technologies for molecular diagnostics are still at an early stage but are rapidly evolving. We are in the process of extensive clinical validation and utility evaluation of different array designs and technical platforms. CNVs of currently unknown importance will be a rich source of novel discoveries.
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Human DNA mutations range from single-nucleotide changes to whole-chromosome alterations. At the small-size end of the mutation spectrum, de novo changes in single base pairs occur at the rate of about 1.7×10^{-8} per base pair per generation *(1)*. It is estimated that there are 2 nonsilent point mutations in each newborn *(2)*. At the large-size end of the mutation spectrum, alterations in chromosome number causing aneuploidy affect about 0.3% of live births *(3)*. In between are microscopic and submicroscopic genomic rearrangements involving different segments of chromosomes that may affect many genes or exons of a gene. The de novo rate of mutation of copy number variants (CNVs)[5] in each newborn has been estimated as 1 in 8 for deletion and 1 in 50 for duplication *(2)*, which are between the rates for de novo point mutations and chromosomal aneuploidy. Recent studies have shown that the human genome contains many CNVs *(4–7)*, which are the result of a distant (inherited) or current (de novo) loss or gain of genomic sequences.

A portion of these alterations will produce a change in the human phenotype and in extreme cases will cause abnormal development and medical conditions. The purpose of clinical genetic diagnostics is to detect such mutations effectively and to correlate these changes with corresponding medical conditions. Various methods have different resolutions that reveal different sizes of genomic imbalance (Fig. 1).

Point mutations can be detected effectively with the Sanger sequencing method after specific amplification of target regions by the PCR. The detection of chromosomal aneuploidy and large rearrangements are within the repertoire of traditional cytogenetics.

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[5] Nonstandard abbreviations: CNV, copy number variant; FISH, fluorescence in situ hybridization; MLPA, multiplex ligation-dependent probe amplification; MGDP, microarray-based genomic DNA profiling; CGH, comparative genomic hybridization; SNP, single-nucleotide polymorphism; BAC, bacterial artificial chromosome; UPD, uniparental disomy; ASD, autism spectrum disorder; pCNV, pathogenic CNV; bCNV, benign CNV; uCNV, CNV of unknown importance.

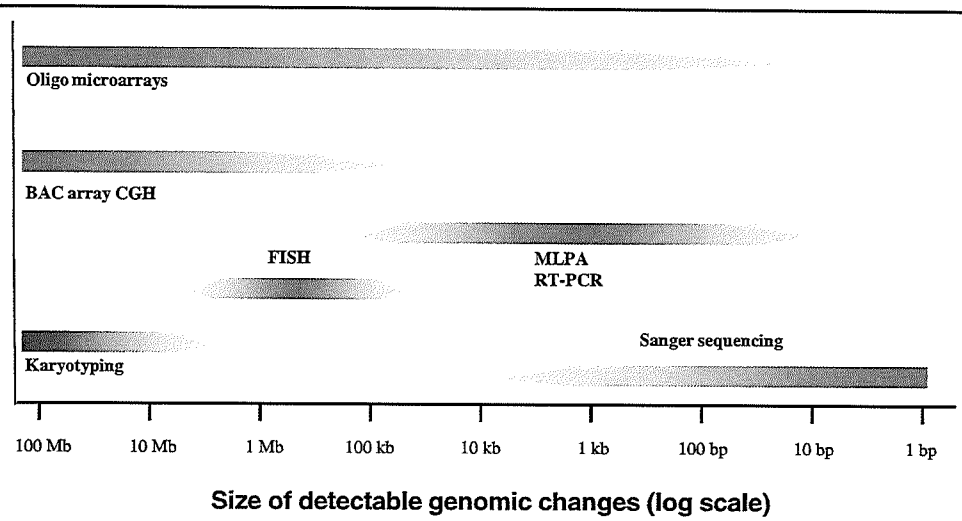

Fig. 1. Various methods with different resolutions detect different sizes of genomic changes.
Point mutations (changes in single or multiple base pairs up to kilobase pairs) can be detected with Sanger DNA sequencing. Karyotyping can detect large chromosomal rearrangements with low resolution (down to 5 Mb). FISH provides much higher resolution (down to 100 kb) for detecting submicroscopic genomic imbalances, but it interrogates only selected loci at one time. MLPA and real-time PCR (RT-PCR) complement FISH and DNA sequencing, but all of these methods suffer from a low information content, limited throughput, and the requirement of choosing candidate targets before the test. Microarrays [BAC and oligonucleotide (oligo)] are capable of detecting both microscopic and submicroscopic copy number changes for the whole genome in a single assay. The high-resolution microarrays (oligo CGH arrays and SNP hybrid arrays) provide the most efficient means to identify genomic imbalance widely (down to kilobase pairs and approaching base pair resolution).

Fluorescence in situ hybridization (FISH) represents a merging of cytogenetic techniques with molecular technology. Such a merger and its applications in the clinical setting were necessitated by the discovery of a set of submicroscopic genomic imbalances associated with specific clinical manifestations (collectively known as microdeletion and microduplication syndromes), but this method interrogates only selected loci at a time. Other techniques designed to detect copy number changes, such as multiplex ligation-dependent probe amplification (MLPA) and real-time PCR, are complementary to those of FISH and DNA sequencing, but all of these methods suffer from low information content, limited throughput, and the requirement of choosing candidate targets before the test.

Microarray-based genomic DNA profiling (MGDP) technologies are capable of detecting both microscopic and submicroscopic copy number changes for the whole genome in a single assay. They provide unprecedented sensitivity and cost-effectiveness for a large group of mutations that have evaded conventional approaches, and they are changing clinical practices. MGDP may be used as a first-tier tool in clinical genetics for many conditions previously evaluated via conventional cytogenetic approaches. We review the current status and some considerations regarding the clinical applications of MGDP technologies.

Development of Genomic DNA Profiling Microarrays

Microarrays have been widely used for gene expression analysis in the past decade; however, it is worth mentioning that DNA microarrays were initially designed for interrogating vast amounts of genomic sequence polymorphisms and variants. The intention to use microarrays for diagnostic purposes was deeply rooted from the very beginning. There are 2 major microarray platforms for genomic DNA profiling—comparative genomic hybridization (CGH) arrays and genotyping arrays—which were developed in parallel yet interactively. Regardless of the technical differences in chip manufacturing and probe types used with these platforms, they share the same principle and rely on the specific hybridization of target and probe sequences. CGH arrays use a 2-color scheme. The method infers the copy number changes in a test sample by comparing it with a reference sample. Genotyping arrays, on the other hand, do not use a control sample; rather, they use the intensity of the hybridization signal to in-

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dicate the relative DNA copy number. In addition, genotyping arrays provide information on single-nucleotide polymorphism (SNP) genotypes.

TRANSFORMATION OF CGH MICROARRAYS FROM LOW-RESOLUTION TARGETED BAC ARRAYS TOWARD WHOLE-GENOME HIGH-DENSITY OLIGONUCLEOTIDE ARRAYS

CGH was first reported by Kallioniemi et al. in 1992 to interrogate cancer genomic DNA with metaphase chromosomes as probes *(8)*. The basic strategy of the technique is to differentially label the DNA from cancer cells and the DNA from healthy reference cells with different fluorochromes and to cohybridize the labeled samples to a metaphase spread from a healthy reference cell. The ratio of the intensities of the 2 fluorochromes reflects the copy number differences between cancer cells and healthy cells. Because this technique uses metaphase chromosomes as probes for hybridization, it is now also called "chromosome CGH." In essence, chromosome CGH is similar to FISH painting, which uses labeled DNA from the entire genome as the probe set. This technology permits a genome-wide survey and identifies the genomic imbalance at different regions of specific chromosomes. Although chromosome CGH has demonstrated its effectiveness in detecting larger genomic imbalances in the cancer genome, the detection power has ultimately been limited by the resolution of a metaphase chromosome.

The major breakthrough in CGH came from microarray technology about a decade ago. Instead of hybridizing labeled genomic targets to metaphase chromosomes, the new scheme uses cloned genomic DNA as probes in a microarray format. Because these probes contain sequence information that permits their specific localization in the human genome, the regions with a genomic imbalance can be delineated by data-visualization software that denotes all of the probes along the genome. The resolution of microarray-based CGH, which is determined by the density and size of the probe, is a substantial improvement over chromosome CGH.

The first CGH microarray, termed matrix CGH, was developed in 1997 by Solinas-Toldo et al. with cosmid and plasmid artificial chromosome clones as probes *(9)*. Subsequently, bacterial artificial chromosome (BAC) clones *(10)* and cDNA clones *(11)* were used to construct CGH microarrays. Subsequent improvements were focused on increasing the probe density and array coverage, as well as improving the signal-to-noise ratio.

The most recent advancement in CGH microarrays has been the use of oligonucleotide sequences as probes *(12)*. Compared with BAC arrays, oligonucleotide arrays have several advantages, including:

1. Reproducibility. As opposed to BAC arrays, in which the content of the probe (i.e., PCR product of a BAC clone) varies from batch to batch, the probe sequences in oligonucleotide arrays are uniformly defined and devoid of highly repetitive sequences. Consequently, oligonucleotide arrays are more reproducible.
2. Sensitivity and specificity. The smaller interprobe spacing of oligonucleotide arrays offers a much higher probe density for better detection of smaller genomic imbalances and more accurate breakpoint mapping, therefore providing much improved sensitivity over BAC arrays. The fact that oligonucleotide probes are selected from the reference human genome sequence allows all users to use any sequence of interest as a potential target, providing a specificity that is impossible with BAC arrays, in which clones can be selected only from existing libraries and need to be validated for their physical location.
3. Customization. Oligonucleotide probes are synthesized in situ on the arrays, allowing for easy customization of content. In addition, many commercial manufacturers offer a large number of preselected array CGH oligonucleotide probes and computer interfaces, making custom design and updating of CGH arrays quite feasible and fast for clinical laboratories, whereas BAC arrays are cumbersome to update and time-consuming for printing.
4. Robustness and reliability. The substantially increased capacity of oligonucleotide arrays enables multiprobe confirmation for a single event (the detection sensitivity and specificity are a function of consecutive probes), as well as increased robustness because the higher signal-to-noise ratios provide higher confidence in CNV diagnosis *(13–15)*.

IMPROVEMENT OF GENOTYPING ARRAYS FROM LOW-RESOLUTION SNP ARRAYS TO HIGH-RESOLUTION HYBRID ARRAYS THAT INTEGRATE SNP AND CNV PROBES

Whereas CGH arrays successfully combine microarray technology with CGH, genotyping arrays for CNVs arose from SNP arrays that were originally designed for sequencing *(16)*, genotyping *(17)*, and gene expression *(18)*. These SNP arrays with short oligonucleotides were developed by Affymetrix with their proprietary photolithographic technology *(19)*. Since then, the density of SNP arrays has been growing constantly. The DNA chips designed to genotype 10×10^3, 50×10^3, 100×10^3, 500×10^3, and even more SNPs have allowed improved detection resolution.

At about the same time that Agilent Technologies developed long oligonucleotide–based whole-genome CGH arrays with their ink-jet technology *(20)*, Affymetrix SNP arrays demonstrated their ability to detect changes in genomic copy number in addition to genotyping *(21–23)*. Similarly, Illumina used a

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Table 1. Major commercial oligonucleotide array platforms and their current products.

Company	Array platform	Resolution (median probe spacing)	Probe number	Oligonucleotide probe type	Detection
Agilent Technologies, Santa Clara, CA	4x44K CGH array	43 kb	43 000+	60-mer	CNV
	8x60K CGH array	41.4 kb	55 000+		
	2x105K CGH array	21.7 kb	99 000+		
	4x180K CGH array	13 kb	170 000+		
	244K CGH array	8.9 kb	236 000+		
	2x400K CGH array	5.3 kb	411 000+		
	1 Million CGH array	2.1 kb	963 000+		
Affymetrix, Santa Clara, CA	Genome-Wide Human SNP Array 6.0	0.7 kb	906 600 SNP probes and 946 000 CNV probes	25-mer	CNV, genotype, and LOH[a]
Illumina, San Diego, CA	HumanCNV370-Quad DNA analysis BeadChip	4.9 kb	320 000 SNP probes and 60 000 non-SNP probes for CNVs	50-mer	CNV, genotype, and LOH
	Human610-Quad DNA analysis BeadChip	2.7 kb	550 000 SNP probes and 60 000 non-SNP probes for CNVs		
	Human1M-Duo BeadChip	1.5 kb	1.1×10^6 SNP and CNV probes targeting exons		
NimbleGen, Madison, WI	HG18 CGH 4x72K WG Tiling v2.0	40 kb	72 000	50- to 75-mer	CNV
	385K WG Tiling, single array	6.27 kb	385 000/array		
	385K WG Tiling, 4-set array	1.57 kb			
	385K WG Tiling, 8-set array	713 bp			

[a] LOH, loss of heterozygosity.

different manufacturing approach in commercializing BeadChips for genotyping and copy number analysis *(24)*.

Table 1 lists the currently available oligonucleotide-array platforms for genomic DNA profiling. Reduced manufacturing costs, unprecedented detection power, and the feasibility of custom design/updates have made these arrays attractive in basic and translational research.

The major advance in genotyping arrays for genomic profiling is the development of hybrid genotyping arrays, i.e., the Affymetrix Genome-Wide Human SNP Array 5.0 and the latest 6.0 version, which combines SNP probes for genotyping with CNV probes for detecting changes in copy number *(25)*. The SNP Array 5.0, which is the prototype of the hybrid array, contains 500 000 SNP probes for genotyping and 420 000 nonpolymorphic probes for CNV analysis. Among the nonpolymorphic probes, 320 000 probes were chosen to provide even spacing across the genome, concentrating on areas not represented by SNPs, and the remaining 100 000 probes covered 2000 known CNVs (50 probes per CNV). The SNP Array 6.0 has 906 600 SNP probes, 744 000 copy number probes evenly spaced along the genome, and another 202 000 probes that target 5700 previously reported CNV regions. Thus, a single 6.0 array offers a total of 1.8 million probes for simultaneous SNP genotyping, CNV analysis, and loss-of-heterozygosity detection.

Although the Affymetrix SNP Array 6.0 (hybrid arrays) is mainly a research tool at the moment, many diagnostics laboratories are actively validating this platform for clinical applications. Although long-oligonucleotide CGH arrays do not have as many probes as short-oligonucleotide genotyping arrays, the signal-to-background dynamic range is generally better with long-oligonucleotide CGH arrays; however, the rich genotyping information, in addition to the CNV data offered by the hybrid arrays, provides additional value to the platform. For instance, the genotyping data simultaneously provide information regarding

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the parent of origin for the detected de novo CNV if parental samples have also been examined. The most important advantage of the hybrid arrays is their capability of detecting copy number–neutral genomic rearrangements, namely uniparental disomy (UPD), through loss-of-heterozygosity analysis. In the long run, the genotyping information can be used for SNP-association analysis.

Translational Research and Applications of MGDP Technologies for Patient Samples

MGDP technologies have been used extensively in clinical and translational research for interrogating patient samples. These studies provided crucial information about clinical utility before their diagnostic applications. A recent review by Stankiewicz and Beaudet *(26)* summarized the applications of microarrays for CGH, primarily dealing with low-resolution or targeted BAC arrays in the evaluation of patients with dysmorphic features, developmental delay, and/or idiopathic mental retardation. The published data produced with different array platforms and patient cohorts indicate an overall detection rate for pathogenic genomic imbalance of patients with multiple congenital anomalies and/or developmental delay/mental retardation of 12%–18%. Only 3%–5% of such patients can be detected by G-banded karyotyping, and an additional 9%–13% can be detected by microarray-based tests *(26)*. As expected, the detection rate is higher with whole-genome tiling arrays than with targeted arrays. A series of such studies overwhelmingly verified the superior sensitivity and detection power of array CGH compared with karyotyping and FISH analysis. As mentioned above, the most important advance in array CGH is replacing BACs with oligonucleotides. Similarly, short-oligonucleotide arrays have also been validated against and found superior to BAC arrays.

INTERROGATING SAMPLES FROM PATIENTS AND HEALTHY INDIVIDUALS WITH OLIGONUCLEOTIDE ARRAYS

Oligonucleotide CGH arrays became commercially available in 2004 and now include platforms from Agilent Technologies *(20)*, NimbleGen *(27)*, Affymetrix *(28)*, and Illumina *(24)* (Table 1). In-house spotted-oligonucleotide arrays *(29)* and custom-designed oligonucleotide CGH arrays *(13, 14, 30–32)* have also demonstrated high sensitivity and reproducible detection of genomic imbalance in testing with samples from patients and healthy individuals. Consequently, many laboratories have shifted from BAC arrays to oligonucleotide arrays *(15)*. Some diagnostic laboratories that provide large-scale services are now using oligonucleotide arrays for clinical diagnostics.

The initial implementation of oligonucleotide CGH arrays for clinical applications focused on targeted pathogenic genomic regions. Shen et al. developed targeted oligonucleotide arrays that used Agilent custom arrays to interrogate 179 clinically relevant genomic loci *(14)*. The multiplex arrays demonstrated results that were 100% concordant with the findings from BAC arrays, showing very high sensitivity. In addition, smaller genomic imbalances that were not detectable by BAC arrays were reliably detected with this oligonucleotide-array system *(14)*. Similarly, Ou et al. emulated BAC arrays with oligonucleotide probes and created an array of 44 000 oligonucleotides that verified an enhanced ability for detecting copy number changes compared with BAC arrays *(13)*. In addition to showing the superior analytical sensitivity of oligonucleotide arrays compared with BAC arrays, these studies also demonstrated the convenience of oligonucleotide-array design and manufacturing. Aradhya and Cherry used samples from 20 patients with dysmorphic features, developmental delay, or mental retardation to directly compare the performance of whole-genome BAC arrays of 1-Mb resolution (Spectral Chip 2600; PerkinElmer) with that of Agilent whole-genome oligonucleotide CGH arrays of 35-kb resolution *(33)*. All of these patients had a typical karyotype. Ten clinically important genomic-imbalance events were detected with the oligonucleotide arrays, whereas only 6 were detected with the BAC arrays. The study clearly demonstrated the superior effectiveness of the oligonucleotide arrays. Because of the large size of the BAC clones and the widespread nature of CNVs, BAC arrays provide very low specificity for detecting CNVs. Conversely, oligonucleotide arrays avoid repeat sequences via the selection of probes that avoid nonspecific regions.

With the intention of improving the capture of frequent subtelomeric imbalances associated with developmental delay/mental retardation, custom-designed oligonucleotide CGH arrays were developed with improved coverage in subtelomeric regions. Exploiting the design concept used earlier in BAC arrays *(34)*, Toruner et al. *(32)* randomly removed one third of the probes from an off-the-shelf Agilent whole-genome CGH array of 44 000 probes and replaced them with 14 000 subtelomeric probes. The resulting arrays provided 5-kb resolution in subtelomeres and 125-kb resolution in the remaining genome. The oligonucleotide CGH array–based molecular ruler *(31)*, another design that emerged at nearly the same time, provided 50-kb resolution for subtelomeric regions and 75-kb resolution for the rest of the genome. This array detected a subtelomeric genomic imbalance of 10.9% and a pathologic imbalance of 4.7% genome wide in clinical samples *(30)*. These custom designs provide

Table 2. Exon-level DNA-profiling arrays.

Reference	CGH array platform	Target gene(s)[a]	Specific probe no.	Resolution
Rouleau et al. (39)	Agilent 11K array	BRCA1	1679	NA[b]
del Gaudio et al. (40)	Agilent 44K array	DMD	8769	NA
Staaf et al. (41)	Agilent 4x44K array	BRCA1, BRCA2, MSH2, MLH1, PTEN, and CDKN2A	9612	<500 bp, partial exon
Saillour et al. (42)	NimbleGen tiling array	CFTR, SGCA, SGCB, SGCD, SGCE, SGCZ, and DMD	72 500	Single exon and mosaic
Hegde et al. (43)	NimbleGen tiling array	DMD	385 747 (mean spacing, 5 bp)	Single exon and mosaic
Wong et al. (44)	Agilent 44K array	130 Nuclear genes involved in metabolic and mitochondrial disorders	≅44 000 (mean spacing, 250–300 bp)	Single exon and mosaic

[a] BRCA1, breast cancer 1, early onset; BRCA2, breast cancer 2, early onset; MSH2, mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli); MLH1, mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli); PTEN, phosphatase and tensin homolog; CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); CFTR, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7); SGCA, sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein); SGCB, sarcoglycan, beta (43kDa dystrophin-associated glycoprotein); SGCD, sarcoglycan, delta (35kDa dystrophin-associated glycoprotein); SGCE, sarcoglycan, epsilon; SGCZ, sarcoglycan zeta.
[b] Data not available.

enhanced coverage for clinically relevant regions, as well as decent coverage for the rest of genome for better measurement of the sizes of imbalance events.

Compared with oligonucleotide CGH arrays, fewer clinical applications have been studied on genotyping arrays. Friedman et al. used the Affymetrix 100K SNP arrays to investigate genomic imbalance *(28)*. Eight control trios that included the unaffected parents and an affected child with a previously recognized chromosomal abnormality or UPD were studied. The Affymetrix array detected the known abnormalities in all control cases, including 4 cases of UPD. The array also detected de novo deletions as small as 178 kb among 100 idiopathic mental-retardation patients with a typical karyotype. In addition, Ming et al. used the 50K Xba chip from the Affymetrix 100K array set for a similar study of patients with multiple congenital anomalies *(35)*. Although both studies illustrated the sensitivity and detection power of Affymetrix arrays for investigating genomic imbalance, the analytical sensitivity and cutoff for detecting copy number–neutral UPD have yet to be firmly established.

Flexible probe selection permits easy design of oligonucleotide arrays that can detect deletions and duplications at the exon level of specific genes (Table 2). Although PCR products for *NF1*[6] (neurofibromin 1) *(36)*, *NF2* [neurofibromin 2 (merlin)] *(37)*, and other large, clinically important genes *(38)* have been used to develop exon-level microarrays, the current manufacturing technology for oligonucleotide arrays has simplified the process so that it is more accessible to ordinary laboratories. These custom-designed, target gene–specific arrays detect duplication as effectively as deletion at the single-exon level, with more precise breakpoint mapping *(39–44)*. Targeting panels of genes associated with one disease at the exon level is attractive for molecular diagnostics.

[6] Human genes: NF1, neurofibromin 1; NF2, neurofibromin 2 (merlin); PARK2, Parkinson disease (autosomal recessive, juvenile) 2, parkin; DMD, dystrophin.

GENOMIC IMBALANCES IN AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASDs) are complex, pervasive developmental-delay disorders characterized by impairments in communication, social interaction, and behavior. Studies of twins have suggested a strong genetic component to ASD. Thus far, few contributory genes have been identified, but cytogenetic changes have been one of the most consistent identifiable causes of autism. Changes detectable by high-resolution G-banding have been reported in 3%–5% of autism patients *(45)*, involving the chromosomal regions at 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3, and Xp22.2p22.3 *(46)*. MGDP is a powerful new venue for exploring the complex nature of ASD genetics. Jacquemont et al. *(47)* used whole-genome 1-Mb BAC CGH arrays to interrogate 29 syndromic autism patients with typical karyotypes and found that more than a quarter of the patients showed spontaneous pathogenic copy number changes. The size of the imbalances ranged from 2 Mb to 17.3 Mb. This study highlighted the immense effectiveness of array CGH in genetic analysis of patients with autism. It

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Table 3. Detection rate of clinically relevant CNVs in ASDs by array technologies.

Reference	Array platforms	Actual resolution	Patient cohort	De novo CNV detection rate
Jacquemont et al. (47)	BAC CGH array	1 Mb	29 Syndromic autism	27.5% (8 of 29)
Sebat et al. (48)	Oligonucleotide CGH array	35 kb	118 Sporadic autism	10.2% (12 of 118)
			77 Multiplex[a] familial autism	3% (2 of 77)
Szatmari et al. (49)	Affymetrix 10K SNP array	476 kb	173 Families, simplex	5.8% (10 of 173)
Weiss et al. (50)	Affymetrix 5.0 array	30 kb	751 AGRE families	6.7% (50 of 751)
Christian et al. (51)	BAC CGH array	Tiling 19K set	397 AGRE samples	2.3% (9 of 397)
Marshall et al. (52)	Affymetrix 500K SNP array	75 kb	427 ASD families	6.3% mean (27 of 427); 7.1% (4 of 56) in simplex families; 2% (1 of 49) in multiplex families
Morrow et al. (56)	Affymetrix 500K SNP array and targeted BAC CGH array	75 kb	42 Consanguineous samples, multiplex	0
			52 Consanguineous samples, simplex	1.9% (1 of 52)

[a] Multiplex, family with ≥2 ASD patients; simplex, family with 1 ASD patient; AGRE, Autism Genetic Resource Exchange.

also demonstrated that extensive genomic imbalances are an important underlying cause of syndromic ASD.

In another study that used oligonucleotide arrays with much higher resolution, Sebat et al. *(48)* revealed the importance of de novo CNVs as genetic risk factors for sporadic ASD. Subsequent studies have provided further evidence to support the notion that copy number changes are strongly associated with idiopathic ASD *(49–55)*. Array technology has detected pathogenic CNVs in 5.8%–10.2% of sporadic ASD patients (Table 3), whereas the rate is consistently low in familial ASD patients (2%–3%). This finding suggests that there are 2 different ASD genetic mechanisms: sporadic ASDs are more likely to be caused by de novo deletions or duplications, whereas familial ASDs are more likely to be due to other types of inherited mutations. Array studies with consanguineous families further demonstrated the existence of recessive gene mutations that are largely not genomic deletions or duplications *(56)*.

Collectively, these studies clearly demonstrate the clinical value of genomic profiling in the evaluation of autism. It is strongly evident that the detection yield of MGDP is much more consistent and higher than traditional cytogenetic techniques *(57)*.

Clinical Validation and Diagnostic Applications of MGDP Technologies

CLINICAL VALIDATION—SENSITIVITY AND SPECIFICITY

As for any new technology, systematic validation is required before MGDP can be used for routine clinical applications *(14, 58, 59)*. The validation process should test all aspects of a new technology, but most importantly the sensitivity and specificity. Sensitivity and specificity are often tested by means of a positively testing cohort with known mutations that have been identified with a well-accepted technology. In the case of MGDP, results from karyotyping and FISH studies are often used. Typically, sensitivity testing examines the false-negative rate, and specificity testing inspects the false-positive rate; however, several aspects of MGDP validation are unique.

Microarray technology is analog in nature. The change in genomic copy number is reflected by a change in signal intensity or a color shift (red vs green) of the microarray features (probe spots). The first critical step is to define the analytical resolution, i.e., the size of CNV that can be detected reliably. Assuming that the feature-analysis software is well established for each array platform (not necessarily the case), CNV calling depends on the algorithm used. Owing to the different array platforms and probe densities, the analytical resolution of each array needs to be specifically defined. It is important to note that sensitivity and specificity vary, depending on the number of consecutive probes examined. Higher sensitivity and specificity can be achieved when more consecutive probes are used. In addition, the signal-to-background ratio for a deletion or duplication changes with the array platform, the labeling chemistry, and the hybridization protocol. Thus, it is necessary to define an appropriate threshold for each clinical-array design. For example,

Table 4. Clinical utility of diagnostic genomic-profiling microarrays.

Reference	Array platform	Sample size, n	GI[a]/CNV size	pCNV	bCNV	uCNV
Shaffer et al. (65)	Signature Genomic targeted BAC CGH array	8789	≥200 kb	6.90%	1.20%	3.90%
Lu et al. (66)	Baylor CMA V4 targeted BAC CGH array	775	≥1 BAC clone	7.60%	10%	0.60%
	Baylor CMA V5 targeted BAC CGH array	1738	≥1 BAC clone	8.90%	8.1%	1.70%
Pickering et al. (67)	Spectral Genomics Spectral Chip 2600 and Constitutional Chip	1176	≥1 BAC clone	9.86%	3.99%	
Aston et al. (68)	Spectral Genomics Spectral Chip 2600 and Constitutional Chip	669	≥2 BAC clones	10.8%	NA	
Baldwin et al. (30)	Targeted plus whole-genome oligo CGH array	211	>500 kb	15.64% (10.90%, targeted; 4.74%, backbone)	8.53% (6.16%, complex; 2.36% familial)	1.90%
Shen et al. (14)	Agilent 2x11K focused oligo CGH array	211	≥3 Consecutive probes, smallest 23 kb	11.9%	4.26%	
Fan et al. (69)	Agilent 44K whole-genome oligo CGH array	100	≥3 Consecutive probes, smallest 17 kb	15%	0.72/case	
Xiang et al. (70)	Agilent 44K whole-genome oligo CGH array	50	>500 kb	6.00%	12.00%	

[a] GI, genomic imbalance; CMA, chromosomal microarray analysis; NA, data not available; oligo, oligonucleotide.

chromosomal mosaicism is not so rare in patients with birth defects and mental retardation. BAC arrays have found 8% of abnormal findings to be mosaic mutations; however, different array platforms have different sensitivities for detecting mosaicism *(60–62)*.

Because microarray technology has much higher resolution than karyotyping and FISH, its analytical resolution cannot be assessed by karyotyping or FISH. In many cases, additional CNVs not detectable by karyotyping or FISH were identified with array technologies. Therefore, other technologies covering smaller-scale mutations, such as MLPA, real-time PCR, or even PCR/sequencing, should be used for validation purposes *(14)*.

CLINICAL VALIDATION—PLATFORM PITFALLS

It is also necessary to check for missing spots on the array that may be clinically important. For example, some commercial arrays do not cover the pseudochromosomal regions of chromosomes X and Y. On the other hand, poor-performing probes (mismapped or poorly hybridizing probes) should be taken out of the array *(59)*. The latter action is particularly necessary in BAC arrays because one mismapped or poorly hybridizing clone will appreciably affect detection sensitivity and specificity. Most of these poorly hybridizing BAC clones may contain low-copy repeats. Thus, the presence of low-copy repeats is more of a problem in the BAC probe than the oligonucleotide probe, whereas oligonucleotide probes that do not perform well may contain secondary structure. Identifying such bad probes and removing them will improve array performance considerably *(14)*. Although such probes cannot be removed from commercial chips, posthybridization data analysis (data filtering) can provide an alternative solution for amending this problem.

It has been suggested that a minimum of 30 abnormal samples be used for clinical validation *(58)*; however, the region and size of the genomic imbalance that the abnormal cases cover are more important than the actual number. Ideally, every probe should be evaluated with abnormal samples. For this purpose, aneuploidy cell lines, as well as samples with well-defined genomic imbalances of different sizes, are very valuable.

UNDERSTANDING CNVs

The most important issue for array interpretation in clinical genetic testing is to define the nature of the identified CNVs *(63)*. In general, CNVs can be categorized into 3 major groups: CNVs with established pathogenicity (pCNVs), i.e., deletions/duplications that overlap with re-

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gions associated with defined genomic disorders or disrupt known disease genes; CNVs of a benign nature (bCNVs), i.e., deletions/duplications repeatedly observed in nonpathologic individuals and with no evidence of an association with any particular clinical phenotype; and CNVs with unknown importance (uCNVs), i.e., deletions/duplications without an established association with any genomic disorder and involving genes of unclear clinical importance [*(14, 30, 65–70)*, Table 4]. Even when bCNVs and uCNVs are intentionally avoided during array design, a large percentage of the identified imbalances often belong to bCNVs or uCNVs, because a substantial number of novel CNVs are still being discovered (Table 4).

We anticipate that many of CNVs of currently unknown importance (uCNVs) will eventually be confirmed as bCNVs as more precise knowledge about their locations, distributions, and frequencies in healthy populations becomes available. Meanwhile, the accumulating evidence from clinical (phenotyping), genetic (inheritance and segregation), and functional (gene content and expression) studies may reveal a portion of these uCNVs to be pathogenic. We believe that uCNVs are a rich source for identifying novel genetic risk factors for a variety of medical conditions.

Our understanding of CNVs will certainly improve with systematic CNV-discovery efforts *(64)*. At the same time, continued studies of genotype–phenotype association are also essential. Public databases such as DECIPHER (https://decipher.sanger.ac.uk/) represent such an effort. In the meantime, many novel CNVs are being discovered in clinical samples with the help of high-resolution whole-genome arrays. Databases derived from clinical samples are also a rich source for evaluating genotype–phenotype correlations. Eventually, this knowledge will make it possible to minimize the detection of bCNVs and maximize the detection of clinically important regions.

CLINICAL-DIAGNOSTIC UTILITY

The first-generation arrays designed for clinical use were targeted BAC CGH arrays developed in house at the Baylor College of Medicine *(34)* and Signature Genomic Laboratories *(71)*. Both arrays were designed to cover regions of known genomic disorders, as well as all subtelomeric regions. Array-CGH and FISH results showed 100% concordance. In addition, array CGH detected changes missed by prior karyotyping or FISH. A similar design was also available commercially, the Constitutional Chip by Spectral Genomics *(72)*. The Constitutional Chip also included backbone clones to cover the rest of the genome with an interval of about 1 Mb. Since that time, more BAC clones (from 589 to >4600) have been selected to cover additional clinically relevant regions (approximately 40–150 recognized genomic disorders/syndromes), improving detection rates from 5.6% in the first-generation BAC arrays to 10.8% in later arrays *(68, 73)*.

The clinical utility of oligonucleotide arrays has not yet been evaluated in large clinical studies; however, the current data indicate that the detection rate is higher than for BAC arrays (Table 4). High-resolution whole-genome oligonucleotide arrays have identified small novel microdeletions and microduplications in 16p11.2 that are associated with mental retardation/autism *(50, 53)*, have helped to circumscribe critical regions such as 17q21.31 *(74)*, and have identified intragenic deletions/duplications in large genes, such as *PARK2* [Parkinson disease (autosomal recessive, juvenile) 2, parkin] and *DMD* (dystrophin) (Y. Shen et al., unpublished data).

Common clinical indications for MGDP-based diagnostics testing include developmental delay, multiple congenital anomalies, dysmorphic features, unexplained mental retardation, seizure disorders, ASDs, and other neurologic/psychiatric disorders such as schizophrenia, as well as prenatal diagnosis, including spontaneous abortion or fetal demise.

Conclusion and Future Development

Microarray-based clinical molecular diagnostics technologies such as MGDP have gone beyond the stage of "coordinated and concurrent FISH analysis" *(75)* and extended into loss-of-heterozygosity testing and possibly methylation profiling. Current MGDP technologies are capable of detecting genomic imbalances of any size but do not reveal positional or orientational information. FISH analysis has largely been responsible for providing positional information. FISH is also instrumental in testing for translocations in parental samples for de novo CNVs that have been identified in probands. MGDP has much better resolution than FISH, however. The field would benefit immensely from a tool with the localization capability of FISH and the resolution of whole-genome oligonucleotide arrays. Advanced next-generation sequencing platforms may eventually provide digital profiling for detecting copy numbers and positional alterations simultaneously. Therefore, DNA-diagnostics profiling may soon extend beyond microarray-based technology, from analog analysis to digital analysis.

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Reviews

Next-Generation Sequencing: From Basic Research to Diagnostics

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BACKGROUND: For the past 30 years, the Sanger method has been the dominant approach and gold standard for DNA sequencing. The commercial launch of the first massively parallel pyrosequencing platform in 2005 ushered in the new era of high-throughput genomic analysis now referred to as next-generation sequencing (NGS).

CONTENT: This review describes fundamental principles of commercially available NGS platforms. Although the platforms differ in their engineering configurations and sequencing chemistries, they share a technical paradigm in that sequencing of spatially separated, clonally amplified DNA templates or single DNA molecules is performed in a flow cell in a massively parallel manner. Through iterative cycles of polymerase-mediated nucleotide extensions or, in one approach, through successive oligonucleotide ligations, sequence outputs in the range of hundreds of megabases to gigabases are now obtained routinely. Highlighted in this review are the impact of NGS on basic research, bioinformatics considerations, and translation of this technology into clinical diagnostics. Also presented is a view into future technologies, including real-time single-molecule DNA sequencing and nanopore-based sequencing.

SUMMARY: In the relatively short time frame since 2005, NGS has fundamentally altered genomics research and allowed investigators to conduct experiments that were previously not technically feasible or affordable. The various technologies that constitute this new paradigm continue to evolve, and further improvements in technology robustness and process streamlining will pave the path for translation into clinical diagnostics.

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In 1977, 2 landmark articles describing methods for DNA sequencing were published. Allan Maxam and Walter Gilbert reported an approach in which terminally labeled DNA fragments were subjected to base-specific chemical cleavage and the reaction products were separated by gel electrophoresis *(1)*. In an alternative approach, Frederick Sanger and colleagues described the use of chain-terminating dideoxynucleotide analogs that caused base-specific termination of primed DNA synthesis *(2)*. Refinement and commercialization of the latter method led to its broad dissemination throughout the research community and, ultimately, into clinical diagnostics. In an industrial, high-throughput configuration, Sanger technology was used in the sequencing of the first human genome, which was completed in 2003 through the Human Genome Project, a 13-year effort with an estimated cost of $2.7 billion. In 2008, by comparison, a human genome was sequenced over a 5-month period for approximately $1.5 million *(3)*. The latter accomplishment highlights the capabilities of the rapidly evolving field of "next-generation" sequencing (NGS)[3] technologies that have emerged during the past 5 years. Currently, 5 NGS platforms are commercially available, with additional platforms on the horizon. To add to this pace, the US National Human Genome Research Institute (NHGRI) announced funding in August 2008 for a series of projects as part of its Revolutionary Genome Sequencing Technologies program, which has as its goal the sequencing of a human genome for $1000 or less (http://www.genome.gov/27527585). This review describes NGS technologies, reviews their impact on basic research, and explores how they have the translational potential to substantially impact molecular diagnostics.

Fundamentals of NGS Platforms

NGS platforms share a common technological feature—massively parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. This design is a paradigm shift from that of

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[3] Nonstandard abbreviations: NGS, next-generation sequencing; NHGRI, National Human Genome Research Institute; dNTP, deoxynucleoside triphosphate; Mb, million base pairs; Gb, billion base pairs; miRNA, microRNA.

Sanger sequencing, which is based on the electrophoretic separation of chain-termination products produced in individual sequencing reactions. In NGS, sequencing is performed by repeated cycles of polymerase-mediated nucleotide extensions or, in one format, by iterative cycles of oligonucleotide ligation. As a massively parallel process, NGS generates hundreds of megabases to gigabases of nucleotide-sequence output in a single instrument run, depending on the platform. These platforms are reviewed next.

ROCHE/454 LIFE SCIENCES

The 454 technology (http://www.454.com) is derived from the technological convergence of pyrosequencing and emulsion PCR. In 1993, Nyren et al. described a sequencing approach based on chemiluminescent detection of pyrophosphate released during polymerase-mediated deoxynucleoside triphosphate (dNTP) incorporation *(4)*. Refinement by Ronaghi et al. served as the foundation for the commercial development of pyrosequencing *(5, 6)*. On a separate front, Tawfik and Griffiths described single-molecule PCR in microcompartments consisting of water-in-oil emulsions *(7)*. In 2000, Jonathan Rothberg founded 454 Life Sciences, which developed the first commercially available NGS platform, the GS 20, launched in 2005. Combining single-molecule emulsion PCR with pyrosequencing, Margulies and colleagues at 454 Life Sciences performed shotgun sequencing of the entire 580 069 bp of the *Mycoplasma genitalia* genome at 96% coverage and 99.96% accuracy in a single GS 20 run *(8)*. In 2007, Roche Applied Science acquired 454 Life Sciences and introduced the second version of the 454 instrument, the GS FLX. Sharing the same core technology as the GS 20, the GS FLX flow cell is referred to as a "picotiter well" plate, which is made from a fused fiber-optic bundle. In its newest configuration, approximately 3.4×10^6 picoliter-scale sequencing-reaction wells are etched into the plate surface, and the well walls have a metal coating to improve signal-to-noise discrimination. For sequencing (Fig. 1), a library of template DNA is prepared by fragmentation via nebulization or sonication. Fragments several hundred base pairs in length are end-repaired and ligated to adapter oligonucleotides. The library is then diluted to single-molecule concentration, denatured, and hybridized to individual beads containing sequences complementary to adapter oligonucleotides. The beads are compartmentalized into water-in-oil microvesicles, where clonal expansion of single DNA molecules bound to the beads occurs during emulsion PCR. After amplification, the emulsion is disrupted, and the beads containing clonally amplified template DNA are enriched. The beads are again separated by limiting dilution, deposited into individual picotiter-plate wells, and combined with sequencing enzymes. Loaded into the GS FLX, the picotiter plate functions as a flow cell wherein iterative pyrosequencing is performed by successive flow addition of the 4 dNTPs. A nucleotide-incorporation event in a well containing clonally amplified template produces pyrophosphate release and picotiter-plate well–localized luminescence, which is transmitted through the fiber-optic plate and recorded on a charge-coupled device camera. With the flow of each dNTP reagent, wells are imaged, analyzed for their signal-to-noise ratio, filtered according to quality criteria, and subsequently algorithmically translated into a linear sequence output. With the newest chemistry, termed "Titanium," a single GS FLX run generates approximately 1×10^6 sequence reads, with read lengths of ≥400 bases yielding up to 500 million base pairs (Mb) of sequence. A recognized strength of the 454 technology is the longer read length, which facilitates de novo assembly of genomes *(9)*. An outstanding concern has been the accurate determination of homopolymers >3–4 bases in length. A 6-base homopolymer should theoretically yield twice the luminescence of a 3-base homopolymer. Operationally, this luminescence yield varies, and estimates of homopolymer length are less accurate with increasing length *(8, 10)*. 454 has reported that the metal coating of the walls of picotiter wells mentioned above improves the accuracy of homopolymer determination. Sequence coverage depth and accuracy for the 454 technology is discussed below in the NGS Data Analysis section.

ILLUMINA/SOLEXA

In 1997, British chemists Shankar Balasubramanian and David Klenerman conceptualized an approach for sequencing single DNA molecules attached to microspheres. They founded Solexa in 1998, and their goal during early development of sequencing single DNA molecules was not achieved, requiring a shift toward sequencing clonally amplified templates. By 2006, the Solexa Genome Analyzer, the first "short read" sequencing platform, was commercially launched. Acquired by Illumina (http://www.Illumina.com) in 2006, the Genome Analyzer uses a flow cell consisting of an optically transparent slide with 8 individual lanes on the surfaces of which are bound oligonucleotide anchors (Fig. 2). Template DNA is fragmented into lengths of several hundred base pairs and end-repaired to generate 5′-phosphorylated blunt ends. The polymerase activity of Klenow fragment is used to add a single A base to the 3′ end of the blunt phosphorylated DNA fragments. This addition prepares the DNA fragments for ligation to oligonucleotide adapters, which have an overhang of a single T base at their 3′ end to increase ligation efficiency. The adapter oligonucleotides are complementary to the flow-cell anchors. Un-

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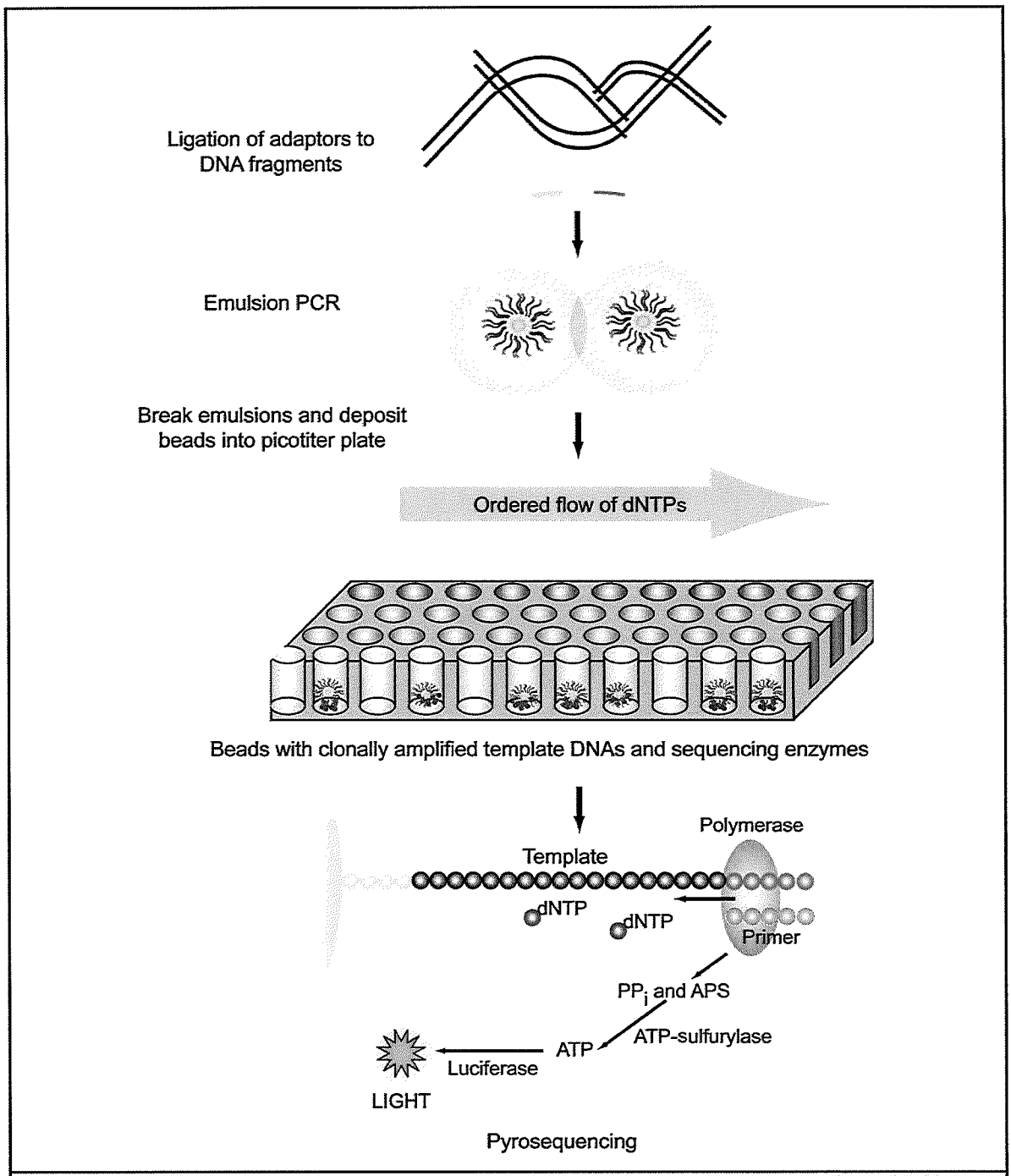

Fig. 1. Roche 454 GS FLX sequencing.

Template DNA is fragmented, end-repaired, ligated to adapters, and clonally amplified by emulsion PCR. After amplification, the beads are deposited into picotiter-plate wells with sequencing enzymes. The picotiter plate functions as a flow cell where iterative pyrosequencing is performed. A nucleotide-incorporation event results in pyrophosphate (PP_i) release and well-localized luminescence. APS, adenosine 5′-phosphosulfate.

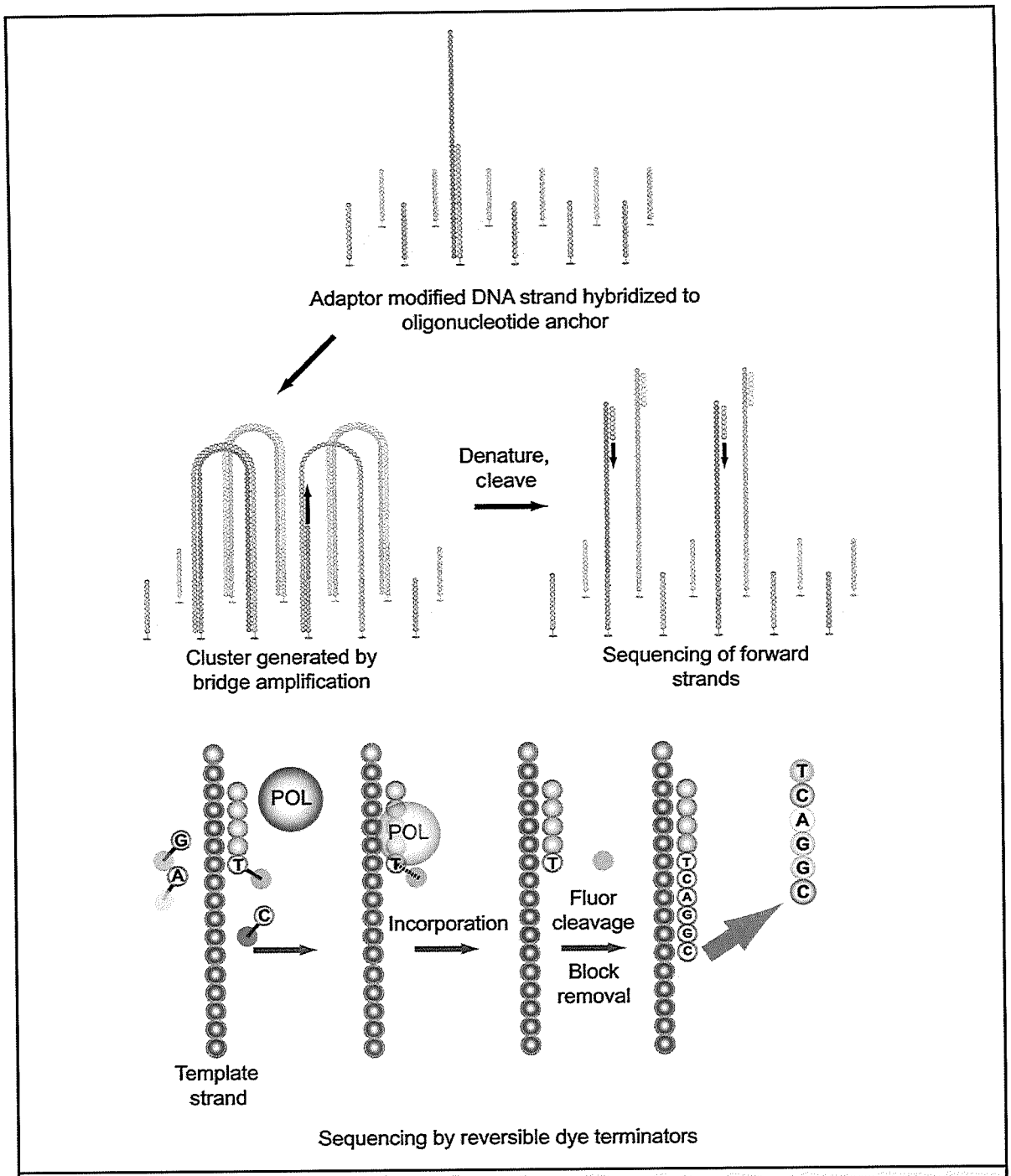

Fig. 2. Illumina Genome Analyzer sequencing.
Adapter-modified, single-stranded DNA is added to the flow cell and immobilized by hybridization. Bridge amplification generates clonally amplified clusters. Clusters are denatured and cleaved; sequencing is initiated with addition of primer, polymerase (POL) and 4 reversible dye terminators. Postincorporation fluorescence is recorded. The fluor and block are removed before the next synthesis cycle.

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der limiting-dilution conditions, adapter-modified, single-stranded template DNA is added to the flow cell and immobilized by hybridization to the anchors. In contrast to emulsion PCR, DNA templates are amplified in the flow cell by "bridge" amplification, which relies on captured DNA strands "arching" over and hybridizing to an adjacent anchor oligonucleotide. Multiple amplification cycles convert the single-molecule DNA template to a clonally amplified arching "cluster," with each cluster containing approximately 1000 clonal molecules. Approximately 50×10^6 separate clusters can be generated per flow cell. For sequencing, the clusters are denatured, and a subsequent chemical cleavage reaction and wash leave only forward strands for single-end sequencing. Sequencing of the forward strands is initiated by hybridizing a primer complementary to the adapter sequences, which is followed by addition of polymerase and a mixture of 4 differently colored fluorescent reversible dye terminators. The terminators are incorporated according to sequence complementarity in each strand in a clonal cluster. After incorporation, excess reagents are washed away, the clusters are optically interrogated, and the fluorescence is recorded. With successive chemical steps, the reversible dye terminators are unblocked, the fluorescent labels are cleaved and washed away, and the next sequencing cycle is performed. This iterative, sequencing-by-synthesis process requires approximately 2.5 days to generate read lengths of 36 bases. With 50×10^6 clusters per flow cell, the overall sequence output is >1 billion base pairs (Gb) per analytical run *(11)*. The newest platform, the Genome Analyzer II, has optical modifications enabling analysis of higher cluster densities. Coupled with ongoing improvements in sequencing chemistry and projected read lengths of 50-plus bases, further increases in output should be realized. Illumina and other NGS technologies have devised strategies to sequence both ends of template molecules. Such "paired-end" sequencing provides positional information that facilitates alignment and assembly, especially for short reads *(12, 13)*. A technical concern of Illumina sequencing is that base-call accuracy decreases with increasing read length *(14)*. This phenomenon is primarily due to "dephasing noise." During a given sequencing cycle, nucleotides can be under- or overincorporated, or block removal can fail. With successive cycles, these aberrations accumulate to produce a heterogeneous population in a cluster of strands of varying lengths. This heterogeneity decreases signal purity and reduces precision in base calling, especially at the 3′ ends of reads. Modifications in sequencing chemistry and algorithms for data-image analysis and interpretation are being pursued to mitigate dephasing *(15)*. Investigators at the Wellcome Trust Sanger Institute, who have extensive experience with the Illumina platform, have published a series of technical improvements for library preparation, including methods for increasing the reproducibility of fragmentation by adaptive focused acoustic wave sonication, enhanced efficiency of adapter ligation by use of an alternate ligase, and reducing the G+C bias that has been observed in Illumina reads via a modified gel-extraction protocol *(16)*.

APPLIED BIOSYSTEMS/SOLiD

The SOLiD (Supported Oligonucleotide Ligation and Detection) System 2.0 platform, which is distributed by Applied Biosystems (http://www.solid.appliedbiosystems.com), is a short-read sequencing technology based on ligation. This approach was developed in the laboratory of George Church and reported in 2005 along with the resequencing of the *Escherichia coli* genome *(17)*. Applied Biosystems refined the technology and released the SOLiD instrumentation in 2007. Sample preparation shares similarities with the 454 technology in that DNA fragments are ligated to oligonucleotide adapters, attached to beads, and clonally amplified by emulsion PCR. Beads with clonally amplified template are immobilized onto a derivitized-glass flow-cell surface, and sequencing is begun by annealing a primer oligonucleotide complementary to the adapter at the adapter–template junction (Fig. 3). Instead of providing a 3′ hydroxyl group for polymerase-mediated extension, the primer is oriented to provide a 5′ phosphate group for ligation to interrogation probes during the first "ligation sequencing" step. Each interrogation probe is an octamer, which consists of (in the 3′-to-5′ direction) 2 probe-specific bases followed by 6 degenerate bases with one of 4 fluorescent labels linked to the 5′ end. The 2 probe-specific bases consist of one of 16 possible 2-base combinations (for example TT, GT, and so forth). In the first ligation-sequencing step, thermostable ligase and interrogation probes representing the 16 possible 2-base combinations are present. The probes compete for annealing to the template sequences immediately adjacent to the primer. After annealing, a ligation step is performed, followed by wash removal of unbound probe. Fluorescence signals are optically collected before cleavage of the ligated probes, and a wash is performed to remove the fluor and regenerate the 5′ phosphate group. In the subsequent sequencing steps, interrogation probes are ligated to the 5′ phosphate group of the preceding pentamer. Seven cycles of ligation, referred to as a "round," are performed to extend the first primer. The synthesized strand is then denatured, and a new sequencing primer offset by 1 base in the adapter sequence (n − 1) is annealed. Five rounds total are performed, each time with a new primer with a successive offset (n − 2, n − 3, and so on). By this approach, each template nucleo-

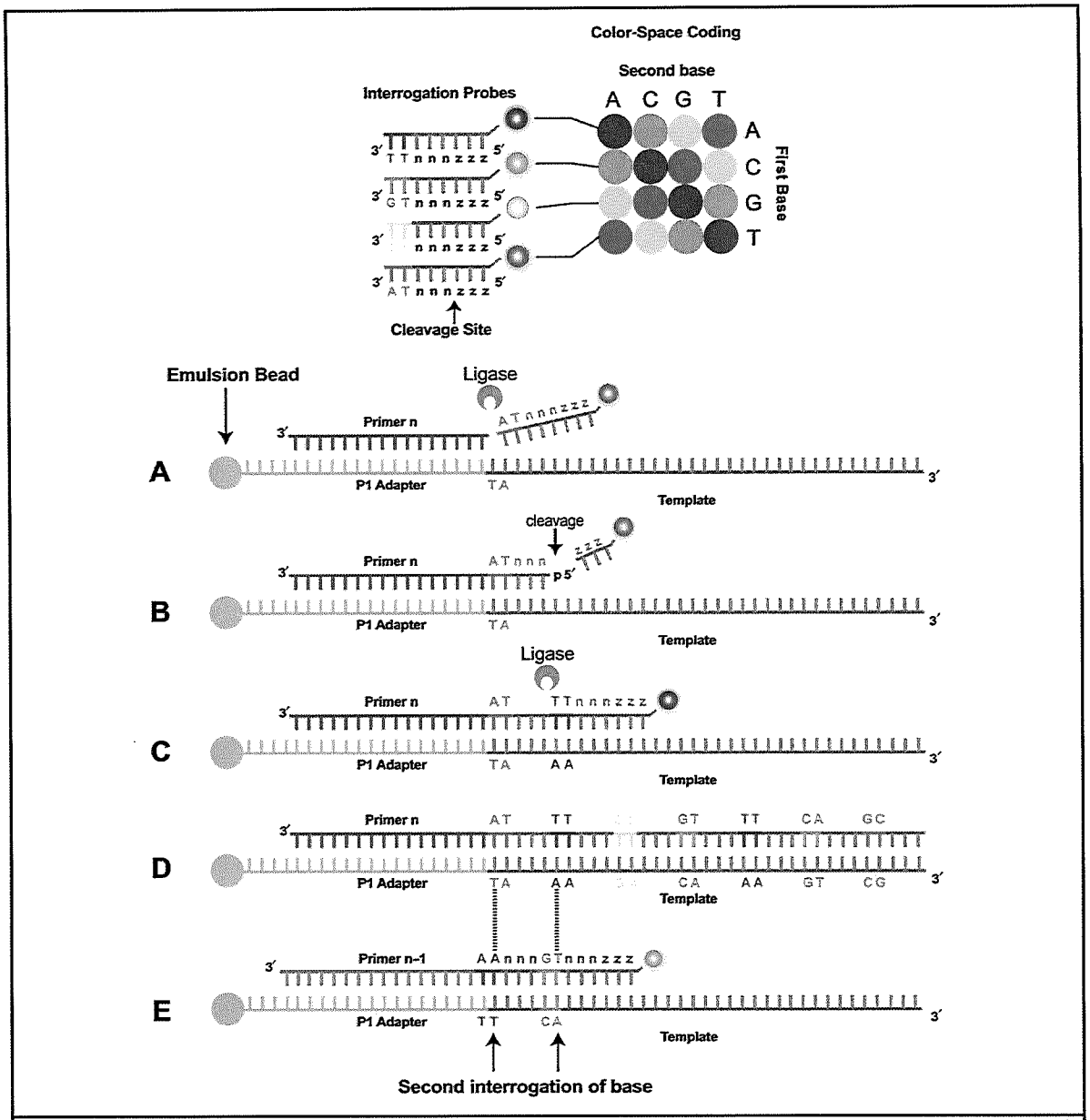

Fig. 3. Applied Biosystems SOLiD sequencing by ligation.

Top: SOLiD color-space coding. Each interrogation probe is an octamer, which consists of (3′-to-5′ direction) 2 probe-specific bases followed by 6 degenerate bases (nnnzzz) with one of 4 fluorescent labels linked to the 5′ end. The 2 probe-specific bases consist of one of 16 possible 2-base combinations. Bottom: (A), The P1 adapter and template with annealed primer (n) is interrogated by probes representing the 16 possible 2-base combinations. In this example, the 2 specific bases complementary to the template are AT. (B), After annealing and ligation of the probe, fluorescence is recorded before cleavage of the last 3 degenerate probe bases. The 5′ end of the cleaved probe is phosphorylated (not shown) before the second sequencing step. (C), Annealing and ligation of the next probe. (D), Complete extension of primer (n) through the first round consisting of 7 cycles of ligation. (E), The product extended from primer (n) is denatured from the adapter/template, and the second round of sequencing is performed with primer (n − 1). With the use of progressively offset primers, in this example (n − 1), adapter bases are sequenced, and this known sequence is used in conjunction with the color-space coding for determining the template sequence by deconvolution (see Fig. 1 in the online Data Supplement). In this technology, template bases are interrogated twice.

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Table 1. Comparison of NGS platforms.

	Roche 454 GS FLX	Illumina Genome Analyzer	Applied Biosystems SOLiD	Sanger
Sequencing method	Pyrosequencing	Reversible dye terminators	Sequencing by ligation	Dye terminators
Read lengths	400 bases	36 bases	35 bases	800 bp
Sequencing run time	10 h	2.5 days	6 days	3 h
Total bases per run	500 Mb	1.5 Gb	4 Gb	800 bp

tide is sequenced twice. A 6-day instrument run generates sequence read lengths of 35 bases. Sequence is inferred by interpreting the ligation results for the 16 possible 2 base–combination interrogation probes. With the use of offset primers, several bases of the adapter are sequenced. This information provides a sequence reference starting point that is used in conjunction with the color space–coding scheme to algorithmically deconvolute the downstream template sequence (see Fig. 1 in the Data Supplement that accompanies the online version of this review at http://www.clinchem.org/content/vol55/issue4). Placing 2 flow-cell slides in the instrument per analytical run produces a combined output of 4 Gb of sequence or greater. Unextended strands are capped before the ligation to mitigate signal deterioration due to dephasing. Capping coupled with high-fidelity ligation chemistry and interrogation of each nucleotide base twice during independent ligation cycles yields a company-reported sequence consensus accuracy of 99.9% for a known target at a 15-fold sequence coverage over sequence reads of 25 nucleotides. On an independent track, the Church laboratory has collaborated with Danaher Motion and Dover Systems to develop and introduce an alternative sequencing-by-ligation platform, the Polonator G.007 (http://www.polonator.org). Table 1 summarizes GS FLX, Genome Analyzer, and SOLiD platform features.

HELICOS BIOSCIENCES AND SINGLE-MOLECULE SEQUENCING

The first single-molecule sequencing platform, the HeliScope, is now available from Helicos BioSciences (http://www.helicosbio.com) with a company-reported sequence output of 1 Gb/day. This technology stems from the work of Braslavsky et al., published in 2003 *(18)*. Having obviated clonal amplification of template, the method involves fragmenting sample DNA and polyadenylation at the 3′ end, with the final adenosine fluorescently labeled. Denatured polyadenylated strands are hybridized to poly(dT) oligonucleotides immobilized on a flow-cell surface at a capture density of up to 100×10^6 template strands per square centimeter. After the positional coordinates of the captured strands are recorded by a charge-coupled device camera, the label is cleaved and washed away before sequencing. For sequencing, polymerase and one of 4 Cy5-labeled dNTPs are added to the flow cell, which is imaged to determine incorporation into individual strands. After label cleavage and washing, the process is repeated with the next Cy5-labeled dNTP. Each sequencing cycle, which consists of the successive addition of polymerase and each of the 4 labeled dNTPs, is termed a "quad." The number of sequencing quads performed is approximately 25–30, with read lengths of up to 45–50 bases having been achieved. The Helicos platform was used to sequence the 6407-base genome of bacteriophage M13 *(19)*. This study demonstrated both the potential and important technical issues that may be relevant to all single-molecule sequencing methods that are based on sequencing by synthesis. First, sequencing accuracy was appreciably improved when template molecules were sequenced twice ("2-pass" sequencing). Second, the accuracy of sequencing homopolymers was compromised by the polymerase adding additional bases of the same identity in a homopolymeric stretch in a given dNTP addition. Helicos has since developed proprietary labeled dNTPs, termed "virtual terminators," which the company reports reduce polymerase processivity so that only single bases are added, improving the accuracy of homopolymer sequencing. Interestingly, the percentage of strands in which longer read lengths can be achieved (e.g., 50 nucleotides) is substantially lower than that obtained with shorter (e.g., 25 nucleotides) read-length sequencing, possibly reflecting secondary structures (e.g., hairpins) assumed by the template molecules.

The Impact of NGS on Basic Research

In the short 4 years since the first commercial platform became available, NGS has markedly accelerated multiple areas of genomics research, enabling experiments that previously were not technically feasible or affordable. We describe major applications of NGS and then review the analysis of NGS data.

GENOMIC ANALYSIS

The high-throughput capacity of NGS has been leveraged to sequence entire genomes, from microbes to humans *(3, 8, 9, 11, 20–24)*, including the recent sequencing of the genome of cytogenetically normal acute myeloid leukemia cells, which identified novel, tumor-specific gene mutations *(25)*. The longer read lengths of the 454 technology, compared with the Illumina and SOLiD short-read technologies, facilitate the assembly of genomes in the absence of a reference genome (i.e., de novo assembly). For resequencing, both long- and short-read technologies have been used successfully. In one comparative study, the 454, Illumina, and SOLiD technologies all accurately detected single-nucleotide variations when coverage depth was ≥15-fold per allele *(20)* (the critical issue of coverage depth is discussed further in the NGS Data Analysis section). The 454 read lengths provide nucleotide haplotype information over a range of several hundred base pairs and are predicted to be better suited for detecting larger insertions and deletions and for producing alignments in areas containing repetitive sequences. Further studies are needed to compare technology performance for detecting insertions and deletions. Each platform has an optional strategy for sequencing both ends of DNA libraries (paired-end sequencing). In addition to effectively doubling sequence output, knowing that reads are associated with each other on a given fragment augments alignment and assembly, especially for short reads. Paired-end sequencing has been used to map genomic structural variation, including deletions, insertions, and rearrangements *(12, 13, 26, 27)*. The ability to sequence complete human genomes at a substantially reduced cost with NGS has energized an international effort to sequence thousands of human genomes over the next decade (http://www.1000genomes.org), which will lead to the characterization and cataloging of human genetic variation at an unprecedented level.

TARGETED GENOMIC RESEQUENCING

Sequencing of genomic subregions and gene sets is being used to identify polymorphisms and mutations in genes implicated in cancer and in regions of the human genome that linkage and whole-genome association studies have implicated in disease *(28, 29)*. Especially in the latter setting, regions of interest can be hundreds of kb's to several Mb in size. To best use NGS for sequencing such candidate regions, several genomic-enrichment steps, both traditional and novel, are being incorporated into overall experimental designs. Overlapping long-range PCR amplicons (approximately 5–10 kb) can be used for up to several hundred kb's, but this approach is not practical for larger genomic regions. More recently, enrichment has been achieved by hybridizing fragmented, denatured human genomic DNA to oligonucleotide capture probes complementary to the region of interest and subsequently eluting the enriched DNA *(30–33)*. Capture probes can be immobilized on a solid surface (Roche NimbleGen, http://www.nimblegen.com; Agilent Technologies, http://www.agilent.com; and Febit, http://www.febit.com) or used in solution (Agilent). Current NimbleGen arrays contain 350 000 oligonucleotides of 60–90 bp in length that are typically spaced 5–20 nucleotides apart, with oligonucleotides complementary to repetitive regions being excluded. For enrichment, 5–20 μg of genomic DNA is fragmented and ligated to oligonucleotide linkers containing universal PCR priming sites. This material is denatured, hybridized to an array for 3 days, and eluted, with the enriched DNA amplified by the PCR before NGS library preparation. In reported studies, up to 5 Mb of sequence has been captured on the 350K array, with 60%–75% of sequencing reads mapping to targeted regions; other reads mapping to nontargeted regions reflect nonspecific capture. In development by NimbleGen is the use of an array of 2.1×10^6 features for capturing larger genomic regions. Agilent's solution-based technology uses oligonucleotides up to 170 bases in length, with each end containing sequences for universal PCR priming and with primer sites containing a restriction endonuclease–recognition sequence. The oligonucleotide library is amplified by the PCR, digested with restriction enzymes, and ligated to adapters containing the T7 polymerase promoter site. In vitro transcription is performed with biotinylated UTP to generate single-stranded biotinylated cRNA capture sequences. For capture, 3 μg of fragmented, denatured genomic DNA is hybridized with cRNA sequences for 24 h in solution. After hybridization, duplexes consisting of single-stranded DNA and cRNA are bound to streptavidin-coated magnetic beads; the cRNA is then enzymatically digested, leaving enriched single-stranded DNA that is subsequently processed for NGS. An alternative enrichment approach developed by RainDance Technologies (http://www.raindancetechnologies.com) uses a novel microfluidics technology in which individual pairs of PCR primers for the genomic regions of interest are segregated in water in emulsion droplets and then pooled to create a "primer library." Separately, emulsion droplets containing genomic DNA and PCR reagents are prepared. Two separate droplet streams are created, one with primer-library droplets and the other with droplets containing genomic DNA/PCR reagents. The 2 streams are merged and primer-library droplets and genomic DNA/PCR reagent droplets are paired in a 1:1 ratio. As paired droplets proceed through the microfluidic channel, they pass an electrical impulse that causes them to physically coalesce. The

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coalesced droplets containing individual primer pairs and genomic DNA/PCR reagents are deposited in a 96-well plate and amplified by the PCR. After amplification, the emulsions are disrupted, and the amplicons are pooled and processed for NGS.

METAGENOMICS

NGS has had a tremendous impact on the study of microbial diversity in environmental and clinical samples. Operationally, genomic DNA is extracted from the sample of interest, converted to an NGS library and sequenced. The sequence output is aligned to known reference sequences for microorganisms that are predicted to be present in the sample. Closely related species can be discerned, and more distantly related species can be inferred. In addition, de novo assembly of the data set can yield information to support the presence of known and potentially new species. Qualitative genomic information is obtained, and analysis of the relative abundance of the sequence reads can be used to derive quantitative information on individual microbial species. To date, most NGS-based metagenomic analyses have used the 454 technology and its associated longer read lengths to facilitate alignment to microbial reference genomes and for de novo assembly of previously uncharacterized microbial genomes. Examples of metagenomic studies include the analysis of microbial populations in the ocean *(34, 35)* and soil *(36)*, the identification of a novel arenavirus in transplantation patients *(37)*, and the characterization of microflora present in the human oral cavity *(38)* and the guts of obese and lean twins *(39)*.

TRANSCRIPTOME SEQUENCING

NGS has provided a powerful new approach, termed "RNA-Seq," for mapping and quantifying transcripts in biological samples. Total, ribosomal RNA–depleted, or poly(A)$^+$ RNA is isolated and converted to cDNA. A typical protocol would involve the generation of first-strand cDNA via random hexamer–primed reverse transcription and subsequent generation of second-strand cDNA with RNase H and DNA polymerase. The cDNA is then fragmented and ligated to NGS adapters. For small RNAs such as microRNAs (miRNAs) and short interfering RNAs, preferential isolation via a small RNA–enrichment method, size selection on an electrophoresis gel, or a combination of these approaches is commonly used. RNA ligase is used to join adapter sequences to the RNA; this step is often followed by a PCR amplification step before NGS processing. After sequencing, reads are aligned to a reference genome, compared with known transcript sequences, or assembled de novo to construct a genome-scale transcription map. Although RNA-Seq is in its early stages as a technology, it has already shown some advantages over gene expression arrays *(40)*. First, arrays depend on tiling existing genomic sequences, whereas RNA-Seq is not constrained by this limitation, allowing characterization of transcription without prior knowledge of the genomic sites of transcription origin. RNA-Seq is capable of single-base resolution and, compared with arrays, demonstrates a greater ability to distinguish RNA isoforms, determine allelic expression, and reveal sequence variants. Expression levels are deduced from the total number of reads that map to the exons of a gene, normalized by the length of exons that can be uniquely mapped. Results obtained with this approach have shown close correlation with those of quantitative PCR and RNA-spiking experiments. The dynamic range of RNA-Seq for determining expression levels is 3–4 orders of magnitude, compared with 2 orders of magnitude for expression arrays. In this context, RNA-Seq has shown improved performance for the quantitative detection of both highly produced transcripts and transcripts produced at low levels. RNA-Seq is being used to confirm and revise gene annotation, including 5′ and 3′, and exon/intron boundaries; the latter is achieved by mapping reads to exon junctions defined by GT-AG splicing consensus sites. Both qualitative and quantitative information regarding splicing diversity can be deduced. RNA-Seq has been applied to a variety of organisms, including *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, mice, and human cells *(40–51)*.

MAPPING OF DNA-BINDING PROTEINS AND CHROMATIN ANALYSIS

The delineation of regulatory proteins associated with genomes was substantially accelerated by the introduction of chromatin immunoprecipitation and microarray hybridization (ChIP-on-chip) technology *(52)*. In this approach, proteins in contact with genomic DNA are chemically cross-linked (typically with mild formaldehyde treatment) to their binding sites, and the DNA is fragmented by sonication or digestion with micrococcal nuclease. The proteins cross-linked with DNA are immunoprecipitated with antibodies specific for the proteins of interest. The DNA in the immunoprecipitate is purified and hybridized to an oligonucleotide array consisting of sequences from the genome, allowing identification of the protein-binding sites. This approach has been successfully used to identify binding sites for transcription factors and histone proteins. ChIP-on-chip technology is now being supplanted in a variety of experimental settings with ChIP-Seq, in which the DNA harvested from the immunoprecipitate is converted into a library for NGS. The obtained reads are mapped to the reference genome of interest to generate a genome-wide protein-binding map *(53–55)*. Studies to date that have exam-

ined the genomic-binding sites of the human NRSF (neuron restrictive silencer factor) and STAT1 (signal transducer and activator of transcription 1) proteins indicate the resolution of ChIP-Seq to be greater than for ChIP-on-chip, as evidenced by confirmation of previously identified binding sites and identification of novel binding sites *(56, 57)*. Analogous to RNA-Seq, ChIP-Seq has the important advantage of not requiring prior knowledge of genomic locations of protein binding. In addition to the study of transcription factors, NGS is being used to map genomic methylation. One approach involves traditional bisulfite conversion of DNA followed by NGS, which has been applied to the study of entire genomes or genomic subregions *(58, 59)*. Ongoing studies are attempting to develop a variant of ChIP-Seq in which genomic methylation is assayed by coupling immunoprecipitation with a monoclonal antibody directed against methylated cytosine and subsequent NGS *(60)*.

NGS Data Analysis

NGS experiments generate unprecedented volumes of data, which present challenges and opportunities for data management, storage, and, most importantly, analysis *(61)*. NGS data begin as large sets of tiled fluorescence or luminescence images of the flow-cell surface recorded after each iterative sequencing step (Fig. 4). This volume of data requires a resource-intensive data-pipeline system for data storage, management, and processing. Data volumes generated during single runs of the 454 GS FLX, Illumina, and SOLiD instruments are approximately 15 GB, 1 TB, and 15 TB, respectively. The main processing feature of the data pipeline is the computationally intensive conversion of image data into sequence reads, known as base calling. First, individual beads or clusters are identified and localized in an image series. Image parameters such as intensity, background, and noise are then used in a platform-dependant algorithm to generate read sequences and error probability–related quality scores for each base. Although many researchers use the base calls generated by the platform-specific data-pipeline software, alternative base-calling programs that use more advanced software and statistical techniques have been developed. Features of these alternative programs include the incorporation of ambiguous bases into reads, improved removal of poor-quality bases from read ends *(62)*, and the use of data sets for software training *(15)*. Incorporation of these features has been shown to reduce read error and improve alignment, especially as platforms are pushed to generate longer reads. These advantages, however, must be weighed against the substantial computer resources required by the large volumes of image data.

The quality values calculated during NGS base calling provide important information for alignment, assembly, and variant analysis. Although the calculation of quality varies between platforms, the calculations are all related to the historically relevant phred score, introduced in 1998 for Sanger sequence data *(63, 64)*. The phred score quality value, q, uses a mathematical scale to convert the estimated probability of an incorrect call, e, to a log scale:

$$q = -10 \cdot \log_{10}(e).$$

Miscall probabilities of 0.1 (10%), 0.01 (1%), and 0.001 (0.1%) yield phred scores of 10, 20, and 30, respectively. The NGS error rates estimated by quality values depend on several factors, including signal-to-noise levels, cross talk from nearby beads or clusters, and dephasing. Substantial effort has been made to understand and improve the accuracy of quality scores and the underlying error sources *(10, 14)*, including inaccuracies in homopolymer run lengths on the 454 platform and base-substitution error biases with the Illumina format. Study of these error traits has led to examples of software that require no additional base calling but that improve quality-score accuracies and thus improve sequencing accuracy *(65, 66)*. Quality values are an important tool for rejecting low-quality reads, trimming low-quality bases, improving alignment accuracy, and determining consensus-sequence and variant calls *(67)*.

Alignment and assembly are substantially more difficult for NGS data than for Sanger data because of the shorter reads lengths in the former. One limitation of short-read alignment and assembly is the inability to uniquely align large portions of a read set when the read length becomes too short. Similarly, the number of uniquely aligned reads is reduced when aligning to larger, more complex genomes or reference sequences because of their having a higher probability of repetitive sequences. A case in point is a modeling study that indicated that 97% of the *E. coli* genome can be uniquely aligned with 18-bp reads but that only 90% of the human genome can be uniquely aligned with 30-bp reads *(68, 69)*. Unique alignment or assembly is reduced not only by the presence of repeat sequences but also by shared homologies within closely related gene families and pseudogenes. Nonunique read alignment is handled in software by read distribution between multiple alignment positions or leaving alignment gaps. De novo assembly will reject these reads, leading to shorter and more numerous assembled contigs. These factors are relevant when choosing an appropriate sequencing platform with its associated read length, particularly for de novo assembly *(9)*.

Error rates for individual NGS reads are higher than for Sanger sequencing. The higher accuracy of

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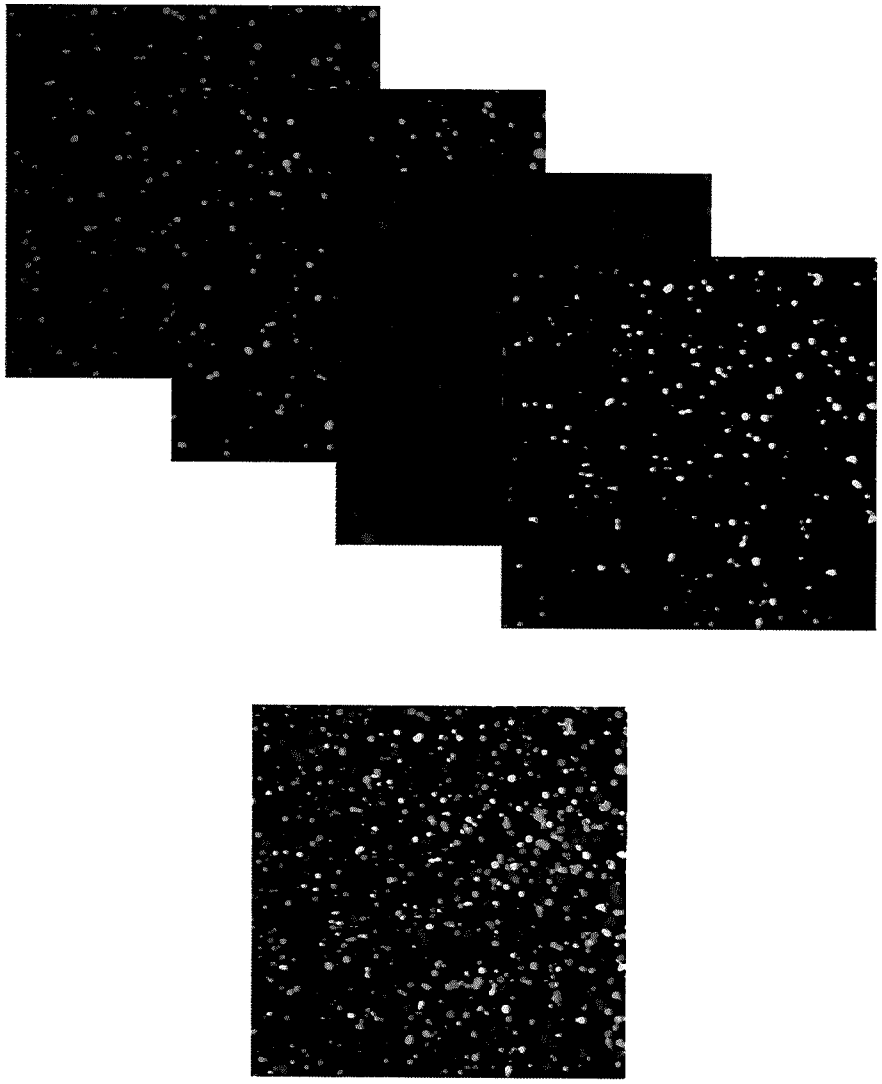

Fig. 4. Pseudocolor image from the Illumina flow cell.

Each fluorescence signal originates from a clonally amplified template cluster. Top panel illustrates 4 emission wavelengths of fluorescent labels depicted in red, green, blue, and yellow. Images are processed to identify individual clusters and to remove noise or interference. The lower panel is a composite image of the 4 fluorescence channels.

Sanger sequencing reflects not only the maturity of the chemistry but also the fact that a Sanger trace peak represents highly redundant, multiple terminated extension reactions. Accuracy in NGS is achieved by sequencing a given region multiple times, enabled by the massively parallel process, with each sequence contributing to "coverage" depth. Through this process, a "consensus" sequence is derived. To assemble, align, and analyze NGS data requires an adequate number of overlapping reads, or coverage. In practice, coverage across a sequenced region is variable, and factors other than the Poisson-like randomness of library preparation that may contribute to this variability include differential ligation of adapters to template sequences and differential amplification during clonal template generation *(11, 70)*. Beyond sequence errors, inadequate coverage can cause failure to detect actual nucleotide variation, leading to false-negative results for heterozygotes *(3, 11)*. Studies have shown that coverages of less than 20- to 30-fold begin to reduce the accuracy of single-nucleotide polymorphism calls in data on the 454 platform *(65)*. For the Illumina system, higher

coverage depths (50- to 60-fold) have been used in an effort to improve short-read alignment, assembly, and accuracy, although coverage in the 20- to 30-fold range may be sufficient for certain resequencing applications *(14)*. As noted above, one comparative study of a yeast genome showed that the 454, Illumina, and SOLiD technologies all accurately detected single-nucleotide variations when the coverage depth was ≥15-fold per allele *(20)*. Coverage gaps can occur when sequences are not aligned because of substantial variance from a reference. Alignment of repetitive sequences in repeat regions of a target sequence can also affect the apparent coverage. Reads that align equally well at multiple sites can be randomly distributed to the sites or in some cases discarded, depending on the alignment software. In de novo–assembly software, reads with ambiguous alignments are typically discarded, yielding multiple aligned read groups, or contigs, with no information regarding relative order.

A large variety of software programs for alignment and assembly have been developed and made available to the research community (see Table 1 in the online Data Supplement). Most use the Linux operating system, and a few are available for Windows. Many require a 64-bit operating system and can use ≥16 MB of RAM and multiple central-processing unit cores. The range of data volumes, hardware, software packages, and settings leads to processing times from a few minutes to multiple hours, emphasizing the need for sufficient computational power. Although a growing set of variations in alignment and assembly algorithms are available, there remains the trade-off between speed and accuracy in which many but not all possible alignments are evaluated, with a balance having to be struck between ideal alignment and computational efficiency.

NGS software features vary with the application and in general may include alignment, de novo assembly, alignment viewing, and variant-discovery programs. In addition some NGS statistical data-analysis tools are being developed (such as JMP Genomics; SAS Institute). Software packages available for alignment and assembly to a reference sequence include Zoom *(71)*, MAQ *(67)*, Mosaik *(72)*, SOAP *(73)*, and SHRiMP (http://compbio.cs.toronto.edu/shrimp/), which supports SOLiD color-space analysis. Software for de novo assembly includes Edina *(70)*, EULER-SR *(74)*, SHARCGS *(75)*, SSAKE *(69)*, Velvet *(76)*, and SOAPdenovo (http://soap.genomics.org.cn/). Recently released commercial software for alignment and de novo assembly includes packages from DNAStar (www.dnastar.com), SoftGenetics (www.softgenetics.com), and CLC bio (www.clcbio.com) that feature data viewers that allow the user to see read alignments, coverage depth, genome annotations, and variant analysis. Fig. 5 presents some examples of NGS data viewed in 2 different software systems.

RNA-Seq data analysis poses unique challenges and requires sequence alignment across spliced regions of transcripts as well as poly(A) tails. Current software has made strong inroads, however, with incorporation of motif recognition at splice junctions and identification of intron–exon borders through regions of low alignment coverage *(41)*. Deciphering multiple transcript isoforms involves mapping reads to known and putative splicing junctions and, in one approach, requires that each isoform be supported by multiple independent splice-junction reads with independent start sites *(51)*. ERANGE software has been used in the analysis of the mouse transcriptome *(43)*. ERANGE maps unique reads to their genomic site of origin and maps reads that match to several sites, or multireads, to a most likely site of origin. Reads that do not map to a known exon are grouped together by homology into candidate exons or parts of exons. The near proportional nature of NGS transcriptome data allows quantification of RNA production from the coverage of the assembled or aligned data. ERANGE uses normalized counts of unique reads, spliced reads, and multireads to quantify transcripts. Additional analytical considerations are needed for miRNA studies, including RNA secondary-structure analysis for hairpins, alignment to known miRNA databases, and searches within the NGS data set for complementary miRNA strands, as described in studies of developing rice grains *(77)* and chicken embryos *(78)*.

Research with ChIP-Seq has led to analysis methods and software that exploit the advantages over ChIP-in-chip, namely a larger, more information-rich data set. The single-base resolution of the data allows improved estimation of binding-site positions in the programs QuEST *(79)* and MACS *(80)*. Aligned data at the protein-binding regions typically have 2 characteristic offset peaks, each of which is populated by only forward or only reverse reads. These peaks are hallmarks of the immunoprecipitated short ChIP-Seq DNA fragments with a binding site near the center and are used by the software to estimate binding-site location near the mean peak position. Additional program features include advancements in statistical analysis to minimize miscalled binding sites, error probability estimation, and motif analysis (see Table 1 in the online Data Supplement).

A Clinical Future for NGS

From the impact that NGS has made at the basic-research level, we can anticipate its translation into molecular diagnostics. Key issues that will need to be addressed in this transition will include complexity of

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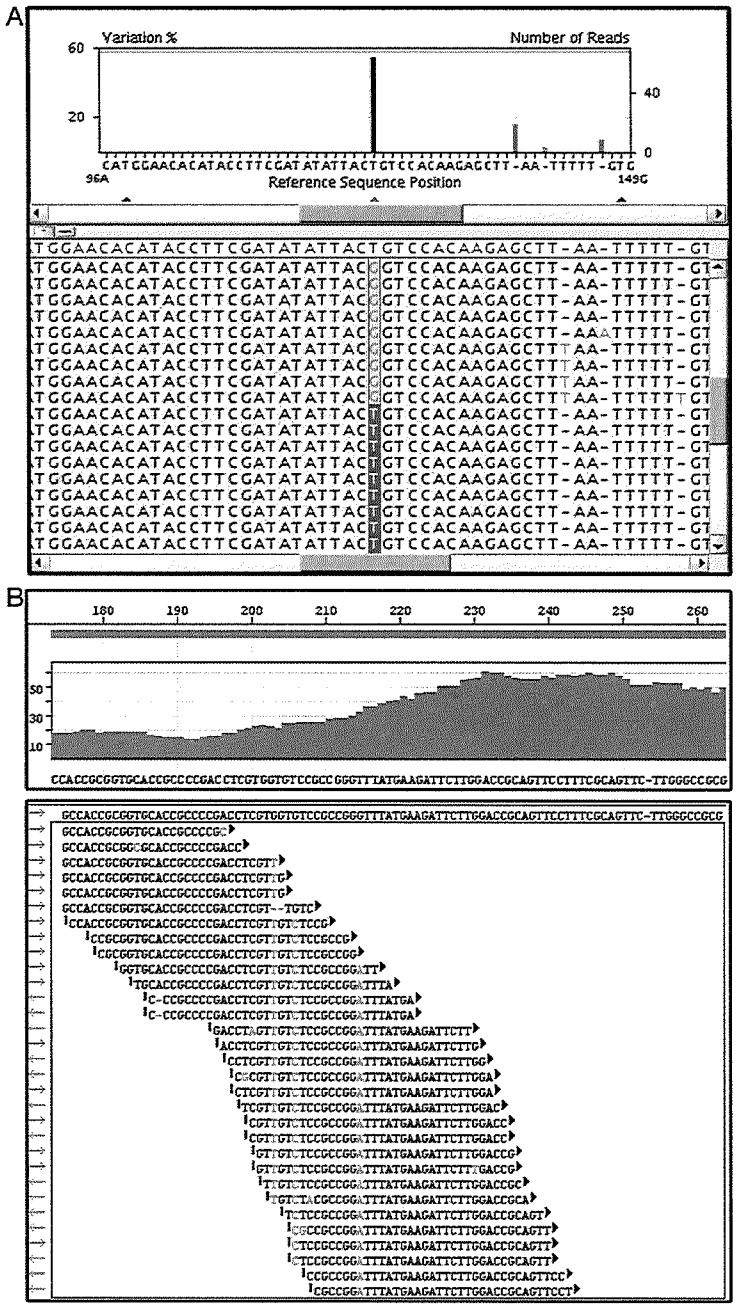

Fig. 5. Examples of NGS data viewed in 2 different software systems.

(A), Roche Amplicon Variant Analyzer software displaying GS FLX data from the CFTR gene [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)]. Lower pane shows reference sequence (green) above 18 of 68 aligned reads. Column highlighted in yellow and blue shows a heterozygous single-nucleotide polymorphism (SNP). Single T/A insertions (red) may represent errors. Upper pane shows percent variation from reference (vertical bars) and coverage (pale blue line). (B), DNAStar SeqMan Pro software displaying Illumina data from Mycobacterium massiliense. Lower pane shows reference sequence above aligned reads. Green and red arrows show direction of sequencing; base calls at variance with reference are indicated (red). Three columns in agreement (red) indicate presumptive SNPs. Other bases in red may be errors. Upper pane shows read coverage, with relative alignment positions above the graph. See the Acknowledgments for disclosure information on the CFTR-gene analysis performed on residual, deidentified DNA.

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technical procedures, robustness, accuracy, and cost. By all these measures, NGS platforms will benefit from continued process streamlining, automation, chemistry refinements, cost reductions, and improved data handling. The cost of NGS is currently substantial in terms of the investment in capital equipment (from approximately $600 000 for the Roche/454 Life Science, Illumina, and Applied Biosystems SOLiD platforms to $1.35 million for the HeliScope platform) and costs of sequencing reagents (from approximately $3500–$4500 for the Illumina, Applied Biosystems, and Roche/454 platforms to $18 000 for the HeliScope platform). Nonetheless, the cost per base is substantially lower than for Sanger sequencing, and combined with the tremendous output, it is straightforward to see why genome centers, core facilities, and commercial contract-sequencing enterprises have readily adopted this new technology. Work flow considerations include the fact that preparation of a sample library requires multiple molecular biology steps and 2–4 days to complete, depending on the platform. In addition to the required molecular biology expertise, data analysis requires expertise in bioinformatics facilitated by a knowledge of Linux operating systems. Leveraging the high-throughput capacity of NGS platforms can be facilitated by analyzing multiple samples with separate flow-cell lanes or compartments. In addition, unique identifier sequences or "bar codes" can be ligated to individual samples, which can subsequently be pooled and sequenced. After sequencing, sequences of individual samples are derived by data deconvolution *(81–83)*.

The transition of NGS into clinical diagnostics is in the early stages of development in large reference laboratories and is being leveraged for applications that require large amounts of sequence information, relative quantification, and high-sensitivity detection. Examples that meet these criteria include the aforementioned detection of mutations in tumor cells from biopsies or in the circulation. In the area of mitochondrial disorders, NGS can be used to sequence the entire 16.5-kb mitochondrial genome, determine mutation heteroplasmy percentage, and analyze nuclear genes whose protein products affect mitochondrial metabolism—all in a single analytical run. In the authors' laboratory, sequencing of mycobacterial genomes is ongoing as an approach to refine organism identification and support clinical epidemiologic investigations. HIV quasi-species detection and relative quantification have been demonstrated and can be used to monitor emerging drug resistance *(84)*. For human genetics, there is an increasing need to analyze multiple genes that, when mutated, lead to overlapping physical findings and clinical phenotypes. For example, 16 different genes are implicated in the pathogenesis of hypertrophic cardiomyopathy *(85, 86)*. For a comprehensive diagnostic evaluation in such settings, it will be necessary to sequence upwards of 100 000 to 200 000 bp. The coupling of NGS with the genomic-enrichment techniques described above offers a promising approach to this technical challenge.

Recently, investigative groups led by Y.M. Dennis Lo and Stephen Quake have applied NGS to the detection of fetal chromosomal aneuploidy *(87, 88)*. Prior work had demonstrated that cell-free fetal nucleic acids (DNA and RNA) are present in maternal blood during pregnancy, along with maternally derived cell-free nucleic acids. Several analytical approaches that use cell-free fetal nucleic acids have been developed to determine fetal aneuploidy, including the analysis of placental mRNA derived from the chromosomes of interest (e.g., chromosome 21) and the determination of relative chromosomal dosage via digital PCR analysis of a large number of target chromosomal loci compared with reference chromosomal loci *(89–91)*. Building on the concept of relative chromosome dosage, the Lo and Quake groups have independently shown the feasibility of converting cell-free DNA from maternal blood into an Illumina library, followed by sequencing and mapping the reads to the reference human genome. Counting the number of reads that map to each chromosome allows the relative dosage of each chromosome to be ascertained. If fetal aneuploidy is present, the number of sequence reads mapping to the affected chromosome would be expected to be statistically overrepresented in the data set. This expectation was confirmed in trisomy 21 pregnancies, with additional supporting evidence obtained for trisomy 18 and 13 pregnancies. These studies open a new avenue for assessing fetal aneuploidy and provide a foundation for NGS-based analysis of cell-free DNA in both nonpathologic and pathophysiological states.

Technologies on the Horizon

New single-molecule sequencing technologies in development may decrease sequencing time, reduce costs, and streamline sample preparation. Real-time sequencing by synthesis is being developed by VisiGen (http://www.visigenbio.com) and Pacific Biosciences (http://www.pacificbiosciences.com). VisiGen's approach uses DNA polymerase modified with a fluorescent donor molecule. Attached to a glass slide surface, the polymerase directs strand extension from primed DNA templates. Nucleotides are modified with fluorescent acceptor molecules, and light energy is used during incorporation to invoke fluorescence resonance energy transfer between polymerase and nucleotide fluorescent moieties, the latter being in the

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γ-phosphate position and cleaved away during incorporation. The company envisions its platform will consist of a massively parallel array of tethered DNA polymerases that will generate 1×10^6 bp of sequence per second.

Pacific Biosciences performs single-molecule real-time sequencing and uses phospholinked fluorescently labeled dNTPs. DNA sequencing is performed in thousands of reaction wells 50–100 nm in diameter that are fabricated with a thin metal cladding film deposited on an optical waveguide consisting of a solid, transparent silicon dioxide substrate. Each reaction well is a nanophotonic chamber in which only the bottom third is visualized, producing a detection volume of approximately 20 zL (20×10^{-21} L). DNA polymerase/template complexes are immobilized to the well bottoms, and 4 differently labeled dNTPs are added. As the DNA polymerase incorporates complementary nucleotides, each base is held within the detection volume for tens of milliseconds, orders of magnitude longer than the amount of time it takes for a nucleotide to diffuse in and out of the detection volume. Laser excitation enables the incorporation events in individual wells to be captured through the optical waveguide, with the fluorescent color detected reflecting the identity of the dNTP incorporated. For sequencing, Pacific Biosciences uses a modified phi29 DNA polymerase that has enhanced kinetic properties for incorporating the system's phospholinked fluorescently labeled dNTPs. In addition, phi29 DNA polymerase is highly processive, with strand-displacement activity. By taking advantage of these properties, Pacific Biosciences has demonstrated sequencing reads exceeding 4000 bases when a circularized single-stranded DNA molecule is used as template. In this configuration, the phi29 DNA polymerase carries out multiple laps of DNA strand-displacement synthesis around the circular template. The mean DNA-synthesis rate was determined to be approximately 4 bases/s. The observed errors, including deletions, insertions, and mismatches can be addressed by developing a consensus sequence read derived from the multiple rounds of template sequencing. Further refinement of the chemistries and platform instrumentation are ongoing, with a 2010 target date for commercial launch *(92–94)*.

Farther out toward the horizon is sequencing based on monitoring the passage of DNA molecules through nanopores 2–5 nm or greater in diameter. Nanopores are being fabricated in inorganic membranes (solid-state nanopores), assembled from protein channels in lipid membranes, or configured in polymer-based nanofluidic channels. In some configurations, current is applied across nanopore membranes to drive the translocation of negatively charged DNA molecules through pores while monitoring changes in membrane electrical conductance measured in the picoampere range. NABsys (http://www.nabsys.com) is pursuing a combination of nanopores with sequencing by hybridization in which single-stranded DNA molecules are hybridized with a library of hexamers of known sequence. The hybridized DNA is interrogated through a nanopore, with the current changes being different in regions of hexamer hybridization. The patterns of hybridization are used to map annealing regions and determine sequence. Oxford Nanopore Technologies (http://www.nanoporetech.com) is developing nanopore-based sequencing that uses an α-hemolysin protein channel in reconstituted lipid bilayers. The nanopores are situated in individual array wells, and single DNA molecules are introduced into the wells and progressively digested by exonuclease. The released single-nucleotide bases enter the nanopore and alter the electrical current, creating a characteristic current change for each individual base *(95, 96)*. Although the technology is seemingly futuristic, considerable NHGRI funding is being directed toward a variety of nanopore technologies under development as part of the goal of achieving the $1000 genome. For further descriptions of nanopore technologies, the reader is referred to recent reviews *(97, 98)*.

Conclusions

The past few years have witnessed the emergence of NGS technologies that share a common basis, massively parallel sequencing of clonally amplified DNA molecules. In 2008, the first NGS platform based on single-molecule DNA sequencing was launched. On the horizon are real-time single-molecule DNA-sequencing technologies and approaches based on nanopores. NGS has had a substantial impact on basic genomics research in terms of scale and feasibility. Over the next several years, NGS is anticipated to transition into clinical-diagnostics use. Essential elements to make this transition successful will be the requirement of streamlining the processes, especially sample preparation, coupled with improvements in technology robustness and characterization of accuracy through validation studies. The large amounts of sequence-data output will pose a bioinformatics challenge for the clinical laboratory. In addition to data processing, the interpretation of sequencing results will require further characterization of the genomic variation present in the regions analyzed. Although considerable work lies ahead to implement NGS into clinical diagnostics, the potential applications are exciting and numerous.

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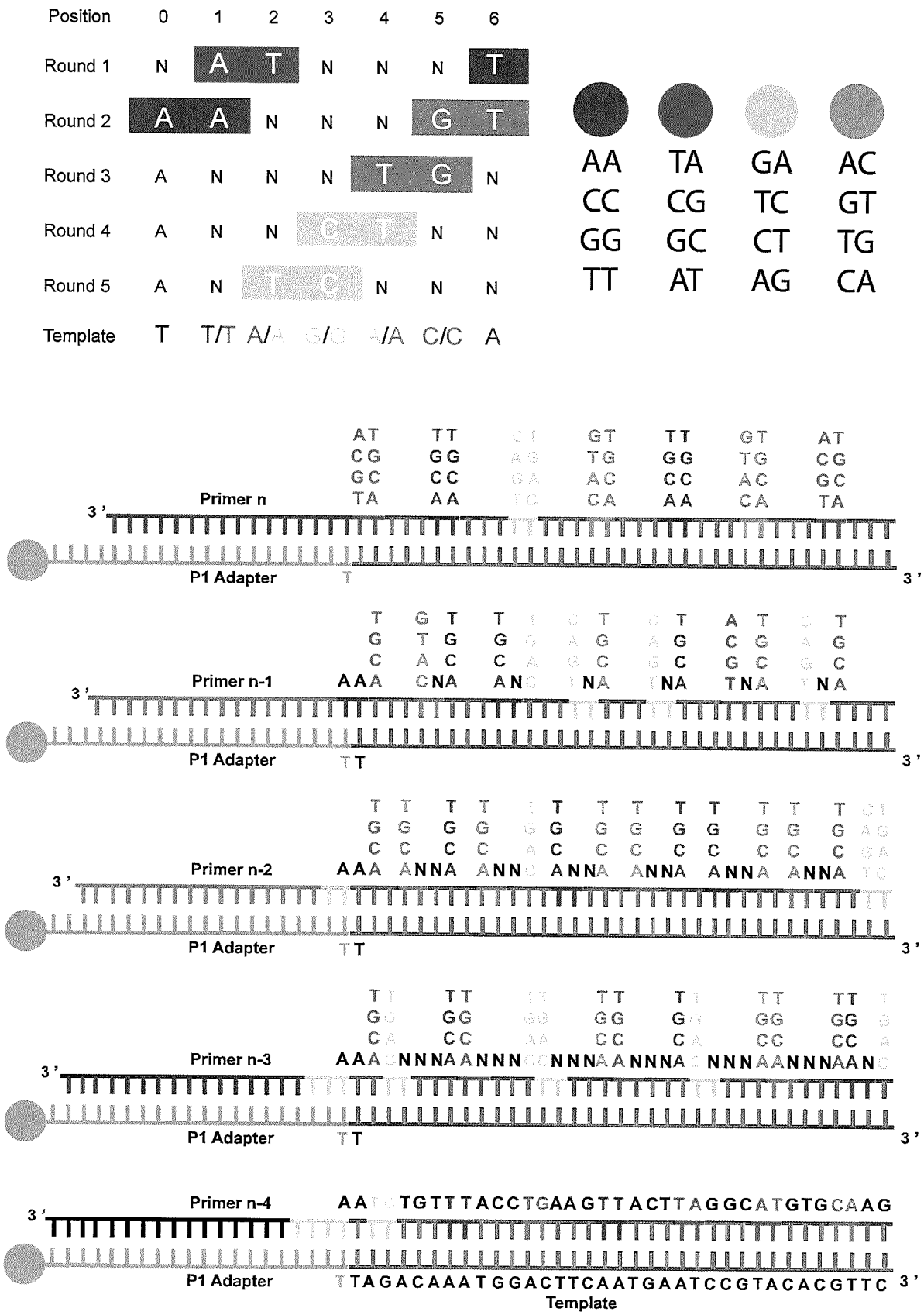
Supplemental Text Voelkerding, Dames, and Durtschi

Supplemental Data Figure 1.



Supplemental Figure 1. SOLiD Color Space Coding and Sequence Deconvolution. Top. Left) Interrogation of a template with sequence TAGACA (positions 1-6 with adapter sequence T at position 0). Right) Color space coding is reshown as reference. Bottom. Sequencing by ligation and deconvolution of template sequence. Extension of primer (n) interrogates template positions 1 and 2, 6 and 7, 11 and 12, and so forth. No sequence information can be determined during the first round since each fluor represents four two base possible combinations. The extension of primer (n-1) allows for the deconvolution of the base at position 1 with the knowledge of the P1 adapter base "T" at position 0. The extension of primers (n-2) and (n-3) provide additional positional color space information prior to deconvolution of the template performed after round 5 (primer n-4). The "N" bases show the positions along the template that have been sequenced twice prior to deconvolution.

Supplementary Data Table 1 Next Generation Sequencing Software Summary

Program Name	Alignment to Reference	De Novo Assembly	SNP Indel Discovery	CHiP/ Bisulphite -Seq	RNA-Seq	Alternate Base Caller	Alignment Viewer	Statistical Analysis	Primary Operating Environments	References	Authors	Web Site
ALLPATHS		X								(1)	Jonathan Butler et al.	
Alta-cyclic						X			Linux, SGE cluster environment	(2)	Yaniv Erlich et al.	hannonlab.cshl.edu/Alta-Cyclic/main.html
Bowtie	X								Linux, Windows, Mac		Ben Langmead and Cole Trapnell	bowtie-bio.sourceforge.net
BS-Seq				X					POSIX OS	(3)	S.J. Cokus et al	epigenomics.mcdb.ucla.edu/BS-Seq/download.html
CHiPSeq				X					Linux, Mac	(4)	D.S. Johnson et al.	woldlab.caltech.edu/html/chipseq_peak_finder
CLCbio Genomics Workbench	X	X	X				X		Windows		CLCbio	www.clcbio.com
EagleView							X		Linux, Windows, Mac	(5)	W. Huang, G. Marth	bioinformatics.bc.edu/marthlab/EagleView
Edena		X							Linux, Windows	(6)		www.genomic.ch/edena.php
ELAND	X								Linux		Anthony J. Cox	bioinfo.cgrb.oregonstate.edu/docs/solexa
Erange				X	X				Linux, Mac	(7)	Ali Mortazavi et al.	woldlab.caltech.edu/rnaseq
Euler-SR		X								(8, 9)	Mark J. Chaisson and Pavel A. Pevzner	
Exonerate	X								Linux	(10)	Guy St, C Slater and Ewan Birney	www.ebi.ac.uk/~guy/exonerate
FindPeaks				X					Java	(11)	Anthony Fejes	vancouvershortr.sourceforge.net
GMAP	X								Unix	(12)	Thomas Wu and Colin Watanabe	
JMP Genomics							X	X	Linux, Win, Mac		SAS Institue Inc.	www.jmp.com/software/genomics
MAQ	X								Linux, Mac	(13)	Heng Li	maq.sourceforge.net/index.shtml
MIRA2	X	X							Linux	(14)	Bastien Chevreux	chevreux.org/projects_mira.html
MOSAIK	X										Michael Strömberg and Gabor Marth	
MUMmer	X								POSIX OS	(15)	Stefan Kurtz, Adam Phillippy, Arthur L Delcher, Michael Smoot, Martin Shumway, Corina Antonescu and Steven L. Salzberg	http://mummer.sourceforge.net
NextGENe	X	X	X				X		Windows		SoftGenetics	www.softgenetics.com
NOVOALIGN	X								Linux, Mac OS X		Novocraft Technologies	www.novocraft.com/products.html
PBShort			X						Linux		Gabor Marth	bioinformatics.bc.edu/marthlab/PbShort
PyroBayes			X						Linux	(16)	A.R. Quinlan, D.A. Stewart, M.P. Strömberg, G.T. Marth	http://bioinformatics.bc.edu/marthlab/PyroBayes

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Program Name	Alignment to Reference	De Novo Assembly	SNP Indel Discovery	CHiP/ Bisulphite -Seq	RNA-Seq	Alternate Base Caller	Alignment Viewer	Statistical Analysis	Primary Operating Environments	References	Authors	Web Site
QuEST				X					Linux	(17)	Anton Valouev	mendel.stanford.edu/sidowlab/downloads/quest
RMAP	X								POSIX OS	(18)	Andrew D. Smith and Zhenyu Xuan	http://rulai.cshl.edu/rmap
Roche 454 analysis tools	X	X	X				X		Linux		Roche	www.454.com/products-solutions/analysis-tools/index.asp
Rolexa						X			R	(19)	Jacques Rougemont	svitsrv25.epfl.ch/R-doc/library/Rolexa/html/00Index.html
SAM							X		Linux		Rene Warren, Yaron Butterfield, Asim Siddiqui and Steven Jones	www.bcgsc.ca/platform/bioinfo/software/sam
SeqMan Ngen with SeqMan Pro	X	X	X				X		Windows	(20)	DNAStar	www.dnastar.com
SeqMap	X								Linux, Windows, Mac	(21)	H. Jiang and W.H. Wong	biogibbs.stanford.edu/~jiangh/SeqMap
SHARCGS		X							Perl	(22)	Juliane C. Dohm, Claudio Lottaz, and Heinz Himmelbauer	sharcgs.molgen.mpg.de
SHRAP		X								(23)	Andreas Sundquist et al.	
SHRiMP	X								Linux, Mac		Michael Brudno and Stephen Rumble	http://compbio.cs.toronto.edu/shrimp
SISSRs				X					Perl		Raja Jothi	dir.nhlbi.nih.gov/papers/lmi/epigenomes/sissrs
Slider	X								Java	(24)	N. Malhis, Y. Butterfield, M. Ester, S.J. Jones	http://www.bcgsc.ca/platform/bioinfo/software/slider
SOAPaligner/ soap2	X								Linux	(25)	T.W. Lam, Alan Tam, Simon Wong, Edward Wu and S.M. Yiu, Ruiqiang Li	soap.genomics.org.cn/soapaligner.html
SOAPdenovo		X							Linux		T.W. Lam, Alan Tam, Simon Wong, Edward Wu and S.M. Yiu, Ruiqiang Li	soap.genomics.org.cn/#
SSAHA2	X								Linux	(26)	Zemin Ning, Anthony Cox and James Mullikin	www.sanger.ac.uk/Software/analysis/SSAHA
ssahaSNP			X						Linux, Solaris, Mac		Adam Spargo and Zemin Ning	www.sanger.ac.uk/Software/analysis/ssahaSNP
SSAKE		X							Linux	(27)	René Warren, Granger Sutton, Steven Jones and Robert Holt	http://www.bcgsc.ca/platform/bioinfo/software/ssake
SXOligoSearch	X								Web Based		Synamatix	http://synasite.mgrc.com.my:8080/sxog/NewSXOligoSearch.php
VCAKE		X							Perl	(28)	W. Jeck	http://sourceforge.net/projects/vcake
Velvet		X							Linux, Mac	(29)	Daniel Zerbino and Ewan Birney	http://www.ebi.ac.uk/~zerbino/velvet/
XMatchView							X		Linux, Windows		Rene Warren and Steven Jones	http://www.bcgsc.ca/platform/bioinfo/software/xmatchview

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Exhibit 42

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A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance

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ABSTRACT

Objectives: To investigate the prospects of newly available benchtop sequencers to provide rapid whole-genome data in routine clinical practice. Next-generation sequencing has the potential to resolve uncertainties surrounding the route and timing of person-to-person transmission of healthcare-associated infection, which has been a major impediment to optimal management.

Design: The authors used Illumina MiSeq benchtop sequencing to undertake case studies investigating potential outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*.

Setting: Isolates were obtained from potential outbreaks associated with three UK hospitals.

Participants: Isolates were sequenced from a cluster of eight MRSA carriers and an associated bacteraemia case in an intensive care unit, another MRSA cluster of six cases and two clusters of *C difficile*. Additionally, all *C difficile* isolates from cases over 6 weeks in a single hospital were rapidly sequenced and compared with local strain sequences obtained in the preceding 3 years.

Main outcome measure: Whole-genome genetic relatedness of the isolates within each epidemiological cluster.

Results: Twenty-six MRSA and 15 *C difficile* isolates were successfully sequenced and analysed within 5 days of culture. Both MRSA clusters were identified as outbreaks, with most sequences in each cluster indistinguishable and all within three single nucleotide variants (SNVs). Epidemiologically unrelated isolates of the same *spa*-type were genetically distinct (≥21 SNVs). In both *C difficile* clusters, closely epidemiologically linked cases (in one case sharing the same strain type) were shown to be genetically distinct (≥144 SNVs). A reconstruction applying rapid sequencing in *C difficile* surveillance provided early outbreak detection and identified previously undetected probable community transmission.

Conclusions: This benchtop sequencing technology is widely generalisable to human bacterial pathogens. The findings provide several good examples of how rapid and precise sequencing could transform identification of transmission of healthcare-associated infection and therefore improve hospital infection control and patient outcomes in routine clinical practice.

ARTICLE SUMMARY

Article focus

- To investigate the prospects of newly available benchtop sequencers to provide rapid whole-genome data in routine clinical practice.
- In particular to investigate the potential of such technology for identification of transmission events of healthcare-associated pathogens.

Key messages

- We demonstrate benchtop sequencing can enhance hospital infection control through high precision support and rejection of transmission using genetic data.
- Whole-genome data provided additional genetic resolution over existing genetic typing strategies.
- We also show this technology offers turnaround times of under a week in a format that, in contrast to molecular typing, is organism independent.

Strengths and limitations of this study

- The case studies presented provide several good examples of how rapid and precise sequencing could transform identification of transmission of healthcare-associated infection.
- Given this is a pilot study, further evaluations of the impact of this technology on hospital infection control are required. However, this study provides a clear rationale for future work undertaking formal comparisons of benchtop sequencing with existing local and national typing schemes.

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BACKGROUND

Uncertainty about the exact route and timing of transmission is a major impediment to management of healthcare-associated infection, particularly for endemic pathogens that are also carried commensally, such as *Staphylococcus aureus* and *Clostridium difficile*. This problem impairs the development and implementation of effective evidence-based measures for infection control.[1]

High-throughput methods using next-generation sequencing (NGS) are revolutionising bacterial genomics, providing sufficient resolution potentially to determine which cases within temporo-spatial clusters are likely to be related.[2–5] With the advent of rapid sequencers, sources of outbreaks have been identified in clinically relevant timescales, demonstrating the potential of NGS to transform infection control practice.[6–8] The latest benchtop machines offer whole-genome sequencing in a format and at a cost accessible to routine hospital laboratories,[9] but the practical prospects for their use are unclear.

Here, we apply benchtop NGS in near real time to four case studies and to a surveillance reconstruction, involving two important healthcare-associated pathogens, *S aureus* and *C difficile*. We demonstrate how NGS supports identification of outbreaks of closely genetically related cases, including highlighting potential genetic links between cases not previously known to be related. In other examples, we show how NGS can refute transmission between cases that are epidemiologically linked, including between cases sharing the same strain type, indicating the additional benefit benchtop sequencing may provide over existing typing strategies.

METHODS

Setting and patients, Oxford-based case studies

The Oxford University Hospitals NHS Trust (OUH) comprises 1600 beds across four hospitals, three in Oxford and one 35 miles north in Banbury. It provides >90% of hospital care, and all acute services, to ~600 000 people in Oxfordshire, UK. The OUH microbiology laboratory provides all diagnostic testing for *S aureus* and *C difficile* for the region. All cases from three suspected outbreaks between July and October 2011 underwent NGS in parallel with routine infection control investigation (figure 1). A cluster of carriers of an atypical methicillin-resistant *S aureus* (MRSA) strain in an intensive care unit (ICU), with an associated bloodstream infection (MRSA cluster 1) was investigated. All *S aureus* isolates from patients in this ICU in the following month were sequenced, first to establish the level of diversity among *S aureus* isolates from the ICU to provide a comparison with the potential outbreak data and second to confirm control of the MRSA outbreak. Isolates were sequenced irrespective of resistance phenotype. The ICU is a 40 bedded unit offering high dependency and intensive care. Briefly, baseline infection control interventions included daily environmental cleaning, cleaning of equipment between patients and use of 'aseptic non-touch technique' for all line insertion, use and care. However, MRSA carriers were not routinely isolated. No other MRSA carriers were identified on the ICU in the month prior to the outbreak and only one in the 2 months following. Two clusters of *C difficile* infection (CDI, *C difficile* clusters 1 and 2) from a medical ward and an elective surgical unit were also investigated. OUH infection control policy targeting *C difficile* is described in a previous publication.[10]

Setting and patients, Health Protection Agency *S aureus* case study

A possible MRSA outbreak reported to the national Health Protection Agency affecting six patients in southern England between July and September 2011 was investigated (figure 1, MRSA cluster 2). All isolates possessed the *lukS* and *lukF* genes encoding Panton–Valentine leukocidin. Two additional local isolates that shared the same staphylococcal protein A (*spa*)-type, but without an epidemiological connection to the cluster, were also included as controls.

Setting and patients, Oxford-based surveillance reconstruction

To investigate the potential of fast turnaround benchtop sequencing as a surveillance tool, all CDI cases from one of the OUH hospitals over a 6-week period (July to August 2010) were sequenced by MiSeq. Although prepared and sequenced together, samples were analysed sequentially in the order originally sent to the routine clinical laboratory to mimic how NGS could support a real infection control investigation. Sequence data obtained on the Illumina (San Diego, California, USA) GAIIx and HiSeq platforms from 1185 of 1460 samples taken between September 2007 and June 2010 from a previously described collection of all Oxfordshire CDI cases[10] were available for comparison.

Samples and sequencing

S aureus isolates were obtained from clinical samples inoculated onto MRSA*Select* agar (Bio-Rad, Hemel Hempstead, UK). Antimicrobial sensitivities were determined by disc diffusion and E testing as per European Committee on Antimicrobial Sensitivity Testing guidelines.[11] *C difficile* isolates were identified following selective culture[12] of toxin enzyme immunoassay-positive diarrhoeal faecal samples.

For both organisms, DNA was extracted using a commercial kit (QuickGene, Fujifilm, Tokyo, Japan), from a single colony subcultured onto a Columbia blood agar plate and incubated for 24–48 h. A combination of standard Illumina and adapted protocols was used to produce multiplexed paired-end libraries (DNA fragments with each sample's DNA tagged with a unique index). Pools of four samples were sequenced at the Wellcome Trust Centre for Human Genetics, Oxford, UK, using sequencing-by-synthesis technology,[13] on the Illumina MiSeq platform, generating 150 base paired-end reads.

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MRSA cluster 1

10 isolates (8 patients), obtained in an intensive care unit, July to September 2011

- 8 nasal screening swabs, from 7 patients, positive over 2 weeks [*S aureus* cases A–G]
- 1 blood culture and 1 nasal swab from bacteraemic patient 8 weeks later [*S aureus* case H]

All 8 methicillin-sensitive *S aureus* isolates from the same unit in October 2011 also sequenced to confirm outbreak control (and assess background diversity)

- 8 nasal screening swabs [*S aureus* cases I–P]

MRSA cluster 2

6 Health Protection Agency isolates associated with a single hospital, July to September 2011

- 3 caesarian section wound infection wound swabs [*S aureus* cases Q, R, S]
- 1 breast abscess – aspirate [*S aureus* case T]
- 1 finger abscess – aspirate [*S aureus* case U]
- 1 nasal swab (case family member) [*S aureus* case V]

2 unrelated cases sharing the same *spa* type included for comparison

- [*S aureus* cases W, X]

***C difficile* cluster 1**

4 CDI cases within 17 days, same ward (medical specialty), September to October 2011

- 4 stool samples [*C difficile* cases A–D]

***C difficile* cluster 2**

3 CDI cases within 22 days, same unit (elective surgical specialty), September to October 2011, preceding CDI case also sequenced

- 4 stool samples [*C difficile* cases E–H]

***C difficile* Surveillance**

All 7 CDI cases in a single hospital over 6 weeks, July to August 2010 sequenced and compared with previously sequenced isolates from September 2007 to June 2010

- 7 stool samples

Figure 1 Clusters and samples. All clusters of cases occurred in the Oxford University Hospitals NHS Trust between July and October 2011, apart from MRSA cluster 2 where samples were obtained by the Health Protection Agency from an outbreak in southern England between July and September 2011. CDI, *Clostridium difficile* infection: ≥3 unformed stools in 24 h, enzyme immunoassay positive, culture positive.

Sequence assembly and analysis

Sequence read data were analysed and assembled using a pipeline developed specifically for bacterial sequencing, as follows: to measure genome-wide sequence similarity, the full set of properly paired reads from each isolate was mapped using Stampy[14] V.1.0.11 (without BWA pre-mapping, using an expected substitution rate of 0.01), to either *S aureus* MRSA252 (GenBank: NC_002952) [15] or *C difficile* 630 (GenBank: AM180355), CD630.[16] Single nucleotide variants (SNVs) were identified across all mapped non-repetitive sites using SAMtools[17] mpileup, after parameter tuning based on bacterial sequences. Known mobile genetic elements in CD630 were excluded from the analysis, as they account for 11% of the genome,[16] and exchange of a single element may result in multiple SNVs in one event. A consensus of at least 75% was required to support an SNV, and calls were required to be homozygous under a diploid model. Only SNVs supported by at least five reads, including one in each direction, which did not occur at sites with unusual depth and were not within 12 bp of other variants, were accepted. Maximum likelihood trees were estimated from the mapped whole genomes using PhyML[18] using a Jukes–Cantor model. To identify variation in gene content, sequence reads were assembled de novo using Velvet V.1.0.18, [19] with parameter values optimised to maximise n50 (50% of the assembly in contigs equal to or larger than this value). These assemblies were used to identify antibiotic resistance genes for *S aureus* and to determine in silico multilocus sequence types (MLST)[12] for *C difficile*.

To assess both MiSeq data quality and its comparability with existing HiSeq data, the MRSA252 and CD630 references and one other isolate of each organism were sequenced on both platforms. Duplicates of two of the *S aureus* isolates from MRSA cluster 1 and an MRSA252 sample were run on MiSeq as controls.

Ethics statement

Ethical approval for sequencing *S aureus* and *C difficile* isolates from routine clinical samples and linkage to patient data without individual patient consent was obtained from Berkshire Ethics Committee (10/H0505/83) and the UK National Information Governance Board

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(8-05(e)/2010). The Health Protection Agency has Patient Information Advisory Group approval to hold and analyse surveillance data for public health purposes under Section 60 of the Health and Social Care Act 2001.

RESULTS

Twenty-six *S aureus* isolates (from 24 patients) and 15 *C difficile* isolates (from 15 patients) (figure 1) were sequenced using the MiSeq benchtop sequencer. Mean read-depths for *S aureus* and *C difficile* were 77.6 and 50.4, respectively, and reference genome coverages were 82.7% and 79.5%, respectively, after quality filtering (see supplementary figures 1–3 for regions called; uncalled regions include repetitive regions which, in contrast to Sanger sequencing, 150 bp reads cannot cover, and mobile elements, as well as other non-core genome). The entire process from commencing DNA extraction to measuring genomic relatedness for each set of sequences was completed within five working days of culture. No sequence differences were detected in the two pairs of replicates: between CD630 and MRSA252 and published reference sequences or between four samples sequenced both with MiSeq and earlier with HiSeq.

NGS supports a ward outbreak, despite discordant antimicrobial and strain-typing data (MRSA cluster 1)

Ten MRSA isolates obtained from eight patients from the same ICU over 4 months belonged to the same *spa*-type (t5973) and were indistinguishable by pulsed-field gel electrophoresis (PFGE) (figure 2A,B). Ward stays and the first positive isolate per patient are shown in

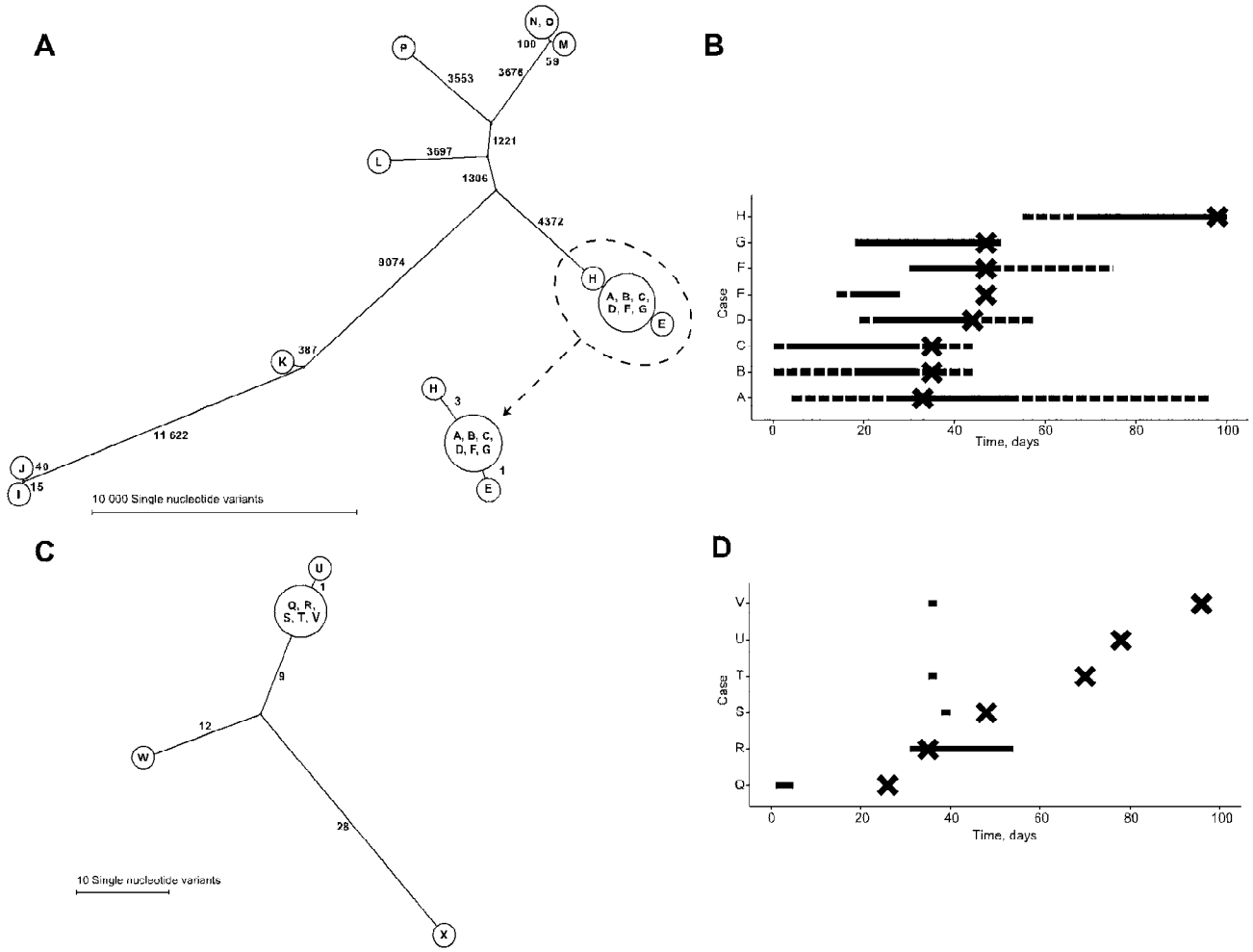

Figure 2 *Staphylococcus aureus* cluster genetic and epidemiological relationships. (A and B) MRSA cluster 1 and related MSSA isolates. (C and D) MRSA cluster 2. Panel A shows all isolates from MRSA cluster 1 (white circles) and all *S aureus* isolates from the following month (grey circles, all methicillin sensitive). The left panels show the genetic relationships between cases as maximum likelihood trees, labelled with the number of single nucleotide variants at which samples differ. Genetically indistinguishable samples are shown in the same circle. The right panels show time spent on the same ward as a horizontal line for each case in both clusters. In panel B, the dashed line indicates time on the same ward and the solid line time in the same bay. In panel D, the solid line indicates time spent on the same ward. The timing of the first positive sample for each case is indicated with a cross. Case H subsequently developed a bloodstream infection.

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Table 1 MRSA cluster 1, comparison of antibiotic susceptibility and associated genetic elements

		Antibiotic susceptibility				Gene presence/ absence	
		Penicillin		Tetracycline			
Patient	Sample date	DD	MIC	DD	MIC	*blaZ*	*tetK*
A	25/7	R	2	S	0.094	+	–
B	27/7	R	2	R	24	+	+
C	27/7	R	4	S	0.094	+	–
D (i)	5/8	R	3	R	24	+	+
D (ii)	10/8	R	2	R	24	+	+
E	8/8	R	4	R	32	+	+
F	8/8	S	0.047	S	0.064	–	–
G	8/8	R	3	S	0.094	+	–
H (i)	28/9	R	4	R	24	+	+
H (ii)*	29/9	R	2	R	24	+	+

Screening swabs were obtained from patient D on two separate dates. All isolates were methicillin heteroresistant, appearing susceptible on routine testing, despite detection of *mecA* by PCR and sequencing. This explains why isolate F appeared phenotypically penicillin susceptible on DD and E testing. The mechanism of heteroresistance for these isolates has not yet been fully elucidated, although the penicillin-susceptible methicillin-resistant phenotype has been described.[20]
*The second isolate from patient H is from a positive blood culture.
DD, disc diffusion (R, resistant; S, susceptible); MIC, minimum inhibitory concentration (milligrams per litre).

figure 2B. The extreme rarity of this *spa*-type in the UK (no t5973 isolates held by the National Reference Laboratory), and the fact they exhibited indistinguishable PFGE types, would normally be considered sufficient evidence for an outbreak. However, the isolates showed methicillin heteroresistance (which impaired initial detection of the outbreak) and differed in penicillin and tetracycline sensitivity (table 1); no common source was identified, casting doubt on the connection between the first seven cases and with the bloodstream infection, which occurred after a further 8 weeks, but in the absence of any contemporaneous patient MRSA isolates.

No sequence differences were detected in the mapped genomes between isolates from six of the carriage cases (A, B, C, D, F and G); case E differed at a single site. The two isolates (nasal swab and blood culture) from the bloodstream infection case (H) were indistinguishable and differed by three SNVs from the other cases. Given previous estimated rates of short-term *S aureus* evolution of 9.6 (95% CI 7.3 to 11.6) [4] and 7.9 (95% credibility interval 4.8–12.8) [21] SNVs/genome/year, these data are consistent with recent acquisition from a common source. In contrast, the mean number of SNVs between all pairs of *S aureus* isolates from the same unit from the following month was 7541 (figure 2A). All the outbreak cases (A–H) were *mec*A positive by PCR and sequencing; however, two plasmids not present in MRSA252, encoding the *bla*Z and *tet*K genes, were detected in seven and five of the isolates, respectively. These genotypic findings matched the phenotypic susceptibility results (table 1).

As sequencing data provided support for an outbreak, this directly led to implementation (and escalation with subsequent cases) of intensive infection control supervision of the unit with visits up to four to five times per day. Observations made resulted in retraining for medical and nursing staff covering administration of intravenous medication and taking blood cultures. Additionally, isolation of all MRSA carriers was implemented and reinforced.

NGS supports transmission of isolates during short periods of shared ward exposure (MRSA cluster 2)

Six Panton–Valentine leukocidin-positive MRSA isolates were identified over 3 months (figure 2C,D). Five had been inpatients on the same ward (Q, R, S, T and V) (with overlapping stays of 1–2 days in four cases) and one (U) was a relative of V. All were *spa*-type t657, which, although relatively rare, has occurred sporadically in this region. The isolates were also indistinguishable by PFGE. Therefore, given the prolonged timescale, it was unclear whether these cases reflected a genuine outbreak or background circulation of related organisms. In fact, only one SNV was detected among all six samples, indicating a recent common source, whereas two isolates originating from the same geographical area and with the same *spa* and susceptibility profiles, but with slightly different PFGE types and no known epidemiological link, differed from the main cluster at 21 and 37 sites, respectively. The sites were distributed throughout the genome ruling out a single recombination event accounting for all the differences (supplementary figure 1). Additionally, no variant sites were detected within the *mut*S, *mut*S2 and *mut*L genes associated with hypermutation.[22] The SNV differences are therefore consistent with a shared ancestry 2–5 years earlier.

NGS refutes transmission between suspected linked cases (*C difficile* cluster 1)

The OUH infection control service identified three CDI cases (B, C and D) occurring over 4 days among

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inpatients on the same ward (figure 3A,B). While UK Department of Health guidance[23] states that a cluster should only be considered an 'outbreak' when the cases share a strain type (eg, by MLST or PCR-ribotyping), such information is slow to obtain. In practice, therefore, such clusters are treated as presumptive outbreaks requiring intensive management. Sequencing showed that all three cases had different computationally predicted sequence types (STs, ST2, ST10 and ST37) and differed at >4000 sites distributed throughout the genome (supplementary figure 2). With short-term *C difficile* evolution rates estimated from serial sampling of 91 patients with samples taken between 1 and 561 days apart at 2.3 SNVs/genome/year (95% credibility interval 1.6–3.0). (Xavier Didelot, personal communication 20 January 2012, manuscript under review), recent transmission between these cases can be excluded with confidence. However, a fourth case (A) that occurred 12 days previously, and was not initially included in the infection control investigation, was indistinguishable from case B; case A was found to have been in an adjacent side room to case B, strongly suggesting ward-based transmission. Presentation of this sequencing data backed transmission event to an outbreak review meeting, resulted in a detailed infection control audit, which in turn led to markedly improved cleaning of equipment.

NGS demonstrates that isolates of the same strain type are not necessarily linked by transmission (*C difficile* cluster 2)

Three CDI cases (F, G and H) occurring over a 3-week period in an elective surgical unit were suggestive of transmission since the most recent previous CDI (E) had occurred 6 months before (figure 3C,D). However, isolates from the three cases were sufficiently genomically diverse to rule out transmission. Notably, two isolates shared a sequence type (ST5) under the strain-typing scheme used in OUH but differed at 144 sites distributed throughout the genome (supplementary figure 3), providing an example of the extra discriminatory power of sequencing over existing typing schemes in ruling out transmission.

NGS-based *C difficile* surveillance, a reconstruction

All seven CDI cases occurring in one of the OUH hospitals over 6 weeks were sequenced on the MiSeq platform and compared with each other and with an 'historical' sequence database comprising 1185 isolates from regional CDI cases occurring in the previous 3 years.

Four of the seven cases (ST3) formed a genetic cluster containing variation at only two SNVs, indicating probable transmission; these cases shared time and space on the same ward around their CDI. The genetically most similar historical CDI case differed from the four

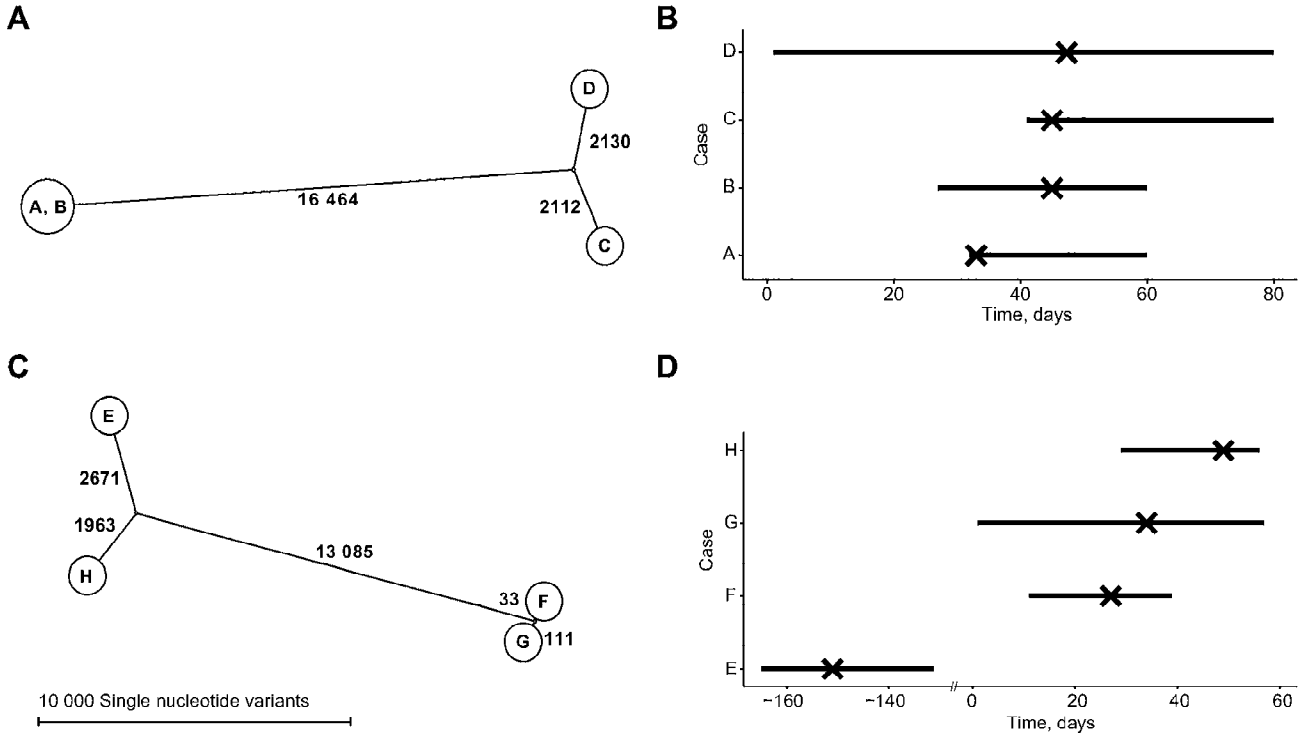

Figure 3 *Clostridium difficile* cluster genetic and epidemiological relationships. (A and B) *C difficile* cluster 1. (C and D) *C difficile* cluster 2. The left panels show the genetic relationships between cases as maximum likelihood trees, labelled with the number of single nucleotide variants at which samples differ. Genetically indistinguishable cases are shown in the same circle. The right-hand panels (B and D) show time spent on the same ward as a horizontal line for each case. The timing of the first positive sample for each case is indicated with a cross.

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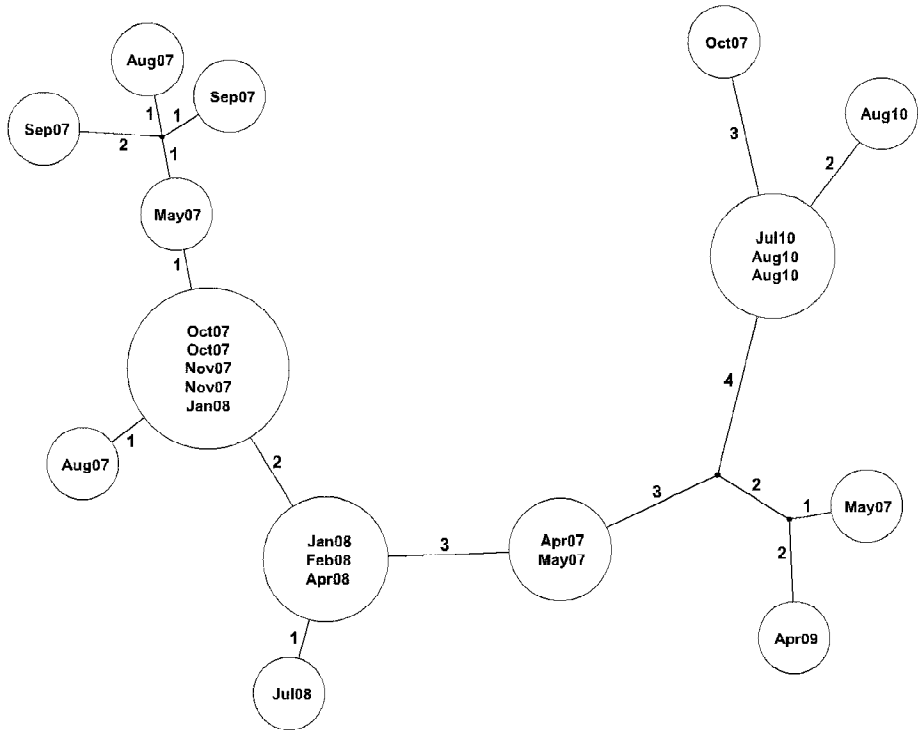

Figure 4 Potential for whole-genome sequencing to enhance *Clostridium difficile* surveillance. Genetic relationships between cases are shown as a maximum likelihood tree, labelled with the number of single nucleotide variants (SNVs) at which samples differ. Genetically indistinguishable cases are shown in the same circle. Cases from an existing database of sequenced isolates are shown in grey circles with the date of isolation. Four cases arising during a 6-week surveillance reconstruction are shown in white circles. Three additional cases sequenced as part of the reconstruction differed at >3000 SNVs from the cases shown and are not shown in the figure. The maximum likelihood tree shown is consistent with data; however, homoplasy was observed at one of the variant sites, 2464157, in the CD630 reference genome.

genetically clustered cases at three SNVs (figure 4); however, it occurred 3 years earlier and 30 miles way. Therefore, no direct relationship could be discerned between the historical cases and the current outbreak.

The three remaining cases in the group of seven reconstruction cases (representing ST1, ST11 and ST13) differed from the other four cases and from each other at >3000 SNVs. The overall mean pairwise SNV difference between all reconstruction cases was 13 012 SNVs. One individual (ST1, ribotype-027), diagnosed on the day of admission and last admitted 8 months previously, yielded a *C difficile* sequence indistinguishable from 11 previous CDI cases, including local strains from 6 months previously. Since this patient had not shared inpatient time with any of the other cases and most of the genetically related cases had occurred outside of OUH hospitals, this may represent previously unsuspected community-based transmission, which could have been investigated had sequence information been available in 2010. No plausible hospital or community patient source could be identified for the other two cases; however, previous cases differing from them at four to 10 SNVs were identified in the previous 6 months to 3 years, consistent with a local strain origin.

DISCUSSION

This study provides strong evidence in two major healthcare-associated pathogens, *S aureus* and *C difficile*, that benchtop sequencing can enhance hospital infection control through high precision support and rejection of transmission using genetic data. We also show this technology offers turnaround times of under a week in a format that, in contrast to molecular typing, is organism independent.

The results obtained in this study were obtained quickly enough to influence cluster investigations and in the outbreaks described were used to inform the hospital's response. Where suspected transmission events were supported by sequencing data in two of the outbreaks infection control supervision of measures to prevent case-to-case spread was significantly enhanced. In contrast, increases in incidence without transmission between infected patients still merit a response, for example, clusters of genetically unrelated *C difficile* cases on wards have prompted a review in our hospitals of antibiotic use and guidance. If such clusters were identified in patients with *S aureus* infection, this might prompt, for example, review of line care or perioperative care. Had the set of cases in the *C difficile* surveillance reconstruction been sequenced in real time, an outbreak would have become apparent after the second case, and it is possible that subsequent transmissions might have been prevented, particularly as compliance with infection control measures was incomplete at the time of the outbreak. Clearly formal evaluation of the use of the technology in an appropriately controlled trial is needed to determine the extent to which the control of these outbreaks was, or would have been, enhanced by the availability of sequence data.

Whole-genome sequencing provides the ultimate resolution of genetic relationships. This offers two clear benefits for inference of transmission events. First, putative transmission events between genetically very distinct isolates can be refuted with confidence. This is of

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particular value by comparison with widely used current typing strategies that are unable to distinguish isolates belonging to a prevalent strain type; for example, PCR-ribotype-027 (NAP-1) accounts for ~35% of *C difficile* strains in UK and North American hospitals.[24 25] Second, close genetic relationships combined with clinical and epidemiological evidence can provide strong evidence in favour of a putative transmission event, justifying infection control intervention, as detailed above. Notably, a genetic match in the absence of an obvious epidemiological link may legitimately prompt investigation of new routes of transmission, such as the possible community transmission identified in our *C difficile* surveillance reconstruction. However, close genetic links cannot be used in isolation to confirm transmission. For example, we identified genetically similar cases separated by both time and space, emphasising the importance of analysing genetic data alongside epidemiological information. The main limitation of this study is it is not large enough to provide a formal comparison with existing technology including a health economic evaluation. Additionally, although we used the Illumina MiSeq platform, other similar benchtop technology exists, for example, Ion Torrent (Life Technologies, Guildford, Connecticut, USA), or is under development, for example, Oxford Nanopore (Oxford Nanopore Technologies, Oxford, UK).

NGS technology is widely generalisable to human bacterial pathogens[26] and has been used previously to investigate transmission of infectious disease.[2–5] Although the benefits of rapid sequencers have been shown in high-profile national outbreaks,[6–8] we provide the first demonstration of rapid sequencing in a benchtop format applied to routine patient care and healthcare-associated pathogens. Existing typing schemes, such as *spa*, PFGE, PCR-ribotyping and MLST, have established a framework in the application of genetic data in outbreak investigation. However, the organism-specific application of these strain-typing methods, often requiring isolates to be sent to a specific reference laboratory, in practice means that many infection clusters remain untyped. Benchtop sequencing builds upon existing typing expertise, offering rapidly available and increased resolution data from an organism-independent platform. This means that a single technology will provide the capacity for individual hospital laboratories to support outbreak investigation for a range of pathogens and allow infection control personnel to efficiently target resources to genuine outbreaks. Sequencing costs are falling rapidly,[27 28] with the prospect within 12 months of being able to obtain complete accurate pathogen sequences in hours, and for as little as US$10 per sequence.[29] It is therefore likely that benchtop sequencing will soon be comparable in price to existing molecular typing while offering consider additional benefits. As well as identifying transmissions, the reproducible and digital nature of locally generated sequence data make these ideal for sharing in national and potentially international surveillance, for sequence-based resistance prediction and for precise generic species identification.[26]

The case studies in this pilot study provide a clear rationale for future work undertaking formal comparisons of benchtop NGS with existing local and national typing schemes. Such comparisons will need to include formal health economic assessments that account for the capital expense of establishing benchtop NGS equipment and expertise in a laboratory, as well as the potential cost-savings associated with more focused cluster investigation, and infection control interventions. The improved accuracy in identifying within-hospital transmission should also lead to better metrics of hospital infection control performance and provide an opportunity for further reductions in the incidence of healthcare-associated infections and hence improvements in patient outcomes.

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Contributors All authors were involved in critical review of the manuscript and have seen and approved the final version. Specific contributions as follows: study conception and design: DWC, TEAP, ASW, PJD, RB, MW and JP; sample acquisition: LO, RL, NCG, AK, AS and JP; sample sequencing: PP and DB; sequence data processing pipeline: RB, TG, EMB and CLCI; analysis of epidemiological and sequence data: DWE, TG, NCG, DJW, XD, TEAP, ASW and DWC; drafting the manuscript: DWE, NCG, TG, ASW, TEAP and DWC. The following two groups of authors contributed equally to this article, DWE, TG, NCG and RB; and TEAP, ASW and DWC. All authors had full access to all the study data and take responsibility for the integrity of the data and the accuracy of the data analysis. DWC is the guarantor.

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Competing interests All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author). The institution of DWC and TEAP received per-case

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funding from Optimer Pharmaceuticals to support fidaxomicin trial patient expenses. DWC and TEAP also received honoraria from Optimer Pharmaceuticals for participation in additional meetings related to investigative planning for fidaxomicin. MHW has received honoraria for consultancy work, financial support to attend meetings and research funding from bioMerieux, Optimer, Novacta, Pfizer, Summit, The Medicines Company, Viropharma and Astellas.

Ethics approval Ethical approval for sequencing *S aureus* and *C difficile* isolates from routine clinical samples and linkage to patient data without individual patient consent was obtained from Berkshire Ethics Committee (10/H0505/83) and the UK National Information Governance Board (8-05 (e)/2010). The Health Protection Agency has Patient Information Advisory Group approval to hold and analyse surveillance data for public health purposes under Section 60 of the Health and Social Care Act 2001.

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A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance

David W Eyre, Tanya Golubchik, N Claire Gordon, et al.

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Diagnosis of Clostridium difficile infection: An ongoing conundrum for clinicians and for clinical laboratories

Carey-Ann D. Burnham
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Diagnosis of Clostridium difficile Infection: an Ongoing Conundrum for Clinicians and for Clinical Laboratories

Carey-Ann D. Burnham and Karen C. Carroll
Clin. Microbiol. Rev. **2013, 26(3):604. DOI: 10.1128/CMR.00016-13.**

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Diagnosis of *Clostridium difficile* Infection: an Ongoing Conundrum for Clinicians and for Clinical Laboratories

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SUMMARY

Clostridium difficile is a formidable nosocomial and community-acquired pathogen, causing clinical presentations ranging from asymptomatic colonization to self-limiting diarrhea to toxic megacolon and fulminant colitis. Since the early 2000s, the incidence of *C. difficile* disease has increased dramatically, and this is thought to be due to the emergence of new strain types. For many years, the mainstay of *C. difficile* disease diagnosis was enzyme immunoassays for detection of the *C. difficile* toxin(s), although it is now generally accepted that these assays lack sensitivity. A number of molecular assays are commercially available for the detection of *C. difficile*. This review covers the history and biology of *C.*

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difficile and provides an in-depth discussion of the laboratory methods used for the diagnosis of *C. difficile* infection (CDI). In addition, strain typing methods for *C. difficile* and the evolving epidemiology of colonization and infection with this organism are discussed. Finally, considerations for diagnosing *C. difficile* disease in special patient populations, such as children, oncology patients, transplant patients, and patients with inflammatory bowel disease, are described. As detection of *C. difficile* in clinical specimens does not always equate with disease, the diagnosis of *C. difficile* infection continues to be a challenge for both laboratories and clinicians.

INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming, rod shaped bacterium that exists in the soil and in the gastrointestinal tract of animals and humans. More than 100 strains exist based upon mutations in the genes on the pathogenicity locus (PaLoc) that encode various toxins (see "Genetics and Pathogenesis" below) (1, 2). There are two forms of the organism, a dormant spore form that is resistant to antibiotics and a vegetative form that can produce toxins and is susceptible to the activity of antibiotics. The organism and its toxigenic potential were discovered in 1935 by Hall and O'Toole, who noted it as a component of the normal fecal microbiota of newborn infants (3). Since it was difficult to cultivate *in vitro*, they named it *Bacillus difficilis* (3, 4). Later, investigators verified that *Bacillus difficilis* produced a toxin that was highly potent when injected intraperitoneally into guinea pigs (5).

Since the organism did not seem to be associated with human disease at that time, it was largely ignored until the antibiotic era. In 1974, Hafiz characterized the organism in more detail, including a description of glutamate dehydrogenase (GDH) (also known as common antigen), and showed that it was widely distributed in nature, including in the stools of many animals (4). Around that time, cases of pseudomembranous colitis (PMC), initially described as early as 1893 (6), became much more prevalent and were attributed primarily to *Staphylococcus aureus* (4). Oral vancomycin provided therapeutic success and became the treatment of choice for this disease (4). Also, in the early 1970s, Tedesco et al. noted that clindamycin treatment for anaerobic infections seemed to cause severe diarrhea in several patients. Those researchers performed a prospective study to assess the frequency of diarrhea associated with clindamycin treatment and to assess patients who developed diarrhea by colonoscopy. Remarkably, 42 of 200 patients developed diarrhea, and 20 patients had endoscopic evidence of PMC, which those researchers called "clindamycin colitis" (7). *S. aureus* was not recovered from the stool samples of these patients. The connection with *C. difficile* as the etiologic agent was not made at that time, nor was it made by Green et al., who studied cecitis induced by penicillin in animals; those investigators did not understand that the toxin that they recovered in tissue cultures in 1974 was produced by *C. difficile* (4).

A series of studies and observations by many groups followed the published observations in 1974. It was shown that oral vancomycin administration could prevent clindamycin colitis when administered simultaneously. Other studies showed that the potential agent of disease could be transferred from ill to healthy animals in cecal contents and that it likely produced a large protein toxin (4). Laughon et al. reported detection of a cytotoxic substance in the stool samples of patients with pseudomembranous colitis (8). Te-Wen Chang and others characterized the morphological changes associated with antibiotic-associated colitis in animals and discovered that the cytopathic effects observed in fibroblast cells in tissue culture could be neutralized with *Clostridium sordellii* antitoxin, thus developing the cytotoxin assay as a potential diagnostic test method (9). In 1978, after multiple experiments, Bartlett et al. published the sentinel paper linking *C. difficile* toxin production to PMC in humans (10). Interestingly, several papers followed in which better diagnostic assays for *C. difficile* cytotoxin were sought, and enzyme immunoassays (EIAs) were developed. However, EIAs were shown to be about 10-fold less sensitive than the cytotoxin assay and were not pursued further by early investigators (4, 8).

During the 1980s and 1990s, *C. difficile* disease was not a major cause of mortality. However, in the new millennium, due to the "perfect storm" of epidemiological factors, host factors, and the emergence of organism traits that facilitate infection and disease, *C. difficile* infections (CDIs) have increased in frequency and severity. These important factors have resulted in reevaluation of diagnostic test methods and have stimulated the development of new assays.

THE ORGANISM—PATHOGENESIS AND DISEASE MANIFESTATIONS

Genetics and Pathogenesis

The emergence of "hypervirulent" *C. difficile* strains in the new millennium has sparked a renewed interest in creating better animal models of human disease and progress in phylogenetic studies to understand the pathophysiology of *C. difficile* infection. At least two strains have been completely sequenced—strain 630, an epidemic, restriction fragment length polymorphism (RFLP)-PCR toxinotype X clinical strain that is virulent and multidrug resistant, and a nontoxigenic strain, CD37 (ribotype 009) (11, 12). Several other isolates have had partial genomic characterization. A detailed discussion of the *C. difficile* genome is beyond the scope of this review, and the reader is referred to other references for information (11, 13). However, it is important to stress that such work has provided insights into how *C. difficile* survives in the environment and causes disease in humans and animals—information that may promote development of diagnostic tests and therapeutic interventions.

In order for *C. difficile* to cause disease, several important conditions must be met. A person must have contact with the spores of a toxin-producing strain of *C. difficile* in combination with alteration of the normal colonic microbiota, permitting colonization of the organism. Both animal models and studies of the human gut microbiome have elucidated the microbial and cellular interactions within this complex intestinal ecosystem (14). Also of importance is the host immune system, as evidenced by the higher rates of infection and worsening disease severity among the elderly and other persons who lack the ability to mount an effective humoral immune response (15, 16). Antitoxin antibodies may be protective and can also explain variations in disease presentations among immunocompetent hosts (15, 17).

The fact that antibiotics alter the gut microbiota has been established since the 1940s, shortly after streptomycin became available and investigators noted the impact of oral administration of this agent on the bacteria present in the feces of mice (14). In subsequent decades, these observations were pursued in animal models

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of enteric diseases, including *C. difficile* infections, where it was discovered that animals who were pretreated with antibiotics prior to challenge with the enteric pathogen succumbed to infection compared to the untreated groups (18–20). In several models, the untreated animals often did not become colonized, or the endogenous microbiota outcompeted the challenge pathogen (18–20), a phenomenon referred to as colonization resistance (14).

Antibiotics are the major risk factor for the development of *C. difficile* disease because of the loss of endogenous microbiota that allows *C. difficile*, when present, to proliferate and invade. Several interesting studies of the gut microbiome using culture-independent methods have elucidated the significant alterations that follow antibiotic administration (21, 22). These studies have also shown that in patients recovering from *C. difficile* disease, recurrence may be related to a failure to restore the normally diverse microbial intestinal community (22, 23). The latter observation explains why fecal transplantation is so efficacious in the management of patients with recurrent *C. difficile* disease (24, 25). The precise protective mechanisms of the normal microbiota in preventing *C. difficile* disease are not completely elucidated. Several hypotheses that are being pursued include (i) the impact of gut microflora on bile acid transformation that impacts *C. difficile* spore germination; (ii) direct antagonism by the normal microbiota through production of bacteriocins, antimicrobials produced by these organisms that directly inhibit the growth of *C. difficile*; (iii) stimulation of innate immune responses by Toll-like receptor 5 signaling produced by intestinal microbiota; and (iv) competition between the microbiota and *C. difficile* for limited nutritional resources (14).

A significant portion of the *C. difficile* genome is involved in encoding factors that ensure survival in the gastrointestinal tract. These coding regions produce enzymes that assist with carbohydrate transport and metabolism, including *p*-hydroxyphenylacetate decarboxylase, which allows the organism to produce *p*-cresol, a bacteriostatic compound that may be inhibitory to intestinal microbes (11, 26). It is this compound that is responsible for the "horse barn" odor characteristic of *C. difficile*, a trait often used to identify the organism when grown in culture. In addition, *C. difficile* produces other compounds that allow it to utilize nitrogen and phospholipids found in the host's diet and to survive in the presence of bile acids (26).

A large segment (19.6 kb) of the genome found in toxigenic strains but lacking in nontoxigenic strains is the pathogenicity locus (PaLoc). The PaLoc contains five genes: *tcdA*, *tcdB*, *tcdC*, *tcdE*, and *tcdR* (1). *tcdA* and *tcdB* are in close proximity, separated by *tcdE*, and encode toxins A and B, respectively. The *tcdE* gene encodes a protein that is important for the release of toxins A and B from the cell (27). Upstream of *tcdB* is *tcdR*, which is a positive regulator of *tcdA* and *tcdB* expression (1, 27). Downstream of *tcdA* is *tcdC*, which functions as a negative regulator of toxin production during the exponential phase of growth (1, 28). We refer the reader to other articles with schematic representations of the PaLoc (26, 29–31). The other genes, *tcdA*, *tcdB*, *tcdE*, and *tcdR*, are expressed during stationary phase (1). Another important regulator of toxin gene expression is CodY, which is a global gene regulator that monitors environmental factors such as the presence of carbohydrates, amino acids, and other nutrients (32). CodY influences *tcdR* expression and, hence, toxin gene production and may be a potential target for future therapeutic agents (32).

In addition to toxins A and B, a third toxin, called binary toxin (see below), is also produced by some, but not all, strains of toxigenic *C. difficile*. The genes that encode binary toxin are not found in the PaLoc (26). Other important virulence factors that contribute to the pathogenesis of *C. difficile* include adhesins, fimbriae, flagella, a capsule, and a paracrystalline S-layer protein (important in cellular adhesion) (11, 26, 29, 33).

Roles of Toxin A, Toxin B, and Binary Toxin in Pathogenesis

Toxin A (TcdA) and toxin B (TcdB) are large clostridial toxins (205 kDa and 308 kDa, respectively). Similar to the toxins of other members of the clostridial toxin family, they cause disease through a variety of cytotoxic mechanisms, most notably the loss of the cytoskeletal structure, leading to cell rounding and cell death (1, 26, 29, 33). Originally, TcdA was believed to be more important in causing *C. difficile* disease. This thinking evolved based upon several observations in a variety of animal models. In both hamsters and mice, intragastric administration of TcdA, but not TcdB, resulted in intestinal fluid accumulation, diarrhea, hemorrhage, and death (29). Because the fluid accumulation in the rabbit ligated ileal loop model was akin to that seen with cholera toxin, TcdA was referred to as an enterotoxin (1, 29). Both TcdA and TcdB, when administered intraperitoneally, were observed to have similar potencies (29). TcdB is referred to as the cytotoxin because it is 100- to 1,000-fold more potent *in vitro* in cultured cells than TcdA (1). It has now been established that both toxins can cause significant disease, as evidenced by outbreaks of severe infection caused by TcdA-negative, TcdB-positive strains (34–36).

Both TcdA and TcdB are large, single-stranded, two-component proteins containing an enzymatically active A subunit and a B subunit that delivers the A subunit into the target cell. X-ray crystallography and small-angle X-ray scattering models have shown that each toxin has four structural domains (29, 37, 38). Production of active toxin involves four steps mediated by their respective domains. The C terminus of both toxins contains the receptor binding domain. This domain has short combined repetitive oligopeptides (CROPs) that, in the case of TcdA, bind to saccharides in the human glycoprotein receptor gp96; the receptor for toxin B binding is unknown (29, 38).

The toxins enter cells by clathrin-mediated endocytosis, where they must escape from the endosome so that glucosyltransferase cleavage and activity may proceed (1, 29). This is achieved by pore formation in the endosomal membrane mediated by hydrophobic segments of the central delivery domain after the region has been structurally changed by endosomal acidification (1, 29). Once in the cytosol, the A component of each toxin is cleaved at a cysteine protease domain adjacent to the glucosyltransferase domain. This cleavage is mediated by host cell inositol hexakisphosphate (InsP6) (26, 29, 39), resulting in release of biologically active toxin, a 63-kDa glucosyltransferase that resides at the N termini of both toxins.

As mentioned above, active toxin inactivates small Rho and other GTPases, affecting their interactions with regulatory molecules and interrupting vital signaling pathways (29). Cells round up, shrink, and die, leading to significant loss of the intestinal epithelial barrier, and tight junctions are disrupted, permitting neutrophil migration. In addition, both toxins stimulate the release of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-8 from activated macrophages. This subsequently leads to neutrophil recruitment, inciting an inflammatory response (40); neutrophil ac-

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cumulation is responsible for the pseudomembrane formation seen in severe colitis.

Approximately 6 to 12.5% of strains of *C. difficile* produce another toxin, called *C. difficile* transferase (CDT), also known as binary toxin, which is encoded by the Cdt locus (CdtLoc) (26). Binary toxin is so called because it is composed of two subunits, namely, CDTa and CDTb, that are produced and secreted from the cell as two separate polypeptides (41). These polypeptides combine into a potent cytotoxin, CDT.

CDT is an ADP-ribosyltransferase that disrupts the cytoskeleton of the cell, leading to cell rounding, loss of fluids, and eventually cell death (40, 41). Like TcdA and TcdB, CDT is a classical AB toxin. The target cell receptors necessary for the binding subunit (CDTb) are unknown. Toxicity of CDT involves cell entry via endocytosis, pore formation that delivers active toxin into the cytosol, and ADP-ribosylation of G-actin (40, 41). The precise contribution of CDT to human disease is still being elucidated. In animal models, CDT-positive (CDT^+) strains that lack TcdA and TcdB do not seem to cause disease, although massive edema was seen in rabbit ileal loops (42). More recent findings indicate that CDT may play a role in intestinal colonization through induction of microtubule formation on epithelial cell surfaces, leading to enhanced adherence (43). Since CDT production appears to be epidemiologically associated with strains producing higher fatality rates (30, 40, 44), this observation and the findings from animal studies have led to the hypothesis that CDT contributes to an increased severity of disease (40).

Multilocus sequencing typing (MLST) and whole-genome sequencing (WGS) of the PaLoc reveal that *C. difficile* can be divided into five genetic groups or clades (designated clades 1 to 5) that continue to evolve at the strain level (44, 45). Within these clades, strains that appear to be associated with more severe disease and increased mortality have emerged—the so called "hypervirulent" types. Examples of these strains include ribotype 027 (toxinotype III, ST-1, BI/NAP1) and ribotype 078 (ST-11) strains. Outbreaks related to these strains increased dramatically at the beginning of the new millennium and continue to cause about 50% of cases in some geographic locations (30, 34). More than a decade of research has determined that there are several factors contributing to the successful dissemination and enhanced pathogenicity of these clones. These factors include (i) fluoroquinolone resistance (30), (ii) higher sporulation rates (46), (iii) rapid internalization and pore formation of TcdB due to divergence in the receptor binding domain (29, 47, 48), (iv) a 20 fold increase in toxin production related to mutations in *tcdC* (49, 50), (v) enhanced cytotoxicity (47), and (vi) production of binary toxin. These and other studies will hopefully further our understanding of the molecular epidemiology of *C. difficile*.

Clinical Manifestations

C. difficile infection can run the gamut from asymptomatic colonization to severe PMC, toxic megacolon, and death. The type of disease and severity of disease are related to the organism factors described above and patient risk factors, including the presence of neutralizing antibody against TcdA and TcdB (16). Patients with clinical symptoms can be stratified into mild-to-moderate illness, severe illness, and fulminant disease (51, 52). This stratification is important in determining the need for treatment, the type of antimicrobial agent that may be required for treatment, whether surgery is needed to control disease, and the need for supportive care such as intensive care unit admission (52). Watery, nonbloody diarrhea, defined as 3 or more stools per 24-h period, is the hallmark of symptomatic illness (51, 52). Mild disease is characterized by diarrhea in the absence of signs and symptoms of colitis. Patients with moderate disease have diarrhea with evidence of colitis characterized by fever and abdominal cramps, usually in the lower quadrants. Laboratory abnormalities in mild and moderate disease include a leukocytosis level of 15,000 cells/μl or lower (51, 52) and a serum creatinine level less than 1.5 times the premorbid level (52). The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines define severe disease as colitis associated with a leukocyte count that is 15,000 cells/μl or higher and a serum creatinine level equal to or greater than 1.5 times the premorbid level (52). Other characteristics of severe disease include markedly elevated temperature reaching 40°C, PMC, and hypoalbuminemia (serum albumin level of <2.5 mg/dl) (51, 52). Fulminant *C. difficile* occurs in <5% of patients and is characterized by severe abdominal pain, profuse diarrhea, or sometimes no diarrhea, as the patient rapidly progresses to development of an ileus or toxic megacolon, a condition in which the colon becomes distended greater than 6 cm and is in danger of perforation (51, 53). This is an ominous development and may indicate the need for emergent surgery. The leukocyte count may be as high as 50,000 cells/μl or higher, and a serum lactate level of >5 mmol/liter portends a poor outcome (53). Several factors have been associated with increased mortality, the most frequent of which is an age of >70 years. Comorbid conditions that contribute to the risk of dying within 30 days include cognitive impairment and liver, renal, and ischemic heart diseases (54).

LABORATORY METHODS FOR *CLOSTRIDIUM DIFFICILE* DETECTION

Methods

Clostridium difficile infection is a clinical diagnosis supported by laboratory findings. The laboratory methods used historically for *C. difficile* detection had prolonged turnaround times, which limited their practicality for diagnostic testing. As a result of this, when enzyme immunoassays (EIAs) emerged for *C. difficile* detection, they were widely adopted by many laboratories because of the speed, convenience, and economy of using these methods. However, it was ultimately demonstrated that these methods lacked analytical sensitivity, which ushered in an era of nucleic acid-based techniques for detection of *C. difficile* in clinical specimens. The next section provides an overview of the laboratory methods used for the diagnosis of *C. difficile* disease.

Cell culture cytotoxicity neutralization assay. The cell culture cytotoxicity neutralization assay (CCCNA) is performed by preparation of a stool filtrate, which is then applied onto a monolayer of an appropriate cell line. A number of different cell lines have been used for this purpose, such as human foreskin fibroblasts, human diploid fibroblasts, Vero cells, McCoy cells, MRC-5 lung fibroblasts, and Hep2 cells. Following 24 to 48 h of incubation, cells are observed for toxin-induced cytopathic effect (CPE); if CPE is observed, a neutralization assay is performed to ensure that the CPE is attributable to *C. difficile* toxins rather than nonspecific toxicity. The neutralization assay is performed by using either *C. sordellii* or *C. difficile* antiserum. Although toxin B is primarily detected in this assay, toxin A is also detected to some extent (55).

CCCNA has historically been considered the gold standard

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(56); however, this method is now considered by most experts to lack the desired sensitivity to be the gold standard for assay comparison studies. The reported sensitivities for CCCNAs are quite variable, ranging from 65 to 90% (52, 57–61). Compared to toxigenic culture, CCCNA has a sensitivity of approximately 75 to 85% (56), and the level of agreement between the two assays can vary considerably between test sites. The performance characteristics of the CCCNA vary depending on a number of factors. The toxin can degrade in the specimen, so if there is a delay in transport, false-negative results can occur. In addition, results can vary depending on the cell line used, the timing between symptom onset and performance of the assay, and pretreatment of the patient with antimicrobial therapy active against *C. difficile*; analytical factors, such as the method of preparation of the fecal filtrate, can also impact the sensitivity of this assay (55, 57, 60, 62 65). CCCNA has fallen out of favor as a routine diagnostic test as a result of inadequate sensitivity, the relatively prolonged turnaround time (24 to 48 h), and the requirement for expertise in maintenance of cell cultures and interpretation of results.

Toxigenic culture. Toxigenic culture for *C. difficile* is based upon isolating the organism from fecal specimens and determining if the recovered isolate is a toxin-producing strain. There have been many different methods reported for this purpose. The essence of all of the methods is based on using anaerobic agar or broth culture with selective and differential agents to inhibit overgrowth of other fecal flora while enhancing the recovery of *C. difficile*. To date, there is no consensus on the best method for recovery of the organism in culture. Some investigators have employed a heat shock or alcohol shock prior to inoculation to enhance spores of *C. difficile* and inhibit growth of other organisms (66–68). A variety of different medium formulations have been described, many of which take advantage of the fact that *C. difficile* can ferment fructose (69, 70). The medium type most commonly used is cycloserine, cefoxitin, and fructose agar (CCFA) (71), but a number of variants on the theme of CCFA have been reported, such as cefoxitin cycloserine egg yolk agar (CCEY), also known as Brazier's medium, and cycloserine cefoxitin egg yolk agar with lysozyme (CCEYL) (72). A variety of agents have been added to these different medium formulations to enhance vegetation of spores, such as lysozyme and taurocholate (73, 74). Broth enrichment prior to plating onto solid agar appears to enhance recovery (67, 74). Regardless of the medium or method used, it is essential that the medium is prereduced prior to inoculation of the specimen to maximize recovery of the organism. Cultures are typically incubated for a minimum of 48 h and are frequently held for up to 7 days before being reported as negative.

Few studies have simultaneously compared different media and culture conditions for recovery of *C. difficile* from samples. A recent systematic evaluation compared different medium types, preincubation steps, and direct plating onto solid medium versus the use of broth enrichment culture (67, 74, 75). Hink and colleagues observed that the most sensitive method for *C. difficile* isolation from both stool and swab specimens was heat shock at 80°C prior to inoculation of the specimen into cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (67).

Once colonies suspicious for *C. difficile* are isolated in culture, the organism is typically identified based on Gram stain, colony morphology, "horse barn" odor, and biochemical testing, such as detection of hydrolysis of L-proline-naphthylamide ("PRO Disk" positive) and spot indole. *C. difficile* isolates will exhibit yellow-green fluorescence under UV light (72). Commercial biochemical methods for identification, such as the RapidANA (Remel, Lenexa, KS) test, may also be used, and identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is used by some laboratories.

When *C. difficile* has been identified, isolates must be tested for the ability to produce toxins. Frequently, this is performed by growing the *C. difficile* isolate in broth (such as prereduced brain heart infusion or chopped meat broth) and subsequently performing CCCNA on the culture supernatant. Alternatively, the supernatant can be tested for toxin production by using commercially available toxin EIAs; this is a rapid and reliable method for toxin detection from cultured isolates when the EIA is positive (76, 77). She et al. reported the Meridian Premier Toxins A & B and the TechLab Tox A/B II assays to be 87.1% and 89.2% sensitive, respectively, for the detection of toxin-producing strains compared to CCCNA performed by using human foreskin fibroblast cells (76).

The fecal specimen for culture may be a stool specimen, rectal swab, or perirectal swab; a recent study suggested that perirectal swab specimens are almost as sensitive as "cup" stool specimens for detection of *C. difficile* in symptomatic patients for whom diagnostic testing for *C. difficile* is sought (95.7% sensitive compared to stool samples) (78). Even for patients with diarrhea, at times it can be difficult to obtain a stool specimen due to a variety of factors, such as the mental status of the patient and the availability of a collection container at the time of defecation.

In addition to conventional culture methods, chromogenic media have been developed for *C. difficile*; these media can expedite recovery of *C. difficile* from fecal specimens. For example, presumptive identification of *C. difficile* can be made based on black colonies on bioMérieux chromID agar. A recent report compared bioMérieux chromID *C. difficile* agar to taurocholate cycloserine cefoxitin agar and bioMérieux CLO medium (79). Four hundred six consecutive diarrheal stool samples from hospitalized patients were cultured; the gold standard for comparison was the recovery of *C. difficile* in the sample by using any of the culture methods. chromID agar was 74% sensitive at 24 h and 87% sensitive at 48 h (79). In a similar study, Perry et al. reported on a prototype of a chromogenic medium for recovery of *C. difficile* from fecal specimens (80); on this medium, *C. difficile* appears as black colonies on a clear background, similar to chromID agar. This prototype agar was compared to bioMérieux CLO medium, BBL *Clostridium difficile* agar, Oxoid *Clostridium difficile* agar with defibrinated horse blood and moxalactam-norfloxacin supplement, and CCEY with and without supplementation with lysozyme. In total, 226 toxin-positive and 113 toxin-negative stool specimens were evaluated under the 5 different culture conditions (toxin status of stool specimens was determined by using the Vidas immunoassay). The sensitivities of the prototype chromogenic agar were 97% and 99% at 24 and 48 h of incubation, respectively (80). The recovery of *C. difficile* was significantly better with the prototype medium than with all other medium types evaluated in this study.

The chromID *C. difficile* medium has also been evaluated for cultivation of *C. difficile* from environmental surfaces within a hospital setting and compared to cefoxitin cycloserine egg yolk agar with lysozyme for this purpose (81). By using a combination of both media, *C. difficile* was recovered from 21% of the sites sampled, but the sensitivity of chromID agar was significantly

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TABLE 1 Examples of studies evaluating the performance characteristics of EIAs for detection of *C. difficile* toxins[a]

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
Meridian Premier Toxins A & B	Microwell EIA	CCCNA	540	96.8	94.3	50.9	99.8	88
Meridian Premier Toxins A & B	Microwell EIA	CCCNA	446	98.7	97.3	ND	ND	94
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	200	48	98	88	87	106
Meridian Premier Toxins A & B	Microwell EIA	Detection of *tcdB* by ≥2 molecular methods	81	42.3	100	100	78.6	90
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	432	58.3	94.7	68.9	87.0	87
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	596	80.8	97.5	78.0	97.9	58
		CCNA		91.7	97.1	78.0	99.1	
TechLab Tox A/B Quik Chek	Lateral flow EIA	Aggregate of EIAs and toxigenic culture	284	59.6	99.2	Not given	Not given	91
TechLab Tox A/B Quik Check	Lateral flow EIA	Toxigenic culture	596	74.4	98.9	88.7	97.2	58
		CCNA		84.3	98.6	86.8	98.3	
ImmunoCard Toxin A & B	Lateral flow EIA	Toxigenic culture	150	47	99.2	88.9	93.6	61
Immunocard Toxin A & B	Lateral flow EIA	Toxigenic culture	200	48	99	91	87	106
Remel Xpect *C. difficile* A/B	Lateral flow EIA	Toxigenic culture	200	48	84	88	87	106
Remel Xpect *C. difficile* A/B	Lateral flow EIA	Toxigenic culture	596	68.8	99.4	92.4	96.6	58
		CCNA		77.8	98.8	87.5	97.6	
Remel ProSpecT	Microwell EIA	Toxigenic culture	596	81.6	93.3	57.4	97.9	58
		CCNA		89.8	92.6	57.5	98.8	
Wampole Tox A/B Quik Chek	Lateral flow EIA	Toxigenic culture	360	43.18	99.68			89
Vidas *C. difficile* Toxin A/B	Solid-phase, enzyme-linked fluorescent assay	Toxigenic culture	150	58.8	89.4	62.5	96.7	61
Vidas *C. difficile* Toxin A/B	Solid-phase, enzyme-linked fluorescent assay	Toxigenic culture	596	80.0	97.3	76.4	97.8	58
		CCCNA		89.8	96.7	75.3	98.8	

[a] CCCNA, cell culture cytotoxicity neutralization assay; ND, not determined.

higher than that of CCEY with lysozyme (87.6% compared to 26.6%) (81).

Chromogenic media are usually more expensive than other medium types; this is typically attributed to the proprietary and chromogenic substrates in the media. However, the studies to date suggest that chromogenic media are sensitive for recovery of *C. difficile* and may speed the time to presumptive identification of isolates.

Toxigenic culture is typically considered to be a reference method rather than a diagnostic method. It is imperative that at least a subset of laboratories maintain expertise in *C. difficile* culture for method comparison and epidemiological studies, but the labor requirement and turnaround time are not usually considered practical for routine diagnostic use. In addition, toxigenic culture detects the ability of a *C. difficile* strain to produce toxin *in vitro* and does not necessarily inform *in vivo* production of toxin in the host. In summary, toxigenic culture is now considered by many to be the gold standard for *C. difficile* detection in fecal specimens (82, 83). The SHEA/IDSA guidelines support the use of toxigenic culture as the gold standard in method comparison studies (52). However, others argue that although toxigenic culture may result in more positive specimens, it might not be a superior test for the diagnosis of clinically actionable disease compared to CCCNA (56).

Toxin immunoassays. Enzyme immunoassays for *C. difficile* diagnostics use monoclonal or polyclonal antibodies directed against *C. difficile* toxins. Until very recently, EIAs for toxin detection have been the most frequently used assays in clinical laboratories for *C. difficile* detection. There are a number of commercially available EIAs for *C. difficile* toxins, including rapid immunochromatographic/lateral flow membrane immunoassays and microwell and solid-phase assays. It should be noted that although some of the early assays detected toxin A exclusively, it is now generally recommended that assays detect both toxin A and toxin B, as toxin A-negative disease-causing strains have been well documented (35, 84–86) (also described above). Overall, the cost per test for these assays is low. The hands-on time required to perform the test varies depending upon the test type. The sensitivity and specificity that have been reported for these assays vary widely, from approximately 40% to 100% (58, 61, 87–94). A summary of several of the studies evaluating different EIAs for detection of *C. difficile* toxins can be found in Table 1. The gold standard used to evaluate these assays is variable and is either CCCNA or toxigenic culture, as indicated in Table 1. A study by Eastwood et al. evaluated a number of EIAs by using both gold standards and highlighted the difference in sensitivity and specificity reported for comparator assays depending upon the gold standard used (58).

Although the poor sensitivity of toxin EIAs for *C. difficile* is well established, the clinical significance of this has not been well documented. In an attempt to address this question, in a retrospective study, Polage and colleagues reviewed the charts of patients at the University of California—Davis Medical Center who had a *C. difficile* toxin test ordered between 1 January 2005 and 21 December 2009 (95). This cohort included 925 toxin-positive patients and 6,121 toxin-negative patients (95). Upon chart review, only 1 out of the 6,121 toxin-negative patients exhibited PMC, and there were no documented cases of complications that could be attributed to *C. difficile* disease in any of these patients, such as toxic megacolon, fulminant colitis, or colectomy. A small proportion (5.3%) of the toxin-negative patients received empirical treatment

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TABLE 2 Examples of results of investigations evaluating the performance characteristics of assays for GDH detection[a]

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
C. Diff Quik Chek	Lateral flow EIA	Detection of *tcdB* by ≥2 molecular methods	81	96.2	76.4	Not given	Not given	90
C. Diff Quik Chek	Lateral flow EIA	Toxigenic culture	401	93.5	96.9	87.9	98.4	110
C. Diff Chek-60	Microwell EIA	Aggregate of EIAs and toxigenic culture	284	100	94.2	Not given	Not given	91
C. Diff Chek-60	Microwell EIA	Toxigenic culture	564	87.6	94.3	78	97.1	58
		CCCNA		90.1	92.9	71.3	98.0	
C. Diff Chek-60	Microwell EIA	Toxigenic culture	497	93.5	98	91.6	98.5	116
C. Diff Chek-60	Microwell EIA	Toxigenic culture and PCR	1,468	93.4	96.6	75.9	99.2	113
C. Diff Complete (GDH component)	Lateral flow EIA	Toxigenic culture	60	100	93.3	93.8	100	115

[a] By definition, GDH assays are screening assays and are not for definitive reporting of *C. difficile*. In this table, specificity is reported for GDH assays performed without confirmatory testing.

for *C. difficile* infection, regardless of the toxin result. This study suggested that the clinical impact of the reduced sensitivity of toxin EIAs for *C. difficile* was minimal, but more studies are needed to fully evaluate this issue.

Although the specificity of the toxin EIAs is generally superior to the sensitivity of this method, specificities vary, and some assays possess inadequate positive predictive values (PPVs) for a diagnostic test (58, 61, 96, 97). An abundance of false-positive results can be just as problematic as false-negative results. This phenomenon was highlighted in a report by Han et al., who observed that a change in the toxin EIA brand used for routine testing (from ProSpecT Toxin A/B [Remel] to *C. difficile* Tox A/B II [TechLab]) resulted in an apparent decrease in *C. difficile* infection from 23.52 to 8.69 cases per 10,000 patient days (97). Using the ProSpecT test, 19.7% of patients were positive with the first test, compared to 9.1% of patients positive with the first test using the TechLab Tox A/B II assay (97). A reduction in anti-CDI therapy followed as a result of fewer positive tests, but no increase in mortality occurred, suggesting that the decreased incidence was a result of improved assay specificity of the toxin A/B assay (97).

There are a number of reasons for the heterogeneity in performance characteristics of these assays, including, but not limited to, geography, circulating strain types (and, therefore, potentially differences in the antigenic features of the toxins that are expressed), the gold standard used for assessment of the assay, host antibody binding to toxin in the gastrointestinal tract, interlaboratory technical variance, institutional variance in ordering practices for *C. difficile* testing and/or specimen transport time, and whether fresh or frozen/stored stool samples are analyzed. To muddy the waters even further, in some studies, investigators have modified manufacturers' recommendations for the assays, such as the optical density (OD) to be considered "positive" for the microwell format EIAs (98). In addition, clinical signs and symptoms have only rarely been taken into consideration when evaluating the performance characteristics of these toxin EIAs (61, 95, 96, 99). Since the lack of sensitivity of *C. difficile* EIAs is a well-known problem within the medical community, common practice has been to order "*C. difficile* testing times three." This approach only compounds the problem; with the poor specificity of these assays, the number of false positives exceeds the number of true positives with each round of testing, especially in a low-prevalence setting.

Due to the inadequate sensitivity of toxin EIAs for *C. difficile* testing, this method is not considered the best way to make a diagnosis of *C. difficile* infection (52, 58, 100, 101). The SHEA/IDSA guidance document outlines that EIAs should no longer be considered adequate stand-alone tests for the diagnosis of *C. difficile* infection (52).

Glutamate dehydrogenase. Glutamate dehydrogenase (GDH) is a metabolic enzyme encoded by *gluD*. This antigen is produced at high levels in all isolates of *C. difficile*, including both toxigenic and nontoxigenic strains. In addition, GDH of *C. difficile* is known to cross-react with that of *C. sordellii*. Therefore, GDH represents a screening test for *C. difficile* disease, and positive assays must be followed up with a confirmatory test, such as a toxin EIA or a molecular test for detection of toxin genes. Overall, the studies performed to date suggest that GDH exhibits high sensitivity as a screening test for *C. difficile* infection and exhibits a favorable negative predictive value (NPV). Some of these studies are summarized in Table 2 (58, 65, 91, 102–116).

Similar to toxin EIAs, GDH assays are available in microwell EIA and lateral flow immunochromatographic formats. The sensitivity (80 to 100%) and NPV for GDH assays appear to be similar regardless of the specific GDH assay type used or the gold standard used to evaluate the assay (108, 113, 116, 117). GDH testing can efficiently provide a rapid turnaround time for negative results, with minimal hands-on time. The cost per test is low, especially compared to the cost of molecular methods.

GDH can be an attractive screening assay to quickly rule out *C. difficile* disease for laboratories that do not have access to or expertise in performance of molecular testing. However, some controversy exists regarding the adequacy of GDH assays to rule out *C. difficile* disease. In order to be a reliable and effective screening method, GDH should be highly conserved among *C. difficile* strains. A report by Tenover et al. raised concern that the sensitivity of GDH assays may vary by strain type; namely, reduced sensitivity for detection of strains of ribotypes 002, 007, and 106 was observed in this study (118). Carman et al. evaluated 104 isolates of *C. difficile*, corresponding to 77 different ribotypes (including both toxigenic and nontoxigenic strains, with ribotypes in common with those in the study by Tenover et al.), from different areas around the world (119). Most of the isolates had been recovered from 2004 to 2011. All of the strains of the ribotypes evaluated carried *gluD*, and predicted amino acid sequences of almost

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TABLE 3 Performance characteristics of EIAs combining GDH detection and toxin EIA for *C. difficile* detection

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
TechLab C. Diff Quik Check Complete	Lateral flow EIA	Detection of *tcdB* by ≥2 molecular methods	81	61.5	100	100	84.6	90
TechLab C. Diff Quik Chek Complete	Lateral flow EIA	Toxigenic culture	200 (all from pediatric patients)	70.8	97.4	89.5	91.4	122
TechLab C. Diff Quik Chek Complete	Lateral flow EIA	Aggregate of EIA and toxigenic culture	284	60.0	99.6	Not given	Not given	91
C. Diff Quik Chek plus Meridian Premier Toxins A & B	Lateral flow EIA/ microwell EIA	Detection of *tcdB* by >2 molecular methods	81	42.3	100	100	78.6	90
C. Diff Chek-60 with Meridian Premier Toxins A & B	Both are microwell EIAs	Toxigenic culture	432	55.6	98.3	87	91.7	87

all of the isolates were identical. All of the isolates produced GDH that was readily detected by using a number of commercial assays, including C. Diff Chek-60, C. Diff Quik Chek, and C. Diff Quik Chek Complete (119). These results suggest that the performance characteristics of commercial GDH assays are not dependent upon the *C. difficile* ribotype.

Goldenberg et al. evaluated the performance of the C. Diff Chek-60 GDH assay by using 64 isolates of *C. difficile* that could be grouped into six ribotypes: ribotypes 002, 005, 023, 027, 078, and 106 (120). Although this study used cultured isolates rather than direct testing of stool samples, there was no significant difference in the performance of the GDH assay for any of the ribotypes studied. Even when the cultures were diluted 1:100, the OD value for the GDH EIA did not vary significantly between any of the ribotypes tested (120).

Based on the studies Carman et al. and Goldenberg et al., it seems unlikely that regional or geographic differences in strain type account for the variance reported for GDH assays, although this cannot be completely excluded (118–121). For method comparison studies, it is not uncommon for the testing to be performed on frozen stool specimens; it is unknown if the freeze-thaw could impact assay results. This could be a confounding factor to consider when evaluating the variance between GDH assay performances in different method comparison studies.

Combination testing—GDH detection and toxin EIA. Some EIAs that combine GDH detection and a toxin EIA in one test have recently come on the market. These combination assays are relatively rapid, and the cost per test is less than that of the molecular methods. The GDH component of these assays appears to have a sensitivity similar to those of stand-alone GDH assays, but the toxin EIA component appears to suffer from the same sensitivity issues as conventional toxin EIAs (87, 90, 91, 122). Some of the studies evaluating these combination assays are described in Table 3. In general, samples that are GDH and toxin negative by these assays can be reported as negative with relatively high confidence, and GDH- and toxin-positive samples can be reported as positive. Samples that are GDH positive but toxin negative should have confirmatory testing performed (such as CCCNA or a molecular assay) to rule out *C. difficile* disease (100, 123, 124).

Molecular platforms for direct detection of *Clostridium difficile* in clinical specimens. The use of nucleic acid amplification methods for the detection of *C. difficile* in fecal samples began to appear in the literature in the early 1990s (125–127). These early assays used conventional PCR methods and targeted a variety of genes, including the *tcdA*, *tcdB*, and 16S rRNA genes. Extractions were cumbersome and time-consuming, and PCR products were detected by gel electrophoresis and/or Southern blot analysis (126, 127). An additional observed pitfall with some of the early primer designs was cross-reactivity with other *Clostridium* species (125). However, sensitivity was found to be significantly better than those of existing anaerobic culture methods (127) and CCCNAs (128).

A decade later, methods of DNA extraction from fecal samples have improved because of the availability of kits that use a mixture of RNases, proteases, and other proprietary reagents in combination with spin columns for nucleic acid concentration and purification to replace cumbersome phenol-chloroform extraction procedures. Nucleic acid amplification techniques (NAATs) have improved, and real-time PCR instruments such as the Cepheid SmartCycler (Sunnyvale, CA), the Roche LightCycler (Pleasanton, CA), and the iCycler IQ (Bio-Rad Inc., Hercules, CA) became available, making laboratory-developed assays easier to verify and implement (59, 88, 129). Several laboratories have reported successful verification and implementation of laboratory-developed quantitative real-time PCR (qPCR) assays for clinical use before the availability of a U.S. Food and Drug Administration (FDA)-cleared platform (59, 106).

Despite these advances, it was 2009 before the first nucleic acid amplification assay for *C. difficile* detection in stool samples, the BD GeneOhm Cdiff assay (BD Diagnostics Inc., Sparks, MD), was approved by the FDA. The BD GeneOhm qPCR assay amplifies conserved regions of *tcdB* and detects the amplified products by using fluorogenic target-specific hybridization probes (molecular beacons) (60). Amplification, detection, and interpretation of the qualitative results are performed by using the Cepheid SmartCycler (Table 4). A built-in internal control alerts the user to problems with inhibition. The current FDA cleared platform has a manual DNA extraction step and several pipetting steps before the vials are loaded onto the instrument. The total assay time ranges from 75 to 90 min depending upon the number of samples. An automated version, BD Max Cdiff, has recently received FDA clearance in the United States (89) and significantly reduces the setup time. The assay involves the use of a 10-μl inoculating loop to transfer liquid or unformed stool to the proprietary sample buffer. The sample buffer tube is placed into the BD Max instrument along with a reagent strip that contains all of the reagents necessary for sample processing and qPCR. The instrument moves a sample from one tube to the next on the reagent strip. Like the manual BD GeneOhm assay, the BD Max Cdiff assay amplifies *tcdB*. However, achromopeptidase is used for bacterial lysis in the newer version of the test (89). Amplified targets are detected with hybridization probes labeled with quenched fluorophores. A disposable microfluidic cartridge is where the amplifi-

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TABLE 4 Available FDA-cleared molecular assays for detection of *Clostridium difficile* in fecal samples[a]

Assay	Target(s)	Chemistry(ies)	Extraction type	Unresolved/invalid rate (%)	TAT (min)
BD GeneOhm	*tcdB*	qPCR/molecular beacons	Manual	0.5 7.3	75 90
BD Max		Achromopeptidase lysis; qPCR/ molecular beacons	Automated		120
ProDesse ProGastro Cd	*tcdB*	qPCR	Automated (Easy Mag)	2.7	180
Cepheid GeneXpert	*tcdB*, *cdtA*, *tcdC* nt 117 deletion	Multiplex qPCR	Automated (Infinity)	NA	29–45
Meridian Illumigene	*tcdA*	Loop-mediated isothermal amplification	Manual Illumipro-10 reader	0.8–4.4	70
Focus Technologies Simplexa	*tcdB*	qPCR, bifunctional fluorescent probe-primers	None	NA	60
Great Basin Portrait Analyzer	*tcdB*	Helicase-dependent amplification; microarray detection		NA	70
Quidel AmpliVue *C. difficile* Assay	*tcdA*	Helicase-dependent amplification; visual evaluation of results using handheld cassette	Manual	0.5	80
Nanosphere Verigene	*tcdA*, *tcdB*, *cdt* nt 117 deletion	PCR combined with gold particle probe capture and silver signal amplification on an array	Automated	2.4	150

[a] *tcdA*, toxin A gene; *tcdB*, toxin B gene; *cdt*, binary toxin gene; nt, nucleotide; NA, not available; TAT, turnaround time.

cation, detection, and interpretation of the signals take place. This cartridge is sealed to prevent contamination. The assay contains an internal sample processing control, which is read on an optical channel of the instrument separate from the patient samples. The overall time to completion is about 3 h, depending upon the total number of samples.

The performance characteristics of the BD GeneOhm Cdiff assay and the BD Max Cdiff assay along with those of the other FDA-cleared assays are summarized in Table 5 (57, 60, 89, 96, 107, 122, 130–134). Only those published studies which used a recognized reference method, such as toxigenic culture or a cell culture cytotoxicity neutralization assay, on all of the samples tested are included in Table 5. In cases where no published papers are available, data have been summarized from the manufacturer's product package insert. It is important to note that very few papers include clinical information, specifically with respect to whether patients met recognized case definitions as specified by clinical practice guidelines (52).

The ProDesse ProGastro Cd assay (Hologic Gen-Probe Inc., San Diego, CA) detects *tcdB* in fecal samples. A portion of the stool sample is diluted in stool transport and recovery (STAR) buffer, followed by centrifugation to remove solid particles (stool clarification). The internal control is added to the clarified stool sample prior to extraction. Nucleic acid extraction and purification occur on the NucliSENS easyMag instrument (bioMérieux, Durham, NC). Amplification and detection using TaqMan chemistry, as well as interpretation of results, which are reported qualitatively, are performed with the SmartCyclerII instrument (Cepheid Inc., Sunnyvale, CA). Of all of the commercially available NAATs, this assay takes the longest to perform (Table 4). Performance characteristics are summarized in Table 5 (130).

Detection of not only *tcdB* but also the binary toxin genes and the deletion at nucleotide 117 on *tcdC* (Δ117) as surrogate markers for presumptive identification of 027/NAP1/BI strains are unique features of the Xpert *C. difficile* Epi assay (Cepheid Inc., Sunnyvale, CA). Users may also buy a version that detects only *tcdB*. This assay is among the simplest to perform and is also the most rapid of the available NAATs. A scored swab containing fecal material is inserted and broken off into an elution vial. After a vortex step, the liquid from the vial is dispensed into the sample port of the test cartridge that contains all of the reagents for amplification and detection as well as the internal control. The cartridge is subsequently loaded into the GeneXpert instrument. The company has a larger and more automated instrument, called the

TABLE 5 Published performance characteristics of currently available NAATs[c]

Assay	Sensitivity (%) (range)	Specificity (%) (range)	NPV (%) (range)	PPV (%) (range)
BD GeneOhm	82.1–100	90.6–99.2	96.7.0–100	58.6–94.4
BD Max Cdiff	97.7	99.7	99.7	97.7
Prodesse ProGastro Cd	77.3–100	93.4–99.2	95.9–100	82.8–94.4
Cepheid GeneXpert	94.4–100	93.0–99.2	99.3–100	78.9–94.7
Meridian Illumigene	86.7–98.1	98–100	98.1–99.5	91.8–98.5
Focus Technologies Simplexa[b]	90.1/79.6[a]	93/95.8[a]	NA	NA
Great Basin Portrait[b]	79.6–90.1	93.0–95.8	95.3–98.4	66–81.4
Quidel AmpliVue *C. difficile* Assay	93.6	94.1	NA	NA
Nanosphere Verigene	98.7/91.8[a]	87.6/92.5[a]	99.9/98.5[a]	42.1/67.3[a]

[a] The first result represents comparison to direct toxigenic culture, and the second represents comparison to enriched toxigenic culture.
[b] No publications to date on the FDA platform; data are from the package insert.
[c] Data were compiled from references 57, 60, 87, 89, 96, 107, 122, 130–136, and 139–143.

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GeneXpert Infinity, that accommodates 48 or even 80 cartridges in a completely random-access format. Table 5 lists the published performance characteristics for *C. difficile* detection in patient samples (87, 133–136). There have been three published reports on the performance of the Xpert CD/Epi assay. Pancholi et al. found 100% accuracy for detection of all 36 027 strains (137). In a comparison of the Xpert assay to ribotyping, Babady et al. (138) found that 42 of 45 ribotype 027 strains were correctly identified. Both of these studies were conducted in areas of the United States where ribotype 027/NAP1/BI strains are prevalent. In contrast, a study from Australia, where disease caused by ribotype 027 strains is rare, reported false-positive results were related to non-027 isolates with unusual mutations that were amplified by the primers in the Xpert CD/Epi assay (139).

The Illumigene assay (Meridian Bioscience, Cincinnati, OH) is the only FDA-cleared NAAT that is based upon the principle of loop-mediated isothermal amplification (LAMP). This assay detects a conserved 5′ sequence of *tcdA* on the PaLoc by using four primers designed to detect six distinct regions (140). The assay, which has seven manual steps before it is loaded onto the reader, is said to detect toxin A-negative, toxin B-positive strains, as it detects a remnant of the gene in the PaLoc. Features of this assay and its performance characteristics derived from several studies are summarized in Tables 4 and 5 (139–141). One important point to make is that while the test is simple to perform, it does require specific training. There are two critical steps that could lead to a higher-than-expected "invalid" rate if not performed correctly. These include filling of the sample collection brush with the right amount of stool sample and adhering to the specified time between preparing the sample and placing it into the Illumipro-10 instrument (142). Another concern is the potential for spore contamination related to all of the manual processing steps.

Focus Diagnostics Inc. (Cypress, CA) has developed the Simplexa *C difficile* Universal Direct assay. This is a real-time PCR system that targets conserved regions of *tcdB*. There are three steps in this assay. First, the liquid or soft stool samples processed in Tris-EDTA (TE) buffer are heat denatured at 97°C for 10 min. After a reagent preparation step, amplification of *C. difficile* DNA and internal control DNA is performed by using fluorescent bifunctional probe-primers together with reverse primers. The specimens and reagent mixtures are loaded onto a Universal Disc for use on the 3 M Integrated Cycler. The instrument software interprets the results and will suppress them if any of the control samples are invalid. The user examines the amplification curves for each patient result along with the DNA internal control to ensure that results are interpreted properly (143). At the time of preparation of the manuscript, there have been no reported studies using this assay.

The Portrait Toxigenic *C. difficile* assay (Great Basin Inc., Salt Lake City, UT) combines helicase-dependent amplification (HDA) with target detection on silicon chip macroarrays (144) to detect the *C. difficile* toxin B gene. The assay uses a proprietary Hot Start approach—RNase-mediated amplification—to prevent primer artifacts during amplification, which is a problem associated with other isothermal amplification systems (144). The array technology is also unique in that the target DNA is biotin labeled. When the target DNA is captured by probes on the array, hybridization is detected in an enzyme-linked immunosorbent assay (ELISA)-type format by the reaction of each bound target with antibiotin/horseradish peroxidase (HRP) conjugates. Reaction of subsequently bound conjugates with a precipitable tetramethylbenzidine (TMB) substrate results in signal amplification, which enhances the sensitivity of the assay. After placing a swab into the patient's specimen, the user inserts it into a tube containing extraction buffer, vortexes it, and then filters it by using a 3-ml syringe. Subsequently, 180 μl is delivered into the sample port of the proprietary cartridge of the assay. After the sample port is closed, the disposable cartridge is inserted into the analyzer, and testing begins. The cartridge contains all reagents necessary to perform the test; amplification and detection both occur within a closed system so as to prevent contamination. The internal control detects the *S. aureus nuc* gene. Results are reported qualitatively within 90 min. In a paper by Hicke et al., the analytical sensitivity was reported to be 10 CFU per fecal sample (144). In a small preclinical study ($n = 130$ specimens) performed by those authors, the Portrait test had a sensitivity of 97% and a specificity of 100% compared to the BD GeneOhm Cdiff assay (144). The results of the clinical trial for FDA approval are provided in Table 5.

Like the Illumigene assay, the AmpliVue *C. difficile* assay (Quidel Molecular, San Diego, CA) targets a highly conserved region of *tcdA*. The assay utilizes helicase-dependent isothermal amplification in a self-contained handheld cassette for amplicon detection. In brief, a swab is used to inoculate a small amount of liquid sample into a dilution tube. Diluted sample (50 μl) is added to a lysis buffer tube, which is heated in a heating block at 95°C. After the 10-min lysis step, 50 μl of sample is added to a reaction tube and heated for 60 min at 64°C. The biotinylated single-stranded DNA amplicons bind to capture probes during the reaction. After incubation, the reaction tube is placed into the amplicon cartridge, and the cartridge is placed into the hinged detection chamber of the handheld cassette. A lateral flow strip within the cassette uses streptavidin-conjugated color particles to visualize the dually labeled probe-amplicon hybrids. These appear as pink-to-red lines on the cassette. There is also a line that corresponds to the process control DNA that must be visible for any valid result (146).

The Verigene *C. difficile* test (Nanosphere Inc., Northbrook, IL) is the most recent assay to obtain FDA clearance. This assay detects both *tcdA* and *tcdB* as well as *cdt* and the deletion at nucleotide 117 on *tcdC*. The Verigene assay requires a sample processing step of a swab placed into the fecal sample, followed by inoculation of the specimen in buffer into the test cartridge. The test cartridge contains a reagent pack, which is a single carrier that sits atop the array, a glass slide that is solid support for the capture oligonucleotides. The cartridge contains all of the reagents needed for amplification and hybridization for each test, and it also captures all waste generated during testing. The testing within the cartridge is performed with an instrument called the Verigene Processor SP.

The Verigene assay has several unique features. The extracted DNA is sheared by a sonication process into 300- to 500-bp fragments to facilitate the hybridization steps. In step 1, the sheared DNA is hybridized to single-stranded DNA capture oligonucleotides on the array and simultaneously to sequence-specific mediator oligonucleotides that detect single-copy DNA regions of each target. In the second hybridization reaction, gold nanoparticles bind to captured target nucleic acid/mediator oligonucleotide complexes. After a wash step, silver signal amplification of the gold nanoparticle probes occurs. A final washing step removes unre-

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acted reagents. When testing is completed, the glass slide is removed and placed into the reader for interpretation. Results are reported qualitatively (147). To date, there have been no publications on this assay, but the results from a five-center clinical trial are summarized in Table 5. Like the GeneXpert *C. difficile* assay, this test may presumptively recognize ribotype 027 strains because it detects the nucleotide 117 deletion on *tcdC* and the binary toxin genes. Although there are no publications available, the data in the package insert show that compared to PCR ribotyping of the isolates recovered from the enriched culture, there was 91.4% positive agreement (147). The assay failed to amplify four specimens that were positive for ribotype 027 by enriched culture (147). One specimen was positive for *tcdA* and *tcdB* but was negative for *cdt* and Δ117, from which a ribotype 027 strain was recovered upon culture (147). Five specimens tested positive for *cdt* and Δ117 by the Verigene assay, but the ribotype recovered was not ribotype 027 (147).

(i) Impact of nucleic acid amplification tests on clinical care. While molecular assays have certainly improved the detection of *C. difficile* in fecal samples, there are numerous questions that have been raised regarding their clinical utility. Some concerns relate to the biology of *C. difficile* and how detection of the genes that encode toxins A and B correlate with expression of toxin. For example, is the gene always expressed, or, given the heterogeneity of *C. difficile*, will strains emerge that are not detected by a particular assay? In terms of the former question, in the majority of the clinical trials, compared to toxigenic culture, there are a few situations where the assays failed to detect a toxigenic organism recovered from a stool sample. It is unclear, however, whether these are related to concentrations of organisms below the limit of detection of an assay, specimen handling problems, or user errors. Few studies have included sequencing or other methods to assess failure of primer-probe binding or problems with completion of amplification cycles. All of the assays discussed above have built-in controls to assess inhibition, and this is one parameter that can be monitored in clinical laboratories as a component of ongoing assay validation.

A few studies have focused on variability in test performance based upon the ribotype of the infecting strain. One study found that the GeneXpert and nonamplified methods, such as an EIA for GDH antigen detection, had equivalent performances for detection of ribotype 027 strains (118). Conversely, significant differences in the performances of the EIAs for toxin A/B and GDH were seen compared to PCR for non-027 isolates (118). However, other studies have not seen this discrepancy based upon strain type (120). It is important to note that almost all of these studies suffered from small numbers of some ribotypes. The reasons for variability in assay performance by strain type for the nonamplified tests are hypothetically related to differences in toxin production among those strains, a cited advantage to molecular testing (121). More data on the impact of strain type on assay performance are needed. The reader is referred to the sections on GDH and epidemiology for more discussion of this topic.

As the circulating strain types causing disease change, it may be important to periodically assess both analytical and clinical performance of molecular tests as a component of ongoing validation. It is unclear if this is the responsibility of the vendor, the user, or public health institutions. Those states, countries, or regions that have an existing surveillance program in place could alert clinical laboratories when a new, predominant strain enters a community. This information could be used to assess assay performance. Since all assays have focused on conserved regions of the toxin A and B genes in the PaLoc found in strains of all ribotypes, it seems less likely that failure to detect a strain in clinical samples is related to mutations within the target sequences, although this remains a theoretical possibility (121).

More important questions concern the clinical utility of NAATs, particularly the specificity and positive predictive values, given that a gene and not an actual toxin is detected. Several studies demonstrating that inappropriate test ordering can certainly impact the specificity of these tests have emerged. In a study by Dubberke and colleagues (96), 36% of patients for whom a *C. difficile* test was ordered did not have clinically significant diarrhea; moreover, 20% of patients had recently taken a laxative. When clinical parameters were used to assess the performance of various test methods, the NAATs had the highest sensitivity but the lowest specificity compared to other modalities (96). In a study to assess the impact of education on *C. difficile* test ordering practices, Redding et al. (273) likewise observed that the presence of diarrhea was recorded in the chart for only 50% of patients for whom testing was ordered, and 44% of patients had received a laxative in the prior 48-h period. Educational intervention succeeded in improving documentation to 71% and reducing testing of patients on laxatives to 21% (Redding et al., submitted).

Although there are few studies that include clinical data when assessing performance characteristics of *C. difficile* testing, the existing data highlight that although NAAT assays for *C. difficile* in fecal specimens are analytically sensitive and specific, clinical specificity may not be optimal; that is, detection of *C. difficile* in a specimen does not always equal disease, as NAAT assays can be positive in both colonization and disease states. It seems plausible that the future of *C. difficile* diagnostics will include detection of not only *C. difficile* but also a biomarker that correlates with active *C. difficile* infection. While several biomarkers have been evaluated (such as fecal lactoferrin and various cytokines [see "Adjunctive tests and biomarkers for diagnosis of *C. difficile* disease" below), to date, an optimal adjunct assay for NAAT testing has not been established. This will likely be an area of active investigation in the coming years.

Just as an example of the great interest in establishing improved diagnostics for *C. difficile* infection, one recent study reported that a dog (2-year-old male beagle) under the care of a professional trainer was able to "sniff out" *C. difficile* in stool samples (148). When the dog was presented with positive and negative stool samples, the dog's sensitivity and specificity for detection of *C. difficile* were 91 to 100% and 83 to 98%, respectively (148). However, it seems unlikely that a sniffing dog will be broadly adopted as the adjunctive assay of the future; not only do logistical concerns exist with keeping a dog in the microbiology laboratory, this would also be completely uncharted territory for regulatory bodies such as the College of American Pathologists (CAP) and the FDA.

Since molecular assays cost up to 2- to 3-fold more than EIAs, the issue of cost-effectiveness has been raised by laboratorians and administrators. To answer this question, the impact on treatment and infection control activities should be considered. A few studies are beginning to emerge to address the impact on pharmacy costs and length of stay in isolation, with favorable results. Peterson and Robicsek (149) were the first to hypothesize that implementation of a NAAT would lead to fewer tests ordered due to enhanced accuracy, which might offset the increased costs of these platforms. Tenover et al. (121) likewise created a hypothetical

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model based upon testing of 1,000 patients, assuming a 10% prevalence of CDI and using published performance characteristics of various test methods. NAAT testing alone, compared to a toxin EIA alone and to various two-step algorithms using GDH detection, resulted in the largest number of patients with disease who would be placed in isolation within 24 h, the largest number of patients who would be removed from isolation more quickly because the rapid NAAT would detect them more rapidly, and the smallest number of patients with false-negative tests who would not be placed in isolation and consequently who would continue to spread the organism within the hospital environment (121).

In actuality, several studies clearly note that *C. difficile* rates doubled when laboratories made the switch from an EIA to molecular testing or incorporating a molecular test into a testing algorithm. This could have a negative impact on public health reporting if the method of testing is not considered (150–153). One study hypothesized that this may be a disincentive to eliminating less sensitive EIAs (151). After a period of increase, rates declined, suggesting better case definition leading to decreased transmission (151). The latter scenario played out at the Johns Hopkins Hospital (JHH). In 2010, the laboratory switched completely to a NAAT that was implemented twice daily 5 days per week and offered once per day on weekends. In the period immediately after implementation, *C. difficile* transmission rates appeared to increase from 11.5 cases/10,000 patient days to close to 15 cases/10,000 patient days for calendar year 2010 (Lisa Maragakis, JHH, personal communication). Rapid test availability allowed for improved isolation. Additionally, the apparent increase in transmission was used to campaign for better hand hygiene and compliance with infection control practices, such as enhanced environmental cleaning. By the second quarter of 2012, the transmission rates were among the lowest seen since 2007: 9 cases/100,000 population (Lisa Maragakis, JHH, personal communication).

In a paper by Catanzaro and Cirone, the authors performed a retrospective study comparing the impact of the switch from a toxin A/B EIA to a PCR method (154). Prior to implementation of the PCR test, the practice was preemptive isolation of symptomatic patients with the caveat that three negative EIA results were required to remove patients from isolation. The latter rule was based upon poor performance of the EIA in their environment, which led to clusters of transmission in some units (154). When the laboratory switched to the PCR test, preemptive isolation was discontinued upon report of one negative test result (154). Postimplementation, the authors observed a significant decrease in health care-associated CDI (4.4 cases/10,000 patient days versus 0.9 cases/10,000 patient days; $P = 0.02$), a reduction in patient isolation days (1,022 versus 364; $P = <0.00001$), fewer tests ordered ($P = 0.02$), and a reduction in the duration of empirical metronidazole therapy for patients with negative *C. difficile* tests ($P = 0.02$) (154). In a study by Sydnor et al. (155), replacement of a two-step algorithm using GDH detection and toxin EIA with an algorithm using GDH detection with PCR resulted in a 54% reduction in empirical antimicrobial use. Unfortunately, those authors did not assess cost savings in either study.

Others are using the assays that detect ribotype 027/NAP1 strains to control the use of more expensive antimicrobial agents such as fidaxomicin. Based upon the original modified intention-to-treat and per-protocol study analyses, fidaxomicin was noninferior to vancomycin with respect to clinical cure rates (156). Significantly fewer patients in the fidaxomicin treatment group had recurrences, unless they were infected with NAP1/ribotype 027 strains, in which case there was no difference between the treatment arms (156). Currently, there are no recommendations to alter infection control practices or to change therapy on the basis of detection of a specific ribotype alone (see the discussion on strain types and disease severity elsewhere in this review).

Few papers have addressed the comprehensive laboratory costs associated with various test methods, and none have measured the impact on overall hospital costs. In one study comparing two-step algorithms predicated upon a GDH result incorporating a cytotoxin test or the Xpert *C. difficile* assay for GDH-positive specimens, performing the Xpert test alone was less expensive when labor costs (accessioning, test performance, and reporting of results) were considered (138). In a study by Larson et al. (104), the authors projected that in making the diagnosis of *C. difficile* 1 day earlier in cases missed by EIA, PCR testing alone could save $200,000 annually by avoiding the costs of repeat testing. Additional research is needed to assess the overall cost-effectiveness of NAATs for the diagnosis of *C. difficile* disease.

Evolution of Testing Methods

As suggested above in the sections describing diagnostic assays for *C. difficile*, the methods used by clinical laboratories have evolved over the last 2 decades, from CCCNA to toxin EIAs to NAATs. Table 6 summarizes the most common assays used for *C. difficile* detection by clinical laboratories subscribing to the CAP proficiency testing program from 2001 to 2012. These data were included with permission from the CAP Microbiology Resource Committee. One of the major differences noticed over time is that a number of laboratories were using an EIA that detected only toxin A in the early 2000s, but gradually, that changed, such that no laboratories were using this type of assay. It is interesting to note the shift away from CCCNAs and EIAs toward the use of molecular testing methods; in 2012, nearly one-third of laboratories reported using a molecular method. Although the methodologies have changed, the overall performance of laboratories in these proficiency testing challenges, as well as the number of laboratories participating in these challenges, has remained relatively stable over this time period (Table 7).

Adjunctive tests and biomarkers for diagnosis of *C. difficile* disease. A variety of fecal biomarkers of inflammation have been developed over the last several decades in an attempt to distinguish inflammatory versus noninflammatory causes of diarrhea and other gastrointestinal complaints. These assays have been embraced by gastroenterologists as noninvasive mechanisms for screening of patients, particularly for inflammatory bowel disease (IBD), where they have been shown to have some utility. Their usefulness in the diagnosis of *C. difficile* disease is less clear. This section only briefly touches upon this subject.

(i) Fecal lactoferrin. Lactoferrin is an iron binding glycoprotein found in neutrophils and in secretions such as breast milk, and therefore, this marker may be present in feces of children who are breast fed, reducing its utility as an enticing marker for bacterial enteritis in children <2 years of age (157). This protein is released following neutrophil activation, and the concentrations in stool and other fluids are proportional to the number of neutrophils recruited (158). Fecal lactoferrin is resistant to proteolysis and is not degraded by intestinal bacteria (159).

Fecal lactoferrin was first measured in stool samples in 1992 by a latex agglutination assay developed by Guerrant et al., who as-

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TABLE 6 Summary of CAP survey results for the most common assays used for *C. difficile* testing, 2001 to 2012

Yr	Survey ID	Total no. of participants	No. (%) of participants using method																	
			Bartels cytotoxicity assay	BD ColorPac Toxin A	bioMérieux Vidas	Meridian Premier Cytoclone A & B	Meridian Premier Toxin A	TechLab/ Wampole Toxin A/B	Thermo BioStar OIA	Meridian Immuno-Card Toxins A & B	Meridian Premier Toxins A & B	Remel Xpect Toxin A/B	TechLab/ Wampole Tox AB II	TechLab/ Wampole Toxin A/B Quik Check	TechLab/ Wampole C. Diff Quik Chek Complete	BD GeneOhm	Cepheid Xpert	Meridian Illumigene
2001	D-C	2,384	33 (1.3)	137 (5.7)	231 (9.6)	204 (8.5)	261 (10.9)	309 (13.0)												
2002	D-A	2,127	31 (1.5)	137 (6.4)	220 (10.3)	226 (10.6)	214 (10.1)	274 (12.9)												
2003	D-B	2,207	25 (1.1)	125 (5.7)	179 (8.1)	353 (16.0)	53 (2.4)	315 (14.2)												
2004	D-B	2,021	22 (1.1)	111 (5.5)	142 (7.0)		93 (4.6)	292 (14.4)	213 (10.5)											
2005	D-A	1,972	17 (0.9)	81 (4.1)	127 (6.4)		71 (3.6)	257 (13.0)	197 (10.0)		573 (28.4)									
2006	D-A	2,041	12 (0.6)	44 (2.1)	83 (4.1)		32 (1.6)	248 (12.1)	141 (7.0)	314 (15.3)	545 (27.6)	92 (4.7)								
2007	D-A	2,304	12 (0.6)	32 (1.4)	54 (2.3)				78 (3.3)	449 (19.4)	563 (27.6)	563 (27.6)								
2008	D-A	2,096		15 (0.7)	37 (1.8)				54 (2.6)	467 (22.3)	570 (24.7)	142 (6.2)	258 (11.2)	79 (3.4)						
2009	D-A	2,170	10 (0.5)	10 (0.5)	33 (1.5)				13 (0.6)	506 (23.3)	488 (23.2)	150 (7.2)	195 (9.3)	145 (6.9)						
2010	D-A	2,239	10 (0.5)	15 (0.7)	42 (1.9)					489 (21.8)	492 (22.7)	155	189 (8.8)	160 (7.3)						
2011	D-B	2,324			62 (2.7)					484 (20.8)	438 (19.6)	137 (1.6)	172 (7.7)	153 (6.8)	126 (5.6)					
2012	D-A	2,189			37 (1.7)					360 (16.4)	367 (15.8)	121 (5.2)	120 (5.2)	131 (5.6)	340 (14.6)	31 (1.3)	253 (10.9)	85 (3.7)		
											224 (10.2)	83 (3.8)	78 (3.6)	96 (4.4)	341 (15.6)	48 (2.1)	332 (15.2)	267 (12.2)		

TABLE 7 Summary of CAP results for *C. difficile* testing, 2001 to 2012

Yr	Survey ID	Total no. of participants	Intended response	No. (%) of participants with correct response
2001	D-C	2,384	Positive	2,220 (93.1)
2002	D-A	2,127	Negative	2,090 (98.3)
2003	D-B	2,207	Positive	2,162 (98)
2004	D-B	2,021	Negative	1,979 (97.8)
2005	D-A	1,972	Negative	1,919 (97.3)
2006	D-A	2,041	Positive	1,970 (96.5)
2007	D-A	2,304	Negative	2,235 (97)
2008	D-A	2,096	Positive	2,077 (99.1)
2009	D-A	2,170	Positive	2,156 (99.4)
2010	D-A	2,239	Positive	2,222 (99.2)
2011	D-B	2,324	Negative	2,310 (99.4)
2012	D-A	2,189	Negative	2,189 (96.6)

sessed the ability of the assay to detect patients with *Shigella* infection (160). Compared to uninfected controls, 96% of patients with shigellosis had fecal lactoferrin titers that ranged from 1:200 to ≥1:5,000 (160). This same group also showed that 92% of patients with *C. difficile* enteritis had elevated latex agglutination titers of fecal lactoferrin of >1:50, with 50% having titers of >1:400 (161). Since then, others have reported on the utility of fecal lactoferrin testing for assessing severity of *C. difficile* infection (162), as a marker for infection associated with moxifloxacin-resistant strains (163), and in testing algorithms as a first step in deciding which patients with diarrhea should be tested for *C. difficile* (159). It has also been used to determine the clinical significance of results using multistep algorithms that involve GDH screening and PCR confirmation (164). In the latter scenarios, lactoferrin levels were higher in patients who were GDH positive/toxin positive than in GDH-positive/toxin-negative/PCR-positive or GDH-negative patients (164). In a study by van Langenberg et al., the authors reported a negative predictive value of 92% when the manufacturer's cutoff was lowered from 7.25 μg/ml to 1.25 μg/ml (159). More prospective studies specifically targeting patients with *C. difficile* disease need to be performed before firm recommendations on the use of this test can be made.

(ii) Fecal calprotectin. Calprotectin is a calcium binding protein found within the cytosol of neutrophils, where it accounts for approximately 60% of their cytoplasmic protein content (158). Under inflammatory conditions of the intestinal tract where neutrophils accumulate, calprotectin is excreted in stool and is resistant to bacterial degradation (158). Studies have shown that it is stable at room temperature in feces for up to 7 days (158). In studies of patients with IBD, cutoffs have been established to differentiate functional from organic bowel disease; values of <50 μg/g indicate functional disease, and values of >150 μg/g of calprotectin are predictive of organic disease and highlight patients who should be referred for colonoscopy (158). In those situations where the values fall between 50 and 150 μg/g, other causes of inflammation, such as infections with enteric pathogens, should be considered (158). It should be noted that nonsteroidal anti-inflammatory agents can also raise fecal levels of calprotectin because of mucosal damage to the small bowel, which recruits neutrophils to the site (158).

Few studies have been performed to evaluate the utility of calprotectin in the evaluation of patients with acute diarrhea. Shastri et al. performed a large, prospective, multicenter, case-controlled

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study to evaluate the accuracy of fecal calprotectin, fecal lactoferrin, and fecal occult blood testing of patients with suspicion of acute enteritis, including *C. difficile* infection (165). Initially, 2,185 patients from three centers were evaluated by routine bacterial stool culture and a *C. difficile* toxin A and B EIA. Of this large cohort, 9.2% of patients ($n = 200$) were positive for a bacterial pathogen and were age and sex matched to a control group that had negative results (165). Commercial assays were used to perform a quantitative fecal calprotectin test (Immunodiagnostik ELISA kit; Immunodiagnostik AG, Bensheim, Germany), a qualitative fecal lactoferrin test (IBD-Chek; TechLab, Blacksburg, VA), and fecal occult blood tests (Haemoccult; Beckman Coulter Inc., Fullerton, CA) (165). There was an overlap of fecal calprotectin levels between the pathogen-positive patients and the control group (0 to 994 mg/liter and 0.1 to 204.7 mg/liter, respectively). Compared to the other tests, the fecal calprotectin specificity and positive and negative likelihood ratios were statistically better in predicting patients with infectious diarrhea (165). Fecal calprotectin levels were highest for the 87 patients with *C. difficile* diarrhea compared to patients with other infectious causes of diarrhea (165). In this group, the sensitivity of the test with a cutoff value of >15 mg/liter was 82.8% (165); however, this value is below the level of acceptability to be used alone as a screening test. The FDA-approved assay available in the United States has an indication for inflammatory bowel disease.

In summary, both fecal lactoferrin and calprotectin are nonspecific markers of intestinal inflammation, and while studies demonstrate that levels of these markers may be significantly elevated in patients with *C. difficile* disease, the sensitivity is too low in most studies to recommend their routine use for screening of patients. However, it does appear to be the case that the absence of fecal lactoferrin or fecal calprotectin is inconsistent with an inflammatory cause of diarrhea.

(iii) Cytokine analysis. There are no commercial assays available for measuring cytokine levels in fecal specimens. Some investigators have shown the correlation of elevated levels of IL-1β and IL-8 in active disease, particularly in cases of moderate to severe infection (162, 166, 167), with subsequent decreases in levels of these cytokines in stool when the patient recovers from acute infection (166). In a study by Jiang et al., the authors demonstrated that a single-nucleotide polymorphism (SNP) (251 A/A) in the IL-8 gene is associated with increased susceptibility to *C. difficile* disease (167). Cytokine analysis of fecal specimens may be an area to be pursued in future studies with respect to assessing severity of infection, potentially to resolve whether a positive NAAT assay is associated with disease or colonization, and perhaps to monitor a patient's response to treatment.

Algorithmic approaches. As alluded to in the sections above, although NAATs for *C. difficile* detection are more sensitive than toxin EIAs and can be used as stand-alone tests, they are more costly than EIAs. The amount of technologist time required to perform the molecular assays can also be longer than for EIAs, depending on the method used. To address these issues, some laboratories have adopted two- or three-step algorithmic approaches to *C. difficile* testing. In the algorithmic approach, samples are screened by using a GDH assay, and if the GDH assay is negative, the specimen is reported as negative without additional testing. If the specimen is GDH positive, confirmatory testing must be performed. In the two-step algorithm, the confirmatory test is typically a molecular assay, and the sample is reported as either positive or negative based on the molecular test result. In the three-step approach, samples are tested for GDH, and if positive, they are tested by using a toxin EIA. Toxin-positive samples can be reported as positive, but GDH-positive/toxin-negative samples are then tested by using a molecular test. Alternatively, the samples can be screened by using a GDH/toxin combination EIA, but since the toxin EIA component of these assays has a sensitivity comparable to those of stand-alone tests (i.e., inadequate sensitivity), GDH-positive/toxin-negative samples should be reflexed to testing (i.e., automatically tested without a physician order when another test is positive in a multitest algorithm) via a molecular method (87, 90, 91, 100, 122–124). These multistep approaches can be especially enticing to laboratories without the equipment or expertise for molecular testing, in order to minimize the number of specimens sent to a reference laboratory for this analysis.

Although algorithmic testing represents a potential cost savings to the laboratory, there are a number of factors to consider when evaluating this approach. The first factor is the prevalence of GDH-positive specimens in a given population. For example, in pediatric patients (discussed below), the rate of GDH-positive specimens may be very high, necessitating the use of two or more assays on a large proportion of specimens submitted. In these cases, cost savings may be negligible. Laboratories will need to consider the clinical impact of the prolonged turnaround time to reporting for specimens requiring multiple tests. Some other practical considerations include the need for inventory, training of staff, quality control, and maintenance of procedures and competency for multiple assay types. There is also the caution that GDH assays appear to be slightly less sensitive than NAATs. These issues are not trivial and must be taken into consideration. Thus, each laboratory must evaluate the utility of multistep algorithms for their patient population.

(i) The European perspective. NAAT assays for PCR detection are highly sensitive for *C. difficile* detection, but they do not differentiate between active infection and asymptomatic carriage. As the antimicrobial agents used to treat *C. difficile* infection can also induce *C. difficile* disease, unnecessary antimicrobial therapy as a result of false-positive assay results is not without consequence. The National Health Services (NHS) laboratories in England are now following a different protocol from what is used in most of the United States. The NHS algorithm uses either a molecular assay or a GDH assay to screen for the presence of *C. difficile*, followed by a "good" or "relatively sensitive" EIA to confirm the diagnosis (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/146808/dh_133016.pdf.pdf [accessed 25 April 2013]) (168). The rationale for this is that the GDH and molecular assays have high NPVs and can be used to expedite the exclusion of *C. difficile* infection. It has been proposed by the NHS that patients who are positive for *C. difficile* by a molecular assay but who are negative for toxin by EIA methods can be declared "potential *C. difficile* excretors," and the decision may be made to apply isolation precautions for *C. difficile* for these patients, but they do not require treatment for disease; other causes of the patient's diarrheal illness should be considered at this point (168, 169). This approach of a NAAT test followed by a toxin EIA is very uncommon in the United States. At the time of preparation of this article, the data supporting these recommendations are under review for publication.

Antimicrobial susceptibility testing. The most commonly pre-

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scribed antimicrobial therapy for *C. difficile* infection is metronidazole or oral vancomycin. As the concentrations of these drugs in the feces are typically high, treatment failures are rarely thought to be due to resistance of the organisms to the antimicrobial therapy (34, 170). However, metronidazole concentrations in the stool can be variable depending on the consistency of the stool and the amount of inflammation in the gut. Some studies have demonstrated that more treatment failures occur with metronidazole therapy than with vancomycin and that higher rates of recurrence are observed in severely ill patients whose *C. difficile*-associated disease (CDAD) is treated with metronidazole (171, 172). In addition, there have been sporadic reports of *C. difficile* isolates with increasing MICs of metronidazole (173–176). The method currently recommended by the Clinical and Laboratory Standards Institute (CLSI) for *C. difficile* antimicrobial susceptibility testing is the agar dilution method using Brucella agar supplemented with hemin, vitamin K, and 5% (vol/vol) laked sheep blood (177). For metronidazole and *C. difficile*, an MIC of <8 μg/ml is considered susceptible. The agar dilution method is very technically demanding and labor-intensive. While this method may be appropriate for epidemiological studies, it is difficult to implement for routine use in the clinical laboratory. Several studies have evaluated the use of disk diffusion and/or Etest/gradient diffusion methods, as these are more amenable for routine use; however, there is much variability in the methods that laboratories use to perform these tests, including the use of both 5-μg and 30-μg disks, different medium formulations, and different incubation times (178–180). No CLSI standardization or guidelines for disk diffusion or Etest for *C. difficile* have been established. In general, these methods exhibit good agreement with one another and with agar dilution methods (178), although the MICs obtained by using the Etest are slightly lower than those demonstrated by agar dilution (179, 180).

EVOLVING EPIDEMIOLOGY

Clinical Implications of Strain Typing

Epidemiology. The epidemiology of *C. difficile* has been in a state of flux over the last 2 decades. There has been an increase in the incidence and severity of CDI in North America and Europe since the early 2000s, and in 2003, the first reports emerged from Quebec, Canada, of a 4-fold increase in CDI rates (26, 181, 182). During 2005, an increase in the rate of *C. difficile* infection was noticed around North America, with a large number of cases being attributed to NAP1/ribotype 027 strains, which exhibited high-level resistance to fluoroquinolones; this trait is thought to be one of the main factors contributing to the success of the rapid spread of this strain type, although the increased toxin production observed *in vitro* has also been postulated to contribute to the high rate of outbreaks of this strain (29, 30, 46–50). Retrospective analysis revealed that ribotype 027 strains were isolated as early as 1958. It appears that different lineages evolved with phenotypic distinctions resulting in different antimicrobial susceptibility patterns and toxin production. Ribotype 027 has an 18-bp deletion and a frameshift mutation due to a single-base-pair deletion at position 117 of *tcdC* (183) that results in a truncated TcdC that lacks function. TcdC is a negative regulator of toxin A and toxin B production, and the resultant hyperproduction of toxin is thought to contribute to increased virulence. Increased sporulation capacity has been hypothesized to contribute to increased transmission of this strain type (46, 49). In contrast, other studies have shown that ribotype 027 is not more virulent than other strain types (184, 185) and that the sporulation rate and toxin production rate are not linked to strain type (170, 186, 187), so this remains an area of controversy.

The increased incidence and severity of *C. difficile* disease have pushed *C. difficile* to the forefront as an important public health problem (34, 182, 188–190). As a result, accurate and timely identification of patients with *C. difficile* infection has been an area of focus for clinical laboratories in recent years.

In addition to the clinical importance of the evolving epidemiology of *C. difficile*, changes in circulating strain types are also of importance for clinical laboratories. For example, with the emergence of toxin A-negative strains associated with severe clinical disease, assays that rely solely on detection of toxin A are generally no longer used (36, 84, 191) (Table 6). It has also been reported that assay performance might vary depending upon the strain type (118).

Community-acquired *C. difficile* infection. Although *C. difficile* is the number one cause of hospital-acquired diarrhea, it is not exclusively a nosocomial pathogen (34, 192). Numbers of cases of community-acquired and community-associated infection are increasing worldwide; around the world, up to 25% of cases are community acquired, and these patients may not have the traditional risk factors for CDI, such as advanced age, antibiotic exposure, and medications to suppress gastric acid (188, 192–195). A pilot study was recently conducted whereby whole-genome sequencing (this method is discussed in more detail below) was performed in almost real time to evaluate *C. difficile* transmission. In this analysis, samples from all of the cases detected over a 6-week period in a single hospital were sequenced and compared with local strain sequences from the previous 3 years. This analysis suggested community transmission, which was not previously suspected (196).

Transmission in food has been proposed as a plausible and enticing explanation for community-acquired disease. While *C. difficile* has been isolated from food, food-borne transmission of disease has not been demonstrated, even though the predominant strain types isolated in food are ribotypes 027 and 078, strains well established to cause human disease (197–201). One recent study reported *C. difficile* recovery from 5 out of 119 seafood and fish samples from a grocery store (all of which were of ribotype 078) (200); in contrast, a large survey that tested for *C. difficile* in 1,755 retail meat samples from across the United States (ground beef, ground turkey, chicken breast, and pork chop) found that no *C. difficile* was isolated from any of these specimens (198), which suggests a low prevalence of contaminated meat products. Thus, the true impact of food as a vehicle for *C. difficile* transmission remains to be clarified.

Strain Typing Methods

A number of characteristics are important for strain typing. Some of the most important properties are the ability of the method to group related isolates, the ability to differentiate unrelated isolates, typeability (i.e., the ability to resolve isolates as similar or distinct in an unambiguous manner), reproducibility (intra- and interlaboratory), and cost-effectiveness, and if possible, the interpretation should be objective (202). The different typing methods employed embody various degrees of each of these characteristics. The "discrimination index" (D) is a metric that has been devised to compare the abilities of different typing methods to discriminate unique strains as different and is based on Simpson's index of diversity (203, 204). Values for D range from 0 to 1, and the closer

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the value is to 1, the more discriminatory the method. Historically, strain typing methods relied on phenotypic patterns such as antimicrobial susceptibility profiles, serotyping with slide agglutination methods, immunoblotting, or bacteriophage patterns. Today, most typing methods for microorganisms are based on molecular analysis.

There has been great interest in strain typing of isolates of *C. difficile* for a number of reasons (205). The first is for epidemiological purposes at a global level, both to evaluate the strain types circulating in different parts of the world and to evaluate the evolution of these strain types. At the local level, strain typing can be used to track transmission and outbreak events. From the perspective of diagnostic testing, it has been proposed that the performance characteristics of some of the available assays may vary depending upon the strain type, which could be of great clinical and public health importance (118). As more and more laboratories make the shift to molecular methods for *C. difficile* detection, it will be important to monitor trends in the circulating strain types to ensure that *C. difficile* variants that may arise have conserved nucleic acid sequences in the regions targeted by these molecular assays, or we may see a shift in the sensitivity of the molecular assays over time.

From the perspective of patient care, it is postulated that strain typing might be important in some circumstances, although routine strain typing of all isolates is not currently indicated. Some strain types may be more correlated with risk of recurrent disease, although more investigation is needed to confirm this (2). In addition, new antimicrobial therapies for *C. difficile* are emerging, such as fidaxomicin. Fidaxomicin is much more costly than traditional therapies for *C. difficile*, such as metronidazole or vancomycin. Early studies evaluating fidaxomicin suggested that the efficacy of this drug and/or risk of relapse on this drug may be variable with the *C. difficile* strain type. For example, Louie et al. reported that the rate of recurrence of non-NAP1 *C. difficile* infection was significantly lower with fidaxomicin (156). Considering the cost of this drug, if these initial findings are replicated in other studies, it could result in an increased demand for real-time strain typing to determine which patients are best suited to receiving this therapy.

In the future, there may be a role for strain typing of isolates from patients with recurrent *C. difficile* disease in order to ascertain if these patients have a relapse of the strain originally causing the infection or if they have acquired or become reinfected with a new strain from the environment. It is possible, with more data, that this type of information may change how clinicians caring for these patients stratify treatment options for individuals with recurrent disease.

A number of different strain typing methods for *C. difficile* have been described and evaluated. To date, only one major study has compared a number of these techniques simultaneously: Killgore and colleagues evaluated restriction endonuclease analysis, pulsed-field gel electrophoresis (PFGE), PCR-ribotyping, multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), amplified fragment length polymorphism analysis, and surface layer protein A gene sequencing typing (206). The analysis included a relatively small sample set of 42 isolates from four countries. While this study was biased toward NAP1 strains, it did provide an assessment of how these typing methods related to one another. The strain typing techniques for *C. difficile* that are used most frequently clinically or for outbreak investigations are described in more detail below.

Pulsed-field gel electrophoresis. PFGE was one of the first molecular typing methods used for *C. difficile*, and it is typically still considered the standard for *C. difficile* typing in North America. PFGE involves digestion of genomic DNA with a restriction enzyme that is a "rare cutter," or infrequently cuts the genomic DNA; for *C. difficile*, SmaI is commonly used (207). The resultant DNA fragments are separated by using agarose gel electrophoresis, but the electric field is repeatedly switched in three different directions during this process (pulsed field). This allows resolution of large fragments of DNA that would not be possible using conventional agarose gel electrophoresis. The resulting banding patterns are referred to as "North American pulsed-field" (NAP) types. Strains with >80% similarity are usually regarded as a single pulsotype, although there can be a great deal of subjectivity in interpretation of the banding patterns, especially when differences between the test strain and the reference strains are slight. In the typing method comparison study by Killgore et al., the *D* value for PFGE was reported to be 0.843 (206).

Ribotyping/PCR-ribotyping. Ribotyping is the strain typing method used most frequently for *C. difficile* in Europe. This method is based on amplification of the intergenic spacer (ITS) region between the 16S and 23S rRNA genes. This operon has several copies in the *C. difficile* genome, and the different copies vary in length. Therefore, a single primer pair can result in a pattern of bands, usually ranging from 200 to 700 bp (208, 209). The bands can be resolved, visualized, and compared on an agarose gel or by using commercial kits or analysis software (206, 208–210).

Two major primer sets have evolved for PCR ribotyping: the "United States" primers and the "United Kingdom" primers (206). The primers described by Stubbs et al. appear to be more discriminatory for *C. difficile* strains than the primers described by Bidet et al. (208, 209). In general, PCR-ribotyping exhibits good discriminatory power but may not be able to differentiate between strains of the closely related ribotypes 027, 106, and 017 (211). In the method comparison study by Killgore et al., the *D* value for PCR-ribotyping using the U.S. method was 0.700, and that using the United Kingdom method was 0.688 (206).

It can be difficult to compare banding patterns between laboratories if analysis software is not used. To circumvent the issues related to manual interpretation of banding patterns, PCR-ribotyping has recently been adapted to high-resolution capillary gel electrophoresis, which has improved the reproducibility of ribotype interpretation (212). In the United Kingdom, work is ongoing to standardize ribotyping protocols across the country using this method.

In the United States, no natural or mandated surveillance system is in place for *C. difficile*. In contrast, England has instituted a *C. difficile* reporting program, whereby isolates are submitted to a central laboratory and a portion of these isolates are ribotyped to monitor epidemiological trends. Figure 1 depicts the presence of different ribotypes in England by quarter from 2007 to 2009 (213). In North America, ribotype 027 is predominant, although ribotype 078 is emerging. New ribotypes of clinical significance continue to emerge; two of the newly described ribotypes, 176 and 198, are closely related to ribotype 027 (and can be mistaken for ribotype 027, depending upon the methodology used) (214). These ribotypes all cluster together by multilocus sequence typing, suggesting coevolution of these strain types and a common ancestry. The fact that North America relies largely on PFGE and Europe relies largely on PCR-ribotyping for strain typing and strain

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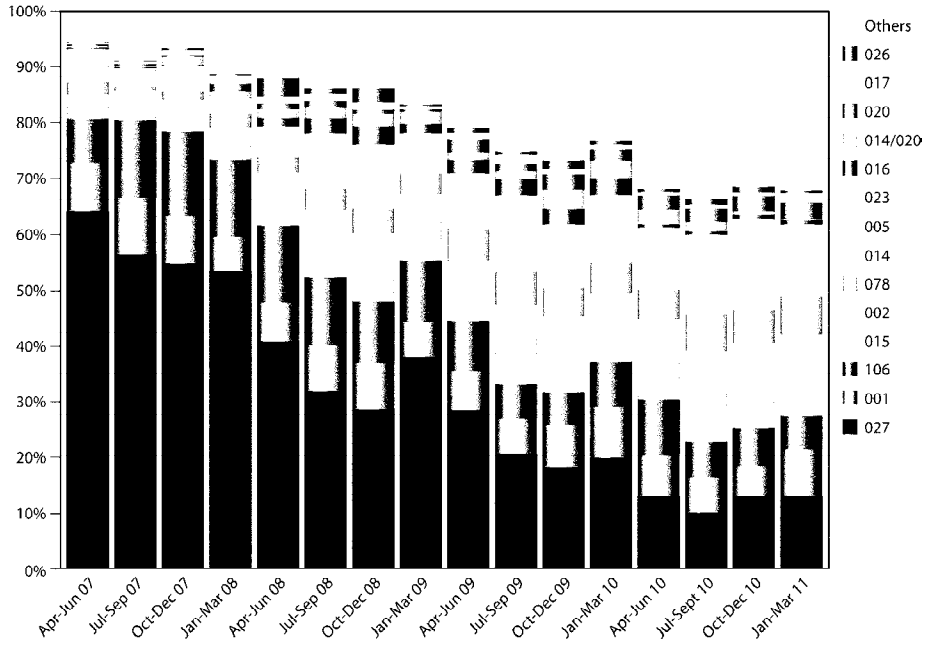

FIG 1 Prevalence of *C. difficile* ribotypes in England, 2007 to 2011. Data are stratified by quarter. (Reproduced from reference 213 with permission from Public Health England.)

designation can at times make interlaboratory exchange and epidemiological comparisons difficult. A limitation of both PFGE and ribotyping methods is that laboratories require a library of reference isolates for comparison in order to provide a "name" for strain types, and outside reference or public health laboratories, relatively few centers have access to such a library of strain types.

Multilocus variable-number tandem-repeat analysis. MLVA is emerging as a typing method for *C. difficile* to be used in outbreak investigations and appears to be discriminatory enough to monitor transmission events in the hospital setting (211, 215–217). This method uses multiple variable-number tandem-repeat (VNTR) loci, which are dispersed throughout the genome and vary with regard to level of diversity to resolve phylogenetic relationships between isolates. The amplicons that are obtained are resolved by using capillary electrophoresis, followed by automated analysis of the fragments. The "summed tandem-repeat difference" (STRD) is used as a measure of genetic difference between isolates. To date, a consensus approach toward MLVA typing for *C. difficile* has not been established. Various approaches to MLVA have been reported in the literature, each evaluating seven loci on the *C. difficile* genome (215, 216, 218). Manzoor and colleagues reported an approach which they term "extended MLVA" (eMLVA), which uses eight novel loci in addition to the seven loci previously reported (211). Using 229 previously ribotyped isolates from diverse locations around the world between 1996 and 2010, this group was able to efficiently subtype all of the isolates in the study while clustering like isolates concordant with PCR-ribotyping. The *D* value reported for this eMLVA method was 0.999, whereas PCR-ribotyping for the same group of isolates resulted in a *D* value of 0.886 (211). Wei et al. evaluated 142 *C. difficile* isolates by MLVA using different numbers of loci to devise a scheme with an adequate discriminatory index but with correlation to established PCR-ribotypes (219). Using various combinations of VNTR loci, they devised MLVA schemes, each with discriminatory indices of 0.99, compared to PCR-ribotyping, which had a *D* value of 0.96 for the same subset of isolates. The reported *D* value for MLVA in the method comparison study by Killgore et al. was 0.964 (206).

MLVA can be useful for outbreak investigations and studies evaluating local transmission events, but as of yet, it remains nonstandardized; there is no established method or naming scheme that is portable across laboratories. MLVA is discriminatory and reproducible, and if agreement can be reached regarding the optimal method, the results could be portable and provide interlaboratory comparability.

Multilocus sequence typing. MLST is now commonly used on many pathogens to assess the general population structure and overall diversity within a species. MLST typically uses allele fragments that are 300 to 600 bp in length; the historical reason for this is that fragments of this size can be reliably and reproducibly read on a single sequencing run using Sanger sequencing methods. Most MLST schemes use between 6 and 12 loci, whereby each unique combination of alleles is assigned its own sequence type (ST), and databases of MLST data and STs are available in the public domain (220, 221). The loci used are typically housekeeping genes, as these are relatively conserved within a species, but variants within these housekeeping genes can be used to distinguish strains. Essentially, isolates are grouped based on their evolutionary proximity. When a novel allele is found, the investigator can apply to have it added to the ST database. This platform therefore allows for portability of results between different laboratories; on the Internet, freely accessible, curated databases of STs exist, making this method portable between laboratories (220).

C. difficile is theoretically well suited to MLST, as the species is relatively genetically heterogeneous. MLST was first described for *C. difficile* typing in 2004 by Lemee and colleagues, who used seven

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housekeeping genes (*aroE*, *ddl*, *dutA*, *tpi*, *recA*, *gmk*, and *sodA*) to analyze a group of *C. difficile* isolates (222, 223). The *D* value for this method was calculated to be 0.958 (222). This was followed by a study by Griffiths et al., who performed MLST on 152 isolates of *C. difficile* isolated in culture, representing 49 ribotypes. The *D* value of the MLST method was 0.90, which was roughly comparable to the *D* value for PCR-ribotyping of the same collection, 0.92 (224). This group also applied this MLST typing scheme directly to stool specimens (rather than cultured isolates of *C. difficile*), with good success. For a small number of the stool specimens, sequencing failed as a result of inadequate amplification directly from the stool specimens. For the specimens where adequate amplification was obtained, the MLST data correlated with the ST of the isolate recovered in culture. The *D* value reported for MLST in the study by Killgore et al. was 0.699, which is lower than that reported by Lemee et al. and Griffiths et al. (207). This may reflect the fact that the study by Killgore et al. contained a larger proportion of NAP1/ribotype 027 strains and therefore inherently represented less diversity than the other two investigations (206, 222, 224).

Whole-genome sequencing. Whole-genome sequencing (WGS) has the potential to be the "ultimate" typing method, and the extra resolution can facilitate evaluation of specific transmission events within clusters or outbreaks (225, 226). Historically, WGS had been considered to be too expensive and cumbersome, with a turnaround time inadequate for real-time outbreak investigations. However, the cost is decreasing consistently, while the speed and throughput of WGS continue to improve (227–229). One challenge or limitation to WGS is still the analysis; adequate bioinformatics are required to make use of the enormous amounts of data that are generated.

Recently, WGS was used to study the transmission of *C. difficile* whereby WGS was performed on 486 samples from cases documented over 4 years in Oxfordshire, United Kingdom (230), using the Illumina platform. This analysis identified instances of direct transmission for a subset of the isolates, but direct transmission was ruled out for the majority of isolate pairs where direct transmission events would have been suggested by using conventional molecular typing techniques. In another pilot study, benchtop WGS was performed in almost real time to evaluate a *C. difficile* outbreak; samples from all cases identified over a 6-week period in one hospital were sequenced and compared with local strain sequences from the previous 3 years. Analysis of these strains illustrated that WGS could provide early outbreak detection and also suggested community transmission, which was not previously suspected (196).

The ribotype 027/NAP1 strains have emerged worldwide and are widespread in health care facilities. While the underlying reasons for this are postulated and described in previous sections of this paper, the reason for the rapid emergence and spread around the globe remain unknown. WGS also has a role in answering this question. He et al. used WGS for phylogenetic analysis and surprisingly found that two distinct lineages of this epidemic strain emerged in North America, termed FQR1 and FQR2 (45); they both acquired the same fluoroquinolone resistance-conferring mutation and a closely related transposon. The FQR2 lineage became more widespread, leading to outbreaks associated with health care institutions in Europe, the United Kingdom, and Australia (45).

WGS is reproducible and portable and does not require a library of established isolates for strain type determination. Although the cost and turnaround time continue to limit the routine use of WGS at the time of preparation of the manuscript, it is likely that WGS will be more widely adopted for *C. difficile* strain typing in the future.

TESTING CONSIDERATIONS FOR THE CLINICAL MICROBIOLOGY LABORATORY

Several professional societies and other organizations have published guidelines for the diagnosis and treatment of *C. difficile* disease (52, 101, 231, 232). These organizations include the SHEA/IDSA in the United States (52), the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (101), and the Australian Society for Infectious Diseases (232). All of the guidelines have a list of patients for whom a diagnosis of *C. difficile* disease should be considered; they also recommend that any hospitalized patient who has ≥3 unformed stools in a 24-h period should be tested. It is recommended that testing be performed only on unformed stool except in rare instances, such as an ileus (232). Laboratories should have specimen rejection policies that restrict testing to watery, liquid, or unformed stools that take the shape of the container. Asymptomatic patients should not be tested, even for test of cure.

Repeat testing is of limited value but is still somewhat controversial. The practice of sending "stools for *C. difficile* times three" became the established norm in many clinical laboratories after the widespread implementation of insensitive enzyme immunoassay methods (233) (the reader is referred to the section above on EIA testing for more details). Physicians would send multiple specimens to the laboratory to compensate for the poor performance of these tests. Numerous studies have demonstrated that regardless of the testing method, EIA (149, 234–236), cell culture cytotoxicity neutralization assays (237, 238), or NAATs (149, 239, 240), and the patient population tested, sending of repeat samples does not increase the yield and can be misleading, as the positive predictive value drops with each subsequent test (149). One systematic review of the literature noted that the yield with a second EIA after an initial negative result dropped to 1.5 to 4.7% in hospitalized patients (241). In an outbreak situation, this review and other studies noted that the yield of repeat testing may be higher (5%), suggesting that outbreak situations may be the one situation where repeat testing may be helpful (234, 241). At least one study has demonstrated that substantial cost savings may be incurred by eliminating duplicate testing without adversely affecting patient care (239). Since longitudinal studies suggest that after 7 days, the frequency of positive results begins to increase again, most likely reflecting new infections as opposed to false-negative tests (101, 234), many laboratories have implemented a 7-day rule; that is, repeat tests following an initial negative result will not be performed within a 7-day period of time.

SPECIAL PATIENT POPULATIONS

Children

High rates of *C. difficile* colonization have been well documented in infants less than 1 year of age (242–245). Infants who are breast-fed appear to have lower carriage rates (14%) than formula-fed infants (30%) (246). One study illustrated that infants were usually colonized with a single clone of *C. difficile* and that they were colonized for several consecutive months (242). Once new foods are introduced to infants, they may acquire new strain types of *C. difficile* (242).

Infants are almost invariably asymptomatic, although it can be

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difficult to discriminate asymptomatic carriage from true disease in infants <1 year of age. The mechanism and pathobiology of this asymptomatic colonization in neonates are unknown, but many hypotheses, including passive transfer of protective maternal antibody and a protective fecal microbiome in infancy, have been offered. There is some thought that infants lack the receptor for the toxin, but considering that the receptor for toxin B is as yet uncharacterized, this remains unproven.

Both nosocomial and community-associated *C. difficile* infections are on the rise in the pediatric population (247–250). Between 2001 and 2006, one study reported an increase in the incidence of *C. difficile* disease in children from 2.6 to 4.0 cases per 1,000 hospitalized children (247); similarly, another study evaluated *C. difficile* in hospitalized pediatric patients and found that from 1997 to 2006, the incidence of *C. difficile* disease was 35 per 10,000 hospitalizations in children 5 to 9 years of age (249). NAP1 strains have been recovered from pediatric patients (251, 252).

Many pediatric institutions are converting or are in the process of converting to molecular methods for detection of *C. difficile* infection. When molecular testing is used on pediatric samples, the increased sensitivity leads to an apparent increase in the incidence of *C. difficile* disease in pediatric patients, just like adults. Few studies have evaluated the performance characteristics of various diagnostic assays for pediatric patients specifically. One of the first studies using a NAAT for pediatric patients was conducted by Luna et al., who tested 157 samples from 96 pediatric patients; toxigenic culture was the gold standard in this investigation. The laboratory-developed PCR assay used in this study was found to be 95% sensitive (253), and once the PCR assay was implemented (2007 to 2010), the overall volume of testing remained constant, but the *C. difficile* positivity rate nearly doubled compared to when the TechLab Tox A/B II EIA was used (2003 to 2006) (253).

Selvaraju et al. examined 200 stool samples from pediatric patients using toxigenic culture as the gold standard. Those researchers evaluated the C. Diff Quik Check Complete, BD GeneOhm, and ProGastro Cd PCR assays. The sensitivity and specificity of each of these assays were as follows: 97.9 and 82.2% for C. Diff Quik Chek Complete, 89.6 and 96.7% for GeneOhm PCR, and 100 and 93.4% for ProGastro PCR, respectively (122). In this study, a retrospective chart review was performed for all patients who were positive for *C. difficile* by PCR methods only; for 3 of these patients, *C. difficile* was a plausible diagnosis, but for the remaining 8 patients, the clinical picture was not compatible with a diagnosis of *C. difficile*. The authors of this study concluded that future prospective studies of pediatric patients are needed to fully elucidate the clinical significance of a positive NAAT assay for *C. difficile* as well as the economic impact of NAAT testing in this patient population (122).

A study by Ota and McGowan evaluated GDH, toxin EIA, and NAAT assays, alone and as part of multistep algorithmic approaches. This was a prospective study including 141 samples from patients aged 1 to 18 years (64). Similar to the findings of Selvaraju et al., and similar to assay evaluations of adult patients, the conclusions of this study were that toxin EIA had the lowest sensitivity for *C. difficile* detection (56%) and that a molecular assay (the Illumigene *C. difficile* assay) exhibited increased sensitivity (89%), although the sensitivity of this assay in this population is somewhat lower than what has been reported in other studies (64). This study does not reveal an assay that is clearly superior for the diagnosis of *C. difficile* infection in pediatric patients.

Although molecular methods may be problematic for *C. difficile* diagnosis in pediatric patients, toxin EIAs may also produce false-positive results. Toltzis and colleagues performed toxigenic culture on 112 stool specimens from pediatric patients that had previously tested positive for *C. difficile* by the Meridian Premier Toxin A & B assay (254). Those authors found that toxigenic *C. difficile* could be isolated from only 72 of these specimens. The average age of the children with EIA-positive, culture-negative samples was significantly lower than that of the children with EIA-positive, culture-positive samples (0.53 years versus 2.86 years; $P = 0.002$), but otherwise, no differences between the two groups of patients were detected (254). Thus, the PPV of a positive EIA for *C. difficile* in pediatric patients is also suboptimal, which is thought to be related to the low pretest probability for disease in the patients. No methodology is clearly superior for making the diagnosis of *C. difficile* disease in this patient population.

It should be noted that not all of the FDA-cleared *C. difficile* assays have been evaluated or are approved for testing of specimens from patients <2 years of age. It is incumbent upon laboratories performing these tests to confirm the indications for their assay and perform additional validation and verification if appropriate.

The American Academy of Pediatrics has recently released a policy statement on *C. difficile* infection in infants and children (255). The guidelines caution that testing for *C. difficile* should be performed only for children who meet the criteria for clinically significant diarrhea and that test results for infants <1 year of age can be difficult to interpret due to high rates of asymptomatic colonization. Some of the general recommendations of this document are that for children >3 years of age, diagnostic testing can be performed as it would be for adult patients. In children 1 to 3 years of age with diarrhea, causes other than *C. difficile* (such as viral causes) should be considered and tested for in addition to *C. difficile* when *C. difficile* is suspected. The guidelines also caution that no diagnostic test should be used as a test of cure, just as is the case for adult patients.

Oncology Patients (Nontransplant)

Risk factors for *C. difficile* infections among oncology patients include chemotherapeutic agents; antibiotics, especially cephalosporins and clindamycin; frequent hospitalizations; and prolonged neutropenia (256–258). While it might be anticipated that cancer patients receiving chemotherapy will have a severe course and increased risk of death compared to other hospitalized patients, in at least one study, this was not the case (259). In a study by Stewart et al., patients with hematological malignancy and *C. difficile* disease had zero colectomies to control disease, compared to four colectomies in the group without malignancy (259). No differences were noted in terms of other markers of disease severity, such as the need for intensive care unit admission (259). The only difference noted was a longer length of stay for patients with *C. difficile* colitis and malignancy (259). This study was limited by the small numbers of patients.

Solid-Organ Transplant Recipients

Longitudinal studies have shown a dramatic increase in the incidence of *C. difficile* among solid-organ transplant (SOT) recipients that parallels that of the disease in the general population and other at-risk groups. In a study by Boutros et al., the incidence of *C. difficile* disease was 4.5% in 1999, peaked at 21.1% in 2005, and plateaued at 9.5% in 2010 (260). These authors also noted that signifi-

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cant independent risk factors for *C. difficile* disease included a transplant other than kidney, age of >55 years, and induction with antithymocyte globulin (260). Once a patient with a SOT develops *C. difficile* disease, there is an independent association with greater mortality; longer length of stay, with the associated increased costs of hospitalization; more complications associated with the transplanted organ; and an increased need for colectomy (261).

Hematopoietic Stem Cell Transplant Patients

Diarrhea is common among hematopoietic stem cell transplant (HSCT) recipients, but until recently, *C. difficile* was relatively uncommon (262). A recent review reports the incidence of *C. difficile* disease among HSCT patients to be as high as 20% (263). In most studies, there do not appear to be any unique risk factors among HSCT recipients that are not shared by other nontransplant at-risk patients (256, 263). However, in a large study by Dubberke et al., patients who received a third- or fourth-generation cephalosporin were more at risk for development of *C. difficile* disease (264). This study also demonstrated that the receipt of growth factors was associated with decreased risk (264). These findings, especially the potentially protective effect of growth factors, need to be verified by prospective studies.

Once patients develop symptomatic *C. difficile* disease, the incidence of graft-versus-host disease (GVHD) greater than grade 2 and mortality not related to relapse of the underlying malignancy are higher than in uninfected patients (264, 265). In a study by Alonso et al., there was a strong relationship between early *C. difficile* disease and the development of GVHD in the year following infection. More prospective studies assessing the relationship between *C. difficile* and GVHD are needed (266).

Patients with Inflammatory Bowel Disease

As is the trend among the general population, studies have shown that the frequency of *C. difficile* disease among patients with inflammatory bowel disease (IBD) has increased by 2- to 3-fold since 2000 (267–269). In contrast to what is observed for the non-IBD population, two studies demonstrated that 66 to 75% of IBD patients acquired infection in the outpatient setting and that only 61% had recent antibiotic exposure (267, 268). Risk factors for *C. difficile* infections in IBD patients from these and other studies include increasing age, comorbidities, malignancies, and immunomodulatory therapy (267, 268). Immunosuppression increases the risk 2-fold (270). Several studies reported an increased risk of disease in patients with ulcerative colitis (UC) (colonic disease) compared with those with Crohn's disease as well as a greater risk with pancolitis than with distal disease (267, 270, 271). These observations have resulted in the recommendation that any IBD patient with symptoms severe enough to result in hospitalization should be tested for *C. difficile* upon admission, even in the absence of traditional risk factors such as antibiotic exposure (269, 271). Since there are no distinguishing clinical features that discriminate a flare of inflammatory bowel disease from diarrhea associated with *C. difficile*, testing is warranted for any IBD patient with significant diarrhea.

Many studies also demonstrated that *C. difficile* can contribute substantially to increased morbidity and mortality among IBD patients compared to non-IBD controls (270, 272). Mortality, especially among patients with UC; the need for bowel surgery; and duration of hospitalization are all substantially increased (267, 270, 272).

While there appears to be a substantial impact on the course of IBD in patients with active *C. difficile* disease, more definitive literature is needed to prove that *C. difficile* actually causes a flare of IBD. Likewise, there are no data to support that the various testing methods for *C. difficile* diagnosis perform differently among these patients than among controls. That said, as with non-IBD patients, patients with IBD can be asymptomatically colonized with *C. difficile*, and therefore, the same criteria for testing, namely, significant diarrhea, should be applied.

SUMMARY AND CONCLUSIONS

C. difficile continues to evolve, and the changing biology and growing lists of patient groups at risk have resulted in an unprecedented surge in the incidence and burden of disease. This review details the methods for diagnostic testing for *C. difficile*. New strain types have emerged, which have led to a marked increase in the incidence of *C. difficile* disease since the early 2000s. The increased incidence of *C. difficile* disease has highlighted the lack of sensitivity of the toxin EIAs that are used by most laboratories for the diagnosis of *C. difficile* and has ushered in a new era of molecular assays for diagnosis. At present, eight FDA-approved molecular assays for *C. difficile* are on the market. However, these assays do not solve the diagnostic uncertainty surrounding *C. difficile*, as detection of *C. difficile* in a fecal specimen does not automatically imply disease. *C. difficile*-associated disease is a clinical diagnosis supported by laboratory findings; this diagnosis continues to be a challenge for clinicians and laboratories alike.

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Exhibit 44

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Diverse Sources of *C. difficile* Infection Identified on Whole-Genome Sequencing

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ABSTRACT

BACKGROUND

It has been thought that *Clostridium difficile* infection is transmitted predominantly within health care settings. However, endemic spread has hampered identification of precise sources of infection and the assessment of the efficacy of interventions.

METHODS

From September 2007 through March 2011, we performed whole-genome sequencing on isolates obtained from all symptomatic patients with *C. difficile* infection identified in health care settings or in the community in Oxfordshire, United Kingdom. We compared single-nucleotide variants (SNVs) between the isolates, using *C. difficile* evolution rates estimated on the basis of the first and last samples obtained from each of 145 patients, with 0 to 2 SNVs expected between transmitted isolates obtained less than 124 days apart, on the basis of a 95% prediction interval. We then identified plausible epidemiologic links among genetically related cases from data on hospital admissions and community location.

RESULTS

Of 1250 *C. difficile* cases that were evaluated, 1223 (98%) were successfully sequenced. In a comparison of 957 samples obtained from April 2008 through March 2011 with those obtained from September 2007 onward, a total of 333 isolates (35%) had no more than 2 SNVs from at least 1 earlier case, and 428 isolates (45%) had more than 10 SNVs from all previous cases. Reductions in incidence over time were similar in the two groups, a finding that suggests an effect of interventions targeting the transition from exposure to disease. Of the 333 patients with no more than 2 SNVs (consistent with transmission), 126 patients (38%) had close hospital contact with another patient, and 120 patients (36%) had no hospital or community contact with another patient. Distinct subtypes of infection continued to be identified throughout the study, which suggests a considerable reservoir of *C. difficile*.

CONCLUSIONS

Over a 3-year period, 45% of *C. difficile* cases in Oxfordshire were genetically distinct from all previous cases. Genetically diverse sources, in addition to symptomatic patients, play a major part in *C. difficile* transmission. (Funded by the U.K. Clinical Research Collaboration Translational Infection Research Initiative and others.)

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MOST EPISODES OF *CLOSTRIDIUM DIFFICILE* infection are believed to result from recent acquisition within a health care setting. Prevention efforts have therefore focused on symptomatic patients, their immediate environment, and judicious use of antimicrobial drugs.[1,2] Person-to-person transmission of *C. difficile* infection and surrounding contamination have been well documented.[2-5] However, there are multiple other potential sources, including patients with asymptomatic colonization[6,7] and sources in the wider environment, such as water, farm animals or pets, and food.[8] The contribution of cases acquired from these sources to the overall burden of disease is unclear, particularly with increasing reports of community-associated *C. difficile* infection.[9]

Previous studies combining data from hospital admissions and genotyping have shown that transmission through hospital-based contact with patients with *C. difficile* infection accounts for less than 25% of new cases.[10,11] However, such studies have not definitively described the role of symptomatic patients in transmission, nor do they account for potential spread across hospital wards by the movement of patients, staff, and equipment[12] or for potential spread from community contacts. Horizontal transmission from symptomatic patients continues to be viewed as the source of most cases of infection and is the basis for recent prevention guidelines.[13]

The assessment of hospital-wide transmission with the use of multilocus sequence typing or ribotyping is hampered by the large numbers of patients who share a genotype and hospital-based contact. However, whole-genome sequencing shows that substantial genetic diversity exists, even within isolates of the same genotype.[14] To quantify the role of symptomatic patients in the transmission of *C. difficile* leading to infection and to determine how such transmission has varied over time, we examined whole-genome sequences in isolates obtained from all patients with *C. difficile* infection in a defined geographic area during a 3.6-year period.

METHODS

POPULATION

The Oxford University Hospitals, comprising four hospitals with a total of approximately 1600 beds (mostly in 4-bed bays within discrete areas of wards containing 20 to 30 beds), provide all acute care and more than 90% of hospital services in Oxfordshire, United Kingdom (approximate population, 600,000). During the study, the infection-control practices in this hospital system were in keeping with published guidelines (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[1,2] All inpatients with diarrhea (defined as ≥3 stools within a 24-hour period that took the shape of a container) underwent testing for the presence of *C. difficile*. The hospitals' central microbiology laboratory used enzyme immunoassays for toxins A and B (Meridian Bioscience) to test all samples obtained in the hospitals and the community.

From September 2007 through March 2011, all such samples with positive results on enzyme immunoassay were cultured. Subcultured single colonies from culture-positive isolates underwent multilocus sequence typing[10,15] and whole-genome sequencing. Repeat isolates of the same sequence type from the same patient were not sequenced, except for 148 randomly selected sample pairs that were used to estimate rates of within-host diversity and evolution (see the Supplementary Appendix). We sequenced repeat isolates with different sequence types from the same patient, which allowed us to account for the effect of mixed infections and reinfections on transmission.[16]

Data were available for all patients on hospital admissions, movement throughout the hospital, and home postal-code districts (28 distinct locations) and general medical practices.

SEQUENCING

DNA was extracted and sequenced with the use of Illumina technology.[17] Sequence reads were mapped to a reference genome, and base-pair calls at each position in the genome that were identified after quality filtering were used to identify single-nucleotide variants (SNVs) between pairs of sequenced isolates and in phylogenetic comparisons. (Details regarding sequencing, including error rates, are provided in the Supplementary Appendix.)

EPIDEMIOLOGIC ANALYSIS

Epidemiologic relationships between genetically related cases were classified as "ward contact" if cases occurred in two patients who had been on the same hospital ward at the same time and if this period was consistent with between-patient transmission. For such transmission to occur, it was assumed that cases were infectious from 1 week before diagnosis through 8 weeks after diagnosis,[10,18,19] with an incubation period (the

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time from the acquisition of *C. difficile* to the diagnosis of infection) of 0 to 12 weeks.[1,10] Patients from whom multiple samples had been obtained were considered to be infectious for 8 weeks after their last positive test. If no ward contact existed, patients could be linked by time (as above) within the same hospital (hospital-wide contact) or by exposure to the same ward but with an interval of up to 28 days separating the discharge (or end of infectivity) of the first patient and the admission of the second patient (classified as ward contamination). Patients whose cases were classified as community contact received care from the same general medical practice or lived in the same postal-code district.

A sensitivity analysis was performed in which the definition of hospital contact was not restricted to a specified period of infectivity, incubation, or ward exposure. For patients with genetically related infections who did not have hospital contact, we investigated whether we could link patients with undiagnosed infection or asymptomatic colonization using hospital-movement data from patients with negative results on enzyme immunoassay and inpatients without diarrhea (for details, see the Supplementary Appendix).

STUDY OVERSIGHT

The study was approved by the Berkshire Research Ethics Committee and National Information Governance Board, which did not require that patients provide written informed consent. All authors vouch for the completeness and accuracy of the data and analysis.

RESULTS

STUDY SAMPLES

From September 2007 through March 2011, a total of 40,924 fecal samples were submitted for *C. difficile* testing. Of these samples, 2377 tested positive for *C. difficile* on enzyme immunoassay, resulting in overall rates of 8.5 positive samples per 10,000 inpatient overnight stays and 13.0 per month for outpatients and patients in the community. Of the samples with positive results on enzyme immunoassay, 2283 (96%) were retrieved for culture. Of these samples, 1714 (75%) were culture-positive for *C. difficile*, representing 1250 infections after accounting for repeated samples with the same sequence type. Of these isolates, 1223 (98%) were successfully sequenced (Fig. S2 in the Supplementary Appendix): 862 (71%) from Oxford University Hospitals inpatients, 246 (20%) from general medical practices, 60 (5%) from Oxford University Hospitals outpatients, and 55 (4%) from other hospitals.

The median age at the time of the diagnosis of *C. difficile* infection was 78 years (interquartile range, 66 to 86). The most prevalent sequence types were sequence type 1 (ribotype 027) in 214 samples (17%), sequence type 8 (ribotypes 002 and 159) in 110 samples (9%), and sequence type 2 (ribotypes 014, 020, 076, and 220) in 103 samples (8%).

GENETIC DIVERSITY WITHIN INDIVIDUAL PATIENTS

The analysis of the evolution and genetic diversity of *C. difficile* within individual patients provides a framework for interpreting differences in SNVs among patients. We evaluated the first and last samples obtained from 145 patients at a median interval of 51 days (interquartile range, 28 to 105; simple range, 0 to 561), using a model based on coalescent theory (in which all alleles of a gene that is shared by all members of a population are traced to a single ancestral copy) (for details, see the Supplementary Appendix).[20] On the basis of this evaluation, the estimated evolutionary rate was 0.74 SNVs (95% confidence interval [CI], 0.22 to 1.40) per successfully sequenced genome per year, and the mean within-host diversity was 0.30 SNVs (95% CI, 0.13 to 0.42). Using model-based 95% prediction intervals, we determined that 0 to 2 SNVs would be expected between isolates that were obtained less than 124 days apart, and 0 to 3 SNVs would be expected between isolates that were obtained 124 to 364 days apart (Fig. S3 in the Supplementary Appendix).

GENETIC DIVERSITY AMONG PATIENTS

Of 957 sequenced isolates from patients with *C. difficile* infection obtained from April 2008 through March 2011, a total of 333 (35%) were genetically related to at least one isolate obtained from a previous case from September 2007 through March 2011. (Genetic relationship was defined as ≤2 SNVs between the two isolates, an association that is close enough to support transmission on the basis of the stated prediction intervals.) The 266 isolates that were obtained from September 2007 to March 2008 were included only as potential sources for later *C. difficile* infections, since these patients could have acquired *C. difficile* from a case before the study started. Of the 957 sequenced

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isolates, 428 (45%) had more than 10 SNVs, as compared with all previous sequenced samples obtained since September 2007 (Fig. 1A). Results were similar in a sensitivity analysis after a basic adjustment for recombination (Table S2 and Fig. S4 in the Supplementary Appendix).

Of the 333 patients with *C. difficile* genetically related to a previous infection, 126 (38%) had ward contact with the previous genetically related case, 5 (2%) were linked only by possible ward-based contamination after the discharge or recovery of an infectious patient, 29 (9%) shared time in the same hospital but were never on the same ward, and 21 (6%) had both ward contamination and hospital-wide contact. For the remaining 152 patients (46%), no hospital-based contact could be established. Of these patients, 15 (10%) were patients at the same general medical practice, and 17 (11%) lived in the same postal-code district. Overall, 120 patients (36%) had no record of any hospital or community contact with a previous genetically related case. Even when the infectious, incubation, and ward-contamination periods were unlimited, 68 patients

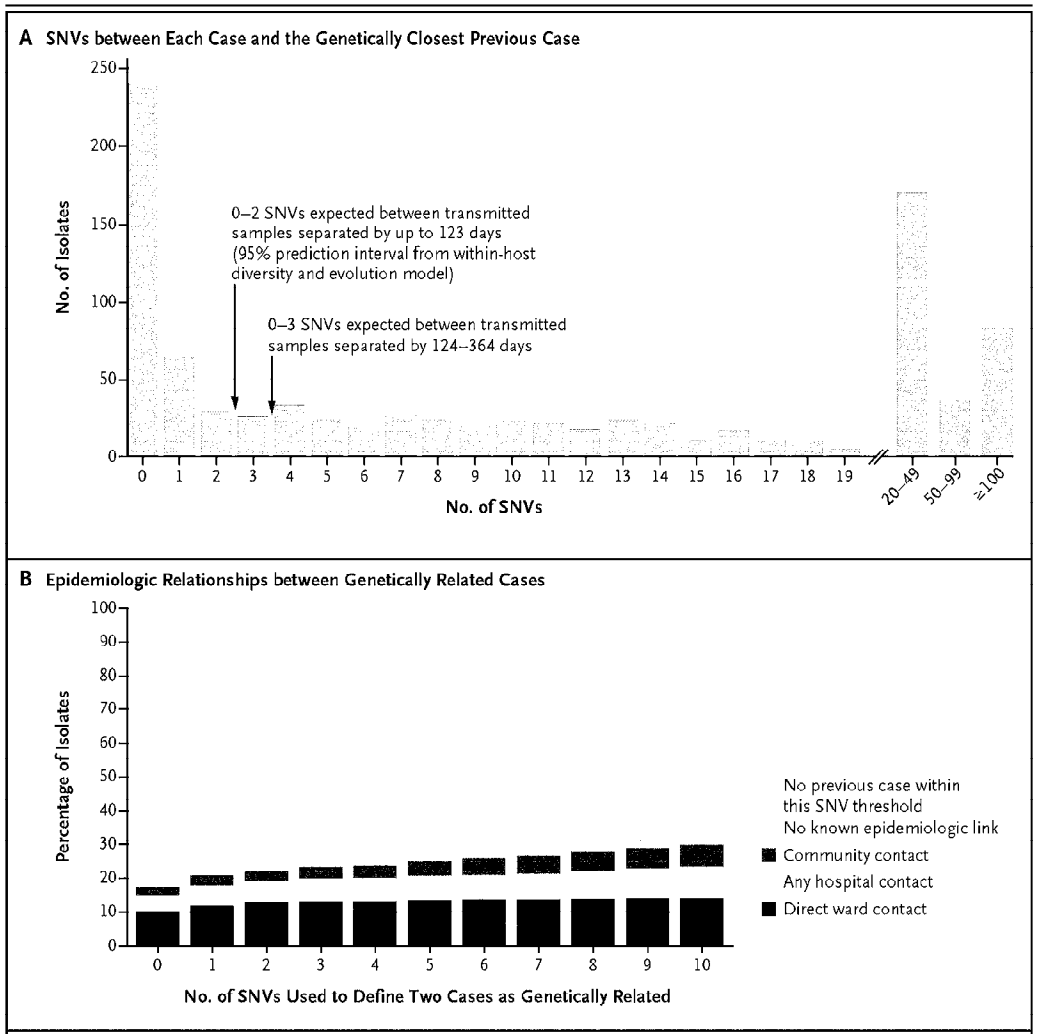

Figure 1. Genetic Variation and Epidemiologic Relationships among 957 Isolates Obtained from Patients with *Clostridium difficile* Infection.

Panel A shows the number of single-nucleotide variants (SNVs) between each sample obtained during the period from April 1, 2008, through March 31, 2011, and the most closely related previous sample obtained after September 1, 2007. Panel B shows the percentages of isolates that were classified as genetically related, according to the different SNV thresholds, along with the epidemiologic links between related isolates.

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(20%) had no hospital or community contact (Table S3 in the Supplementary Appendix). The results were consistent when other SNV thresholds for the definition of genetically related cases were used (Table 1 and Fig. 1B).

EPIDEMIOLOGICALLY UNEXPLAINED CASES

We investigated several possible explanations for the source of the 120 cases for which there was no epidemiologic explanation for the genetic relationship (Table S4 in the Supplementary Appendix). The median time since the most recent previous genetically related case was 113 days (interquartile range, 28 to 281), which suggests that point sources or laboratory contamination (of samples processed in weekly batches) were unlikely. Transmission mediated by health care workers is probably accounted for in the 17 genetically related samples (5%) obtained from patients with physicians in the same medical specialty, which were already excluded from the 120 cases. Of the 120 samples, 32 (27%) were obtained from patients with possible transmission from an intermediate patient who had tested negative for *C. difficile* on enzyme immunoassay but who had known contact with a symptomatic patient with a genetically related strain. However, 27 of 120 date-matched pairs of genetically distinct (>10 SNVs) cases (22%) also had at least one shared contact who had negative results on enzyme immunoassay, a finding that suggests that many of the intermediate contacts that were found between genetically related cases were the result of chance rather than transmission (P=0.55). Similarly, 36 patients (30%) had a hos-

Table 1. **Classification of 957 Cases of *C. difficile* Infection According to the SNV Threshold Used to Define a Genetic Relationship.***

Classification	Main Analysis	Sensitivity Analysis	
	0–2 SNVs	0 SNVs	0–10 SNVs
		number of cases (percent)	
Genetically distinct	624 (65)	717 (75)	428 (45)
Genetically linked to any previous case	333 (35)	240 (25)	529 (55)
Closest genetic link through hospital contact			
Any hospital contact within plausible limits†	181 (19)	144 (15)	224 (23)
Ward contact†	126 (13)	98 (10)	136 (14)
Ward contamination only‡	5 (1)	7 (1)	8 (1)
Shared medical specialty only†	17 (2)	15 (2)	28 (3)
Other hospital-wide contact only†	12 (1)	9 (1)	22 (2)
Ward contamination and hospital-wide contact	21 (2)	15 (2)	30 (3)
Closest genetic link through community contact, with no hospital contact			
Any community contact	32 (3)	23 (2)	63 (7)
Same general medical practice	15 (2)	10 (1)	37 (4)
Same residential postal-code district, but different general medical practice	17 (2)	13 (1)	26 (3)
Genetically related but no known hospital or community contact	120 (13)	73 (8)	242 (25)

* Shown are the proportions of cases that would be classified into various epidemiologic groups according to the SNV threshold used to define genetically related infection as compared with previous cases: the threshold used in the main analysis of the study (≤2 SNVs) or two alternative thresholds used in sensitivity analyses (0 SNVs or 0 to 10 SNVs). In the main analysis, isolates that were obtained from April 1, 2008, through March 31, 2011, were classified as genetically distinct from any other isolate obtained after September 1, 2007, if they were separated by more than 10 SNVs. A sensitivity analysis with no restrictions on the duration of infectious, incubation, and ward-contamination periods is provided in Table S3 in the Supplementary Appendix.

† This category was defined as contact in which patients shared time and space in the same setting (hospital ward, hospital, or medical specialty) that was consistent with between-patient transmission. It was assumed that cases were infectious from 1 week before diagnosis to 8 weeks after diagnosis, with an incubation period of 0 to 12 weeks.

‡ Ward contamination was defined as contamination persisting for up to 4 weeks after a patient's discharge from the ward.

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pital contact who had not been tested by means of enzyme immunoassay (i.e., a possible asymptomatic carrier) in common with a genetically related case, as compared with 35 pairs of genetically distinct cases (29%) (P=1.00). Of these 36 patients, 32 also had an enzyme immunoassay–negative contact. Analyses that allowed an unlimited duration of ward contamination (to account for the possible persistence of spores on wards) accounted for an additional 13 cases (11%). No pair of patients who had 2 SNVs or fewer attended the same outpatient clinic on the same day. Overall, none of these potential transmission routes could link 48 patients with genetically related strains who had no hospital or community contact (40%).

WHOLE-GENOME SEQUENCING VERSUS CONVENTIONAL GENOTYPING

As described above, we classified cases using genetic sequences and then determined the epidemiologic relationships. We also compared the results of previous analyses that were based on combining genotyping (by means of multilocus sequence typing) and epidemiologic data[10] with the number of SNVs between patients (Fig. 2). Among 190 pairs of patients with ward contact who had the same sequence type, 126 (66%) had no more than 2 SNVs (consistent with transmission); 54 (28%) had more than 10 SNVs between them. In comparison, of 302 patients who were linked by other forms of hospital contact (predominantly shared time), only 41 (14%) had no more than 2 SNVs, and 230 (76%) had at least 10 SNVs, which ruled out transmission that was previously thought to be plausible.

TEMPORAL PATTERNS IN DIVERSITY

Given the diversity observed within a host over time, we determined that the probability of observing more than 10 SNVs through evolution during the 3.6-year study period was less than 0.001. To assess the number of genetically distinct clusters of cases that occurred during the study, we defined distinct subtypes as separated by more than 10 SNVs from all other cases (Fig. 3). The number of cases of each subtype varied markedly. In addition to clear clustering of cases over time, we found instances in which cases from the same lineage were separated by long periods without a case. In 12 cases, genetically indistinguishable strains were recovered more than a year apart without any intervening case. Distinct subtypes were identified consistently throughout the study, suggesting that the cases arose from a considerable reservoir (Fig. S5 in the Supplementary Appendix).

To test the hypothesis that interventions that were aimed at reducing transmission might have preferentially affected genetically related cases, we compared the incidence of cases of *C. difficile* infection caused by genetically distinct isolates (those with >10 SNVs from any previous case) with the incidence of cases that were genetically related to a previous case (≤2 SNVs). Despite a marked month-to-month variation in the incidence of genetically distinct and related cases, a downward trend was observed in the two types of cases, with no evidence of a significant between-group difference in reduction (P=0.44 for heterogeneity) (Fig. 4A and 4B). Among the genetically related cases, a marked reduction was seen in cases that involved a known hospital contact, with no change in other cases over time (P<0.001 for heterogeneity) (Fig. 4C and 4D). Genetically related cases that were caused by sequence type (ST) 1 *C. difficile* known as North American Pulsed Field type 1 (NAP1) and polymerase-chain-reaction (PCR) ribotype 027 (ST1/NAP1/027) accounted for much of this decrease (Fig. S6 in the Supplementary Appendix).

Patients with isolates that were genetically related tended to be older (median age, 81 years; interquartile range, 70 to 87) than those with genetically distinct isolates (median age, 76 years; interquartile range, 64 to 85; P<0.001). On the basis of surveillance definitions,[1] 253 of 333 genetically related cases (76%) were classified as having a "health care onset" or being "health care associated," as compared with 282 of 428 genetically distinct cases (66%) (P=0.003).

DISCUSSION

In a 3.6-year study of whole-genome sequencing of isolates from more than 1200 patients with *C. difficile* infection who were living in a defined geographic area with a typical incidence of the infection[21] and standard infection-control practices,[2] we found that a substantial number of patients had acquired *C. difficile* from sources other than symptomatic patients with positive results for *C. difficile* toxin on enzyme immunoassay. Only 35% of cases were genetically related to at least one previous case (i.e., ≤2 SNVs). These data show that in a majority of cases, *C. difficile* infection is not transmitted from another symptomatic pa-

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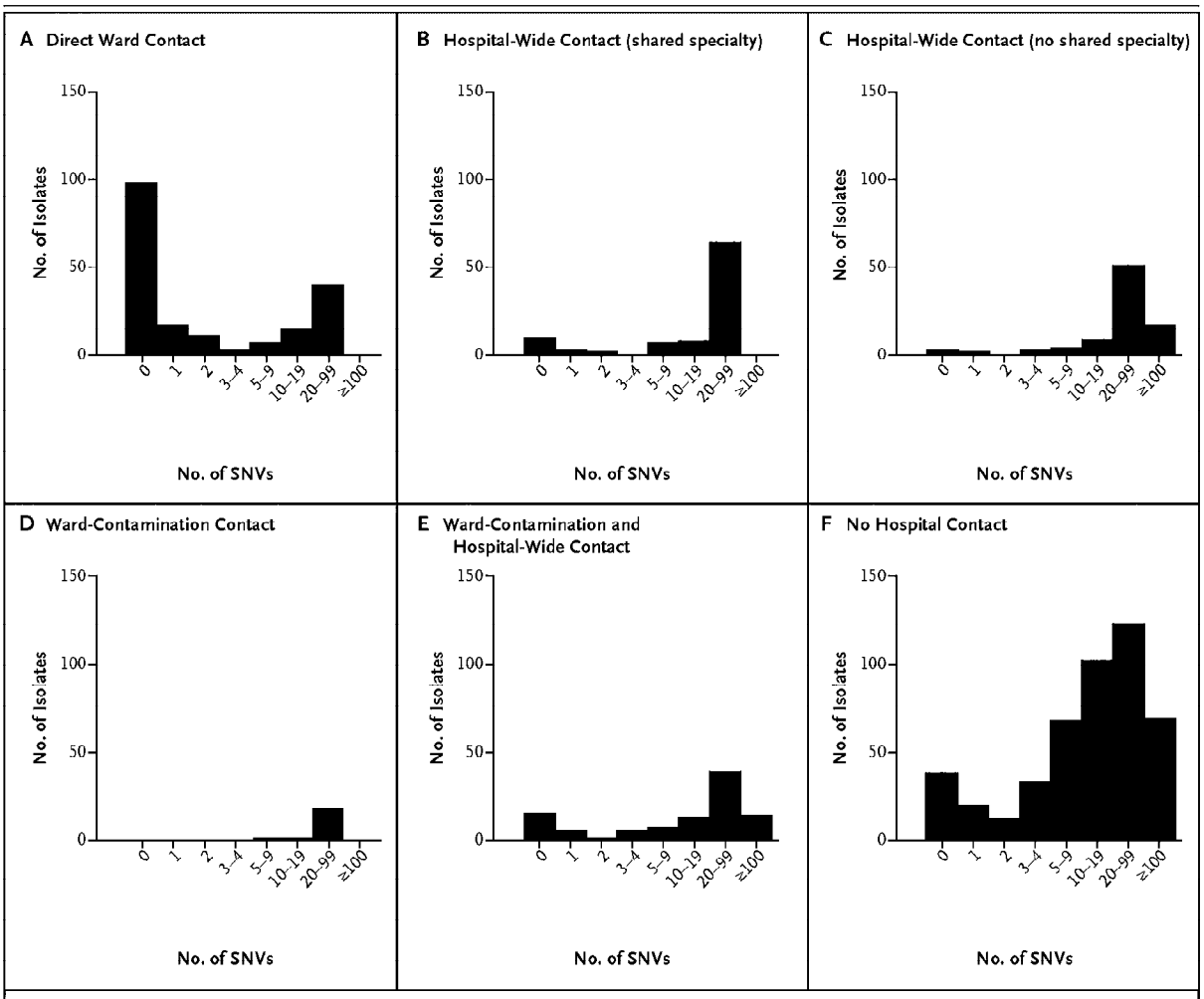

Figure 2. Number of SNVs between Cases on the Basis of Multilocus Sequence Typing and Epidemiologic Data.

Shown is the breakdown of 957 isolates obtained from patients with *C. difficile* infection from April 1, 2008, through March 31, 2011. Patients sharing a sequence type and epidemiologic contact with a case since September 1, 2007, are shown in Panels A through E: direct ward contact (Panel A), hospital-wide contact with a shared medical specialty (Panel B), hospital-wide contact with no shared medical specialty (Panel C), only ward-contamination contact (Panel D), and both ward-contamination and hospital-wide contact (Panel E). Patients with no previous case of or hospital contact with a case of the same sequence type are shown in Panel F.[10] For multiple contacts of the same type that occurred with different potential sources of infection, the lowest number of SNVs associated with that type of contact is shown.

tient. This finding is based only on whole-genome data and therefore does not depend on potentially incomplete epidemiologic data that were a key limitation of previous genotyping-based transmission studies[10,11] that could assess only ward-based transmission.[12] Applying the greater discrimination provided by whole-genome sequencing, we found that 13% of cases were genetically related and involved ward contact, and 19% of cases were genetically related and involved some sort of hospital contact. The remainder of the genetically related cases may represent exposure to at least one intermediate host or source rather than direct contact.

A total of 45% of cases had sufficient genetic diversity (>10 SNVs from any previous cases) to represent transmission originating from sources other than the symptomatic cases that were included in the study. Some patients with genetically distinct isolates may have acquired *C. difficile* infection from sources outside Oxfordshire, but given the patients' median age of 78 years, it is more likely that most organisms were acquired from asymptomatic persons[22] or some other envi-

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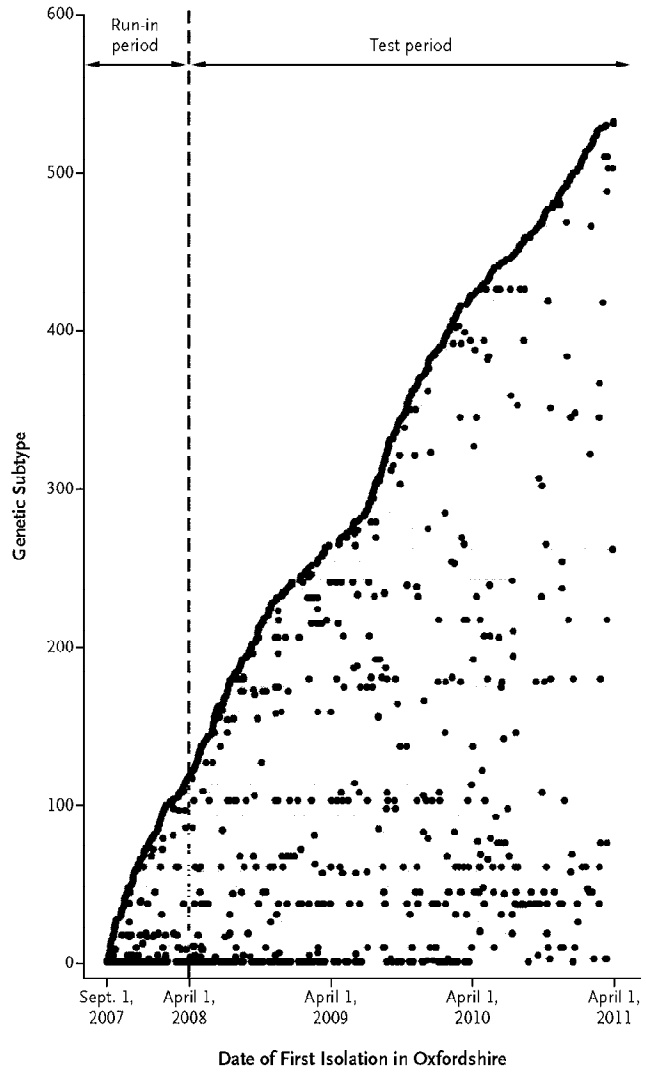

Figure 3. Timing and Size of *C. difficile* Genetic Clusters.
Isolates with more than 10 SNVs from any previous sample were defined as distinct genetic subtypes and are plotted on separate horizontal lines, according to the date of the first isolation in Oxfordshire. Isolates that were obtained from September 2007 through March 2008 (run-in period) were included only as potential sources for later *C. difficile* infections, since patients could have acquired *C. difficile* from a case before the study started. (Models estimating the total population diversity are provided in Fig. S5 in the Supplementary Appendix.)

ronmental reservoir. The ongoing identification of genetically distinct isolates throughout the study period points to diverse alternative sources. This finding might be expected, since *C. difficile* is an ancient organism[23] with diverse strains present in humans, animals, and food.[24] However, this diversity does not necessarily translate into diverse causes of *C. difficile* infection. In general, the organism is likely to have been acquired recently,[25-27] allowing only a limited window for *C. difficile* exposure, and many infections are caused by relatively few lineages, in particular ST1/NAP1/027.[28] It is therefore striking that we observed diverse subtypes in patients with *C. difficile* infection, each representing a separate transmission event from a reservoir or asymptomatic carrier. Typically, one or very few cases arose from each exposure, reflecting both limited secondary transmission and the absence of widespread transmission from a few point sources. Therefore, to prevent exposure, numerous sources need to be targeted.

Whole-genome sequencing also identified 13% of cases that were genetically related (≤2 SNVs) but without any evidence of plausible previous contact through a hospital, residential area, or family doctor. Exploring possible explanations for these cases, we found no evidence of substantial contributions from third-party transmission within hospitals by health care workers or patients. Patients with genetically related strains who did not have hospital or community contact could also have been exposed to a common source; the median of 113 days between such cases makes a short-term point source such as fresh food or water unlikely, unless it was persistently contaminated. Further, detailed studies of these patients in real time may provide insights into the location and nature of such sources.

The incidence of genetically distinct *C. difficile* cases was similar to that of genetically related cases. This finding suggests that interventions to reduce susceptibility to disease in exposed patients (e.g., changes in the use of antibiotics or specific types of antibiotics), rather than just to reduce transmission of *C. difficile* from symptomatic patients, might have played a major role in reductions in the incidence of *C. difficile* infection in the region during the past 5 years. Supporting this possibility, the use of fluoroquinolones and cephalosporins fell significantly between 2007 and 2010 in the treatment of 12,603 British patients with *C. difficile* infection whose cases were reported to the *C. difficile* Ribotyping Network.[29] According to a recent U.K. government report, the rate of use of fluoroquinolones and cephalosporins fell in 175 English hospitals between 2006 and 2009.[30] During a similar period, active restriction of the prescribing of fluoroquinolones and cephalospo-

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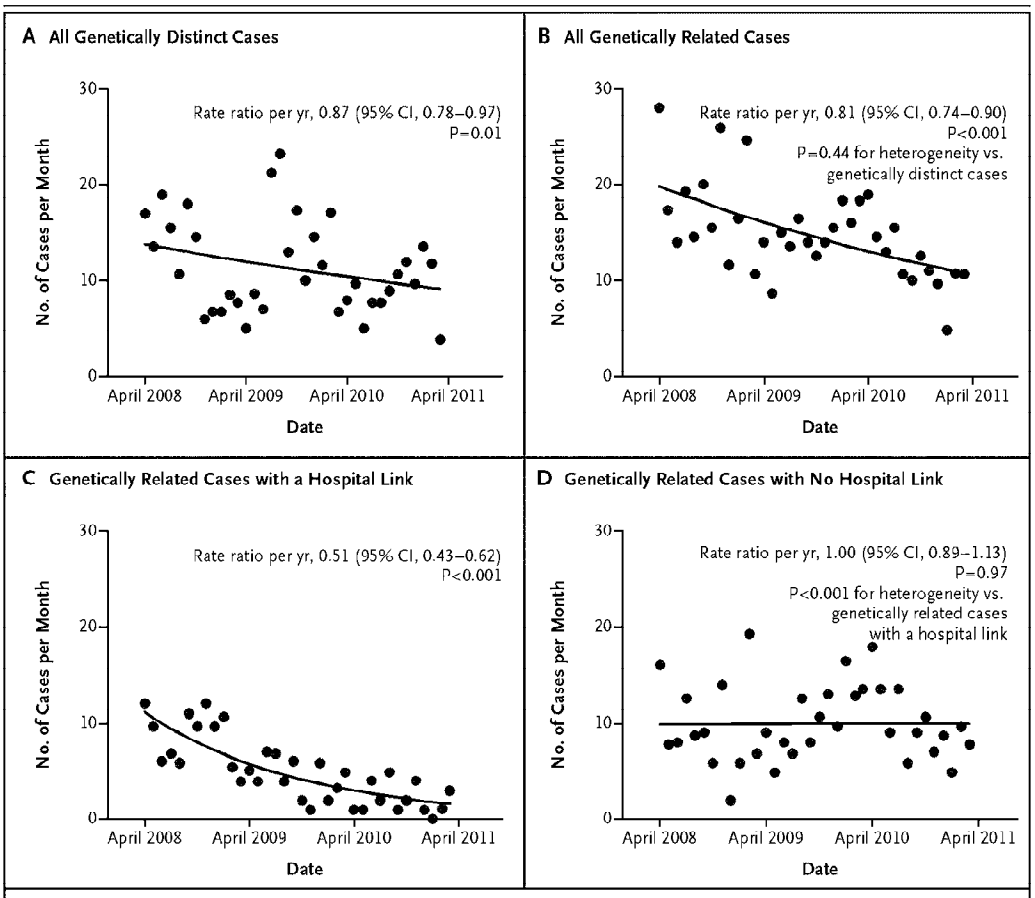

Figure 4. Trends in Genetically Related and Distinct Cases, According to Date.

Shown are cases of *C. difficile* infection that were genetically distinct (>10 SNVs) from all previous cases (on the basis of samples obtained after September 1, 2007) (Panel A), cases that were genetically related (≤2 SNVs) (Panel B), and cases that were genetically related and either had a hospital link (Panel C) or had no hospital link (Panel D). The study population was 600,000 persons, so the rate of 30 cases per month corresponds to 5 cases per 100,000 population per month. The blue lines indicate the model-based estimates of the per-year rate ratio. (Details regarding the regression models that were used in the analysis and trends in genetically related cases grouped according to the presence or absence of ribotype 027 are provided in the Supplementary Appendix.)

rins in a 450-bed Scottish hospital, without any other change in infection-control procedures, was associated with a relative reduction of 77% in the incidence of *C. difficile* infection.[31] Data from a simulation study also suggested that reducing the susceptibility of exposed patients to infection may be more effective at reducing incidence than lowering transmission rates.[32] Interventions to prevent secondary cases in our hospitals from 2007 (Table S1 in the Supplementary Appendix) may underlie the substantially larger decrease in the number of genetically related cases associated with hospital contact, particularly those caused by ST1/NAP1/027, as compared with those not associated with hospital contact. Genotyping and sequencing were performed retrospectively, so these studies did not affect clinical or infection-control practice. Large decreases in the prevalence of this epidemic strain in England have been described recently and are believed to be due to targeted infection-prevention measures.[29] Alternatively, ST1/NAP1/027 may have declined independently of infection-control efforts, as has been observed with strains of methicillin-resistant *Staphylococcus aureus* (MRSA),[33] possibly because of changes in strain fitness, host immunity, or changes in antimicrobial use combined with strain-specific antibiotic resistance.[34] The unchanged incidence of possible secondary cases with no hospital link is an ongoing challenge.

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Our study has several limitations. We ascertained initial cases on the basis of enzyme immunoassay, which has limited sensitivity. However, multiple samples were available from many patients during the study.[35] If the missing cases (a minority) were missed at random, our estimates of the diversity and trends in genetically related and distinct cases are likely to be robust. More sensitive or two-step testing may also identify *C. difficile* carriers with diarrhea, who represent another possible source of transmission.[36] The molecular clock that was used to assess potential transmissions is an approximation, particularly since mutation rates among reproducing vegetative forms may be different from such rates among spores. However, slow or arrested evolution in spores would mean that genetically distinct cases were even more distantly related. Genetically quiescent spores may also complicate attempts to reconstruct transmission events, since related cases may be separated by long intervals. However, such separation was seen infrequently, and indistinguishable strains that were separated by at least 1 year were identified in only 12 of 785 samples obtained after the first year of the study. We accounted for mixed infections that were detected by genotyping samples obtained from patients for whom multiple samples were available.[16] However, undetected mixed infections that were missed by sequencing a single colony subculture and subsequent reinfections with the same sequence type may provide an explanation for some apparently unlinked cases. The widely reported prevalence of mixed infections (<10%)[16] could not explain the diversity that was identified, and minority strains in mixed infections appear to play a limited role in transmission.[37] Samples from asymptomatic adults[25,38-40] and children[41,42] and from the environment[8,24] were not available, so we could not assess these potential sources of transmission. Quantifying their contribution to the total incidence of *C. difficile* infection remains an area for future study.

In conclusion, we found that the transmission of *C. difficile* infection from symptomatic patients accounted for slightly more than a third of such cases in a region with a typical incidence of infection,[21] which suggests that many cases arise from genetically diverse sources. The use of rapid benchtop sequencing allows for the identification of genetically related cases in nearly real time[43] so that cases that are clearly linked by hospital or community contact can be targeted to prevent further spread. Such sequencing also permits more sensitive monitoring of institutional infection-control performance through the counting of genetically related cases, rather than all cases. Data from whole-genome sequencing are also sufficiently discriminatory that genetically related cases without a clear epidemiologic link can be investigated in a highly focused way to discover novel routes of transmission. Such investigations are likely to shed new light on the source of currently unexplained infections and the diverse sources of *C. difficile* infection.

The views expressed in this article are those of the authors and not necessarily those of the National Health Service, the National Institute for Health Research (NIHR), or the Department of Health.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Exhibit 45

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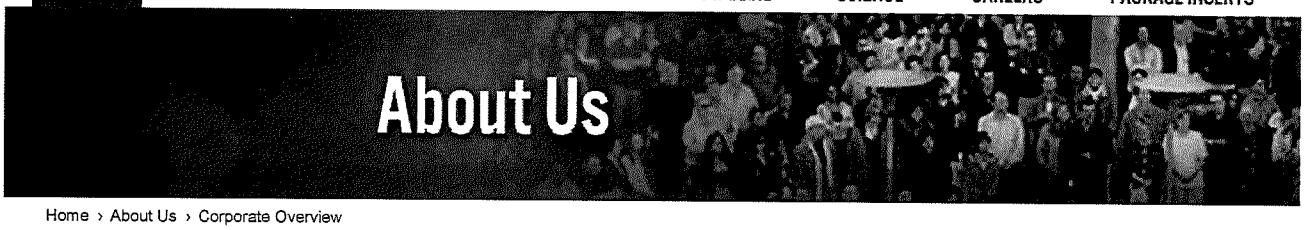

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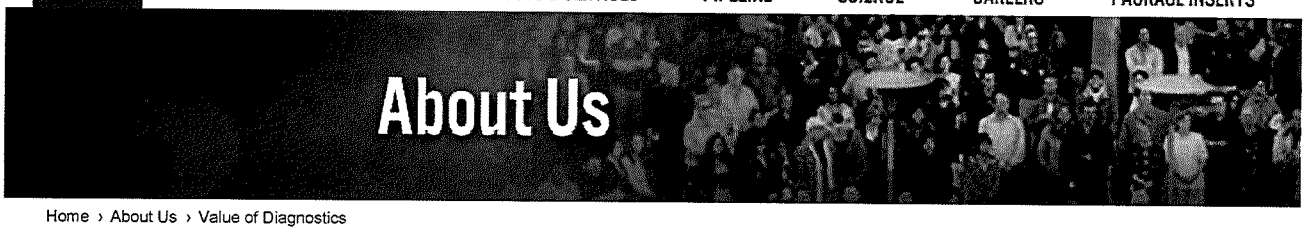

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Value of Diagnostics

Diagnostic tests are an essential part of the health care process and provide critical information to physicians and patients. Diagnostics can help assess a patient's risk for disease, assist in the diagnosis of diseases, and help physicians determine what therapies or treatments are appropriate for a given patient. Not only can diagnostic tests improve health status and patient outcomes, they can also save health care dollars. In short, diagnostics promote early detection, which leads to more appropriate treatment, which in turn generates better medical and economic outcomes. For example, although diagnostics comprise only a small portion of total hospital spending - less than five percent - they influence up to 60-70 percent of health care decision-making.

Gen-Probe develops, manufactures and markets molecular diagnostics - also known as nucleic acid tests (NATs) - that provide fast, accurate, cost-effective results based on the human genome. Gen-Probe's NATs detect the unique genetic sequences of microorganisms that can cause infectious diseases or infect donated blood, as well as the genetic mutations that can cause certain kinds of cancer. By detecting disease-causing agents directly, through their unique genetic fingerprints, molecular diagnostic tests can provide faster, more accurate results than traditional methods such as antibody and culture testing. For example, Gen-Probe's tests for HIV-1 and the hepatitis C virus have helped reduce the risk of contracting these dangerous diseases from a blood transfusion to approximately one in two million.

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We want to hear from you! Please send your questions and comments to webmaster@gen-probe.com.

GEN-PROBE INCORPORATED, 10210 GENETIC CENTER DRIVE, SAN DIEGO, CA 92121, USA

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Exhibit 47

About Us

We are a global life sciences company

We believe in the power of science and appreciate its rigorous discipline. That's what drives our passion for innovation, leading to transformative offerings that support endeavors throughout the world.

Our extensive range of products and services, from instruments to everyday lab essentials, ensures quality and performance for every lab, every application. Customers in more than 160 countries count on us in their quest to improve life in meaningful ways.

$3.7 billion (2011 revenue)
10,400+ Employees
1,500+ Scientists
160+ Countries
50,000+ Products
4,000+ Patents
600,000+ Citations

Corporate Brochure

Research Sciences
Cell Biology
Genetic Analysis
Molecular Biology

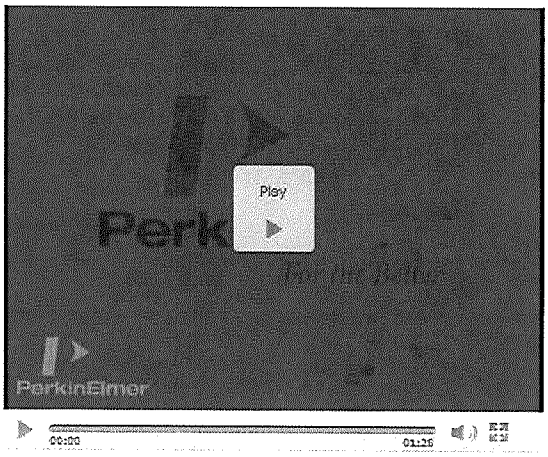
Applied Sciences
Agriculture Biology
Animal Health
Bioproduction

Medical Sciences
Genomic Medicine
Molecular Diagnostics
Regenerative Medicine

ILLUM-0669

Exhibit 48

About Us



Turning knowledge and innovation into action

As a global technology leader, PerkinElmer is taking action to harness the power of insights and transform them into knowledge to deliver innovative, differentiated solutions for our customers. From critical therapeutic and disease research and prenatal screening, to environmental testing and industrial monitoring, we are actively engaged in improving health and advancing quality and longevity of life all around the world.

From multi-vendor laboratory services, software and informatics, to clinical lab testing and unparalleled customer care, we have the people, tools and resources to help customers solve their business and scientific goals. Our core competencies, coupled with our commitment to excellence, provide a strong foundation for delivering optimal service and support. By encouraging exploration, ingenuity and integrity, together with teamwork and employee development, at PerkinElmer we live our mission, every day.

A Leader in Human Health

Whether it's testing newborns for life-threatening disorders, supporting scientists in finding better cures, or helping doctors treat disease, our focus on human health extends beyond the lab and into hospitals and homes. Our leadership position in the emerging field of personalized medicine enables us to provide solutions to meet customer needs across the value chain, from in vitro to in vivo imaging. With our innovative screenings and treatments, and development and knowledge of advanced life science tools, we help generate earlier medical insights, more accurate results, and more effective therapies.

- PerkinElmer Fact Sheet
- Corporate Capabilities Brochure
- Corporate Social Responsibility Report

A Leader in Environmental Health

Our work in environmental health improves the quality and sustainability of our environment and the security of people in the places where we live, work and play. Through a collaborative effort, we provide the insights, analytical instrumentation, and services that ensure clean air and water, and safer food and consumer products - the essential components of a safer, healthier, and better tomorrow.

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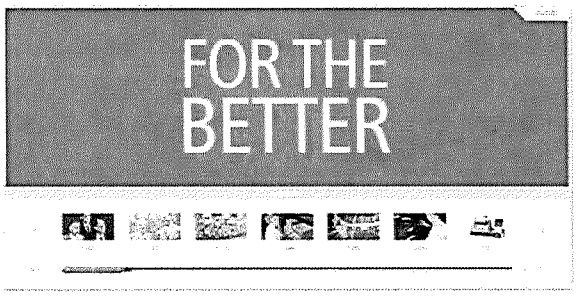


A 75 year history of innovation

April 19, 2012 marks PerkinElmer's 75th anniversary as a brand. A long-time leader in the scientific community, PerkinElmer has a rich history of innovation and making life better through major scientific industry-defining strides in human and environmental health.

VIEW TIMELINE

Key Facts

Mission:
Improving the health and safety of people and the environment

Ticker Symbol:
PKI (NYSE)

Revenue:
Approximately $1.9 billion in revenue

Chairman & CEO:
Robert F. Friel

Global Headquarters:
Waltham, MA

Operations in:
More than 150 countries in the Americas, Europe, Asia and Africa

Total Number of Employees:
Approximately 7,000

Number of Patents:
3,300

Solutions and Services for:

- Biomedical
- Chemicals
- Consumer Products - Manufacturing
- Energy
- Environmental
- Food, Beverage and Nutraceuticals
- Forensics
- Healthcare
- Life Science Research

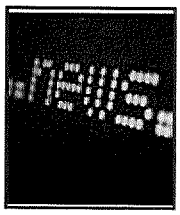
ILLUM-0671

Life Science Research

Translate your discovery into results.

With a growing emphasis on translational insight, it is more important than ever to examine the molecular mechanisms of disease and be able to translate in vitro models into in vivo results. PerkinElmer offers leading solutions and renowned expertise in assays, imaging and informatics that will help you bring it all together.

Whether working in a well, cells, or small animals, now you can focus on your science, gain insight sooner and succeed faster.



Genetic Engineering & Biotechnology News

Read how PerkinElmer further established itself as a player in the personalized medicine arena through its acquisition of Caliper Life Sciences.

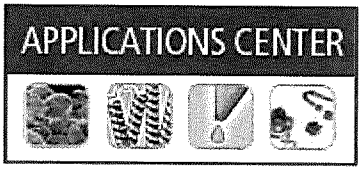
Read More

Events

Join us at PerkinElmer Owners Group

PerkinElmer Owners Group
June 7-8, 2012
Marriott Hotel
Newton, MA

Learn More



Applications Center

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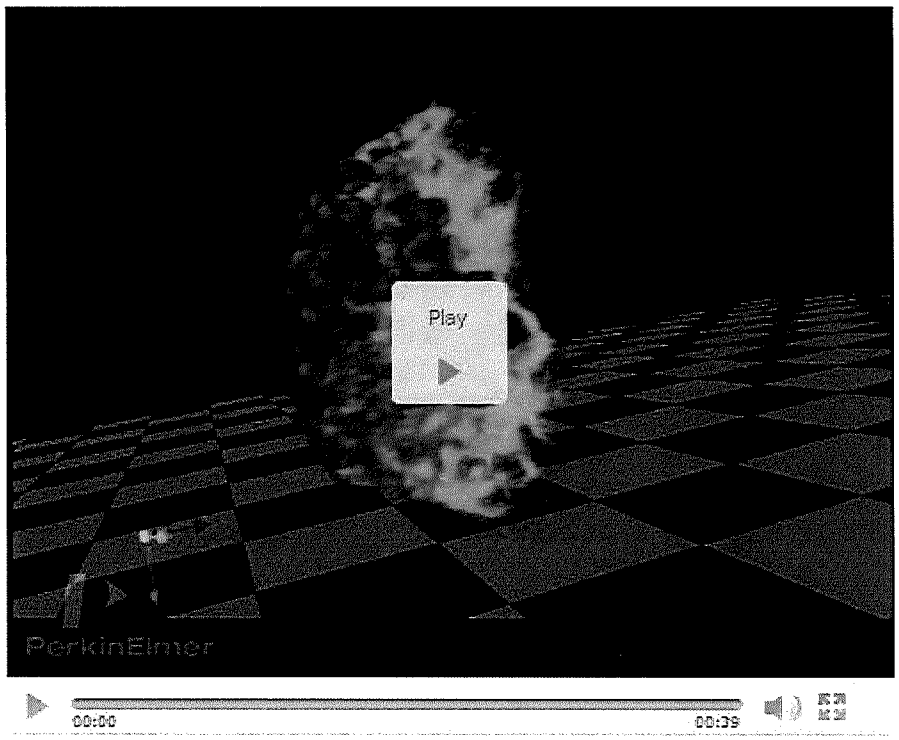
Visit the Life Sciences Applications Knowledge Base

Volocity Video

Volocity® 3D Image Analysis Software is the only solution that provides all the tools you need to visualize, analyze and validate 3D fluorescence images from a wide range of confocal microscopy, widefield and high content screening systems and is fully integrated for a seamless user experience.



Make discoveries and answer complex questions that you could not before. Visualize samples in 3D so they can be viewed from any direction for a more complete picture. Greatly improve your image and data quality with powerful restoration. Measure shapes, volumes and distances with precision. Relate cellular structure to function, and identify trends for a greater understanding.

Learn more about our Volocity 3D Image Analysis Software.

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PerkinElmer
For the Better

Labeled Nucleotides

PerkinElmer is the world's leading supplier of labeled nucleotides, offering the broadest range of standard products as well as rapid custom synthesis options. We offer high purity radioactive, fluorescent and hapten labeled nucleotides to meet any need.

Labeled nucleotides are critical elements in life science research. They provide a means of nucleic acid sequence and are also used to measure cellular activity. Typical applications include:

- Southern and northern blotting for DNA and RNA sequence detection
- DNA sequencing with labeled dideoxynucleotides (Sanger method) as well as various next-generation techniques
- Localization of nucleic acid sequences within cells and tissues using in situ hybridization (ISH or FISH)
- Gene expression profiling with microarrays
- Measurement of cell proliferation by incorporation of tritiated thymidine
- Assessment of kinase activity with [γ-^{33}P]ATP

Products

Fluorescent and Hapten Labeled Nucleotide Analogs

Labeled nucleotides are powerful tools for detection of specific nucleic acid sequences in a wide range... View more

Radioactive Nucleotides

PerkinElmer provides the world's most complete line of 33P, 32P, 35S, 3H and 14C nucleotides, stocked... View more

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Clinical Diagnostics Systems

With reliable diagnostic methods for anemia, thyroid or reproductive dysfunction and diabetes, PerkinElmer helps provide security for members of society throughout their adult lives. In some countries, tumor marker assays for cancer detection and treatment monitoring are supplied.

Products listed here may not be available in all countries. For information on availability, please contact your local representative.

View Technologies

Cancer Screening, Diagnosis & Monitoring

With its unique series of products for PSA detection, PerkinElmer has spearheaded a new approach to... View more

Growth Hormone

Hemoglobinopathy

Instruments & Accessories

Reproductive Hormones & Infertility

Thyroid Screening, Diagnosis and Monitoring

A complete panel of Wallac DELFIA® and AutoDELFIA™ kits for assessment of thyroid function analytes... View more

Type 2 Diabetes Detection and Monitoring

More than 100 million people worldwide are affected by diabetes, but many are undiagnosed. This number... View more

Virology

These products are intended for research purposes only and are not approved for diagnostic use. View more

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Exhibit 49

Solutions for
- Animal Pathogens & Genotyping
- Biomedical Research
- Companion Diagnostics
- Drug Development
- Food Safety Testing
- Human Identity and Forensics
- Molecular Diagnostics

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"Easier and faster — purify DNA in no time!"

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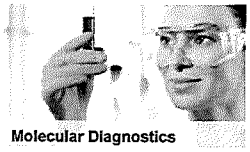
- Technical Support Center
- Exhibitions / Webinars
- Handbooks

Genes & Pathways



Molecular Diagnostics

Automated Solutions

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Ordering: 800-426-8157
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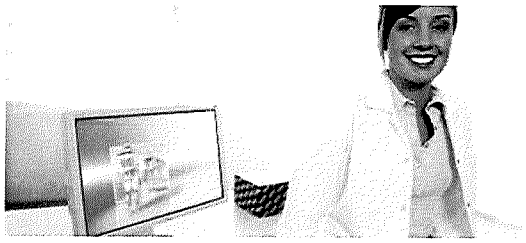

Whole Genome Amplification (WGA)

Researchers often face the problem of limited or insufficient DNA quantity for downstream analysis. QIAGEN whole genome amplification technology solves this problem by providing high yields of whole genomic DNA from small or precious samples. REPLI-g Kits and Service accurately copy the original source DNA — without bias — and the amplified DNA can be directly used in a range of genetic analyses.

This section of our Web site provides the resources you need for accurate and reliable whole genome amplification including a comprehensive WGA tutorial.

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Product Focus

Direct and reliable amplification from paraffin sections with the new REPLI-g FFPE Kit.
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Feature

Find out more about gene copy number analysis in our comprehensive WGA tutorial.
more

Resources

- WGA tutorial
- REPLI-g selection guide
- Application data
- References
- Brochure on genotyping

More Links

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- Handbooks/protocols
- FAQs
- Request more information
- QuantiTect Whole Transcriptome Kit

Contact more

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Ordering: 800-426-8157
E-mail Technical Service

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Sample & Assay Technologies

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  - Molecular Diagnostics
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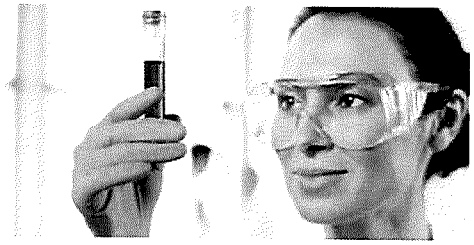
  - Virus Research
- Products by Technology
- Products by Genes



Molecular Diagnostics

At QIAGEN, one of our key goals is to support clinical decision-making and improve patient healthcare. As a leading provider for sample and assay technologies, QIAGEN provides complete solutions for sample collection, nucleic acid purification, pathogen detection, and genetic testing.

The broad range of solutions enables manual or automated sample processing in combination with [illegible]mation

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PAXgene Blood RNA Kit IVD

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Exhibit 50

Products for US Customers

Pharmaceuticals in the US

Roche has brought many highly effective drugs onto the market and is a world leader in innovative cancer drugs. Other areas include viral infections, metabolic, central nervous system disorders and inflammatory diseases.
In the US our pharmaceuticals are marketed by Genentech.

- US product list
- Or visit our local website www.gene.com

Solutions for Diagnostics in the US

As the world leader in in-vitro diagnostics, we supply a wide range of rapid, reliable instruments and tests for disease screening and diagnosis in laboratories, at the point of care, and for patient self-management.

- US product list
- Or visit our local website www.roche-diagnostics.us

Products for Researchers in the US

Roche Applied Science supplies a broad array of instruments and highly specific reagents and test kits for use in the diverse research market.

The portfolio is especially strong in genomics and proteomics.

- www.roche-applied-science.com

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Roche Applied Science

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- Oncology Research
- Cell Imaging Systems
- Transfection
- Cell Culture Reagents
- Mycoplasma Detection and Elimination
- Virus Research
- Reporter Gene Detection
- Apoptosis
- Cytotoxicity
- Cell Proliferation
- Cell Counter and Analyzer
- Real-Time Cell Analysis Systems

Sample Preparation

- Automated Sample Preparation
- Manual Sample Preparation

PCR

- Real-Time PCR Reagents
- PCR/RT-PCR Enzymes
- Real-Time PCR Systems

Microarrays

- Gene Expression
- Instruments
- Chromatin Immunoprecipitation-on-chip (ChIP-chip)
- DNA Methylation
- Software
- Comparative Genome Sequencing (CGS)
- Kits and Consumables
- Sequence Capture
- Comparative Genomic Hybridization (CGH)

Sequencing

- Genome Sequencer FLX System
- GS Junior System
- Assays
- GS FLX+ System

Discovery Reagents

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- Protein Analysis
- Antibodies and Detection Substrates
- Nucleotides and Analogs
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- Laboratory Reagents
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Get answers fast with our online technical support.

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Get in contact with our Sales Representatives

- Sales Representatives

Roche Prime Supply (RPS)

- On-Site Reagent Supply Program

For Technical Questions:
Phone: 800-262-4911

To order or for price information:
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Phone: 800-428-5433
FAX: 800-428-2883

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Roche Applied Science
P.O. Box 50414
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Catalog Products are: For life science research only. Not for use in diagnostic procedures unless otherwise indicated.
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Roche USA

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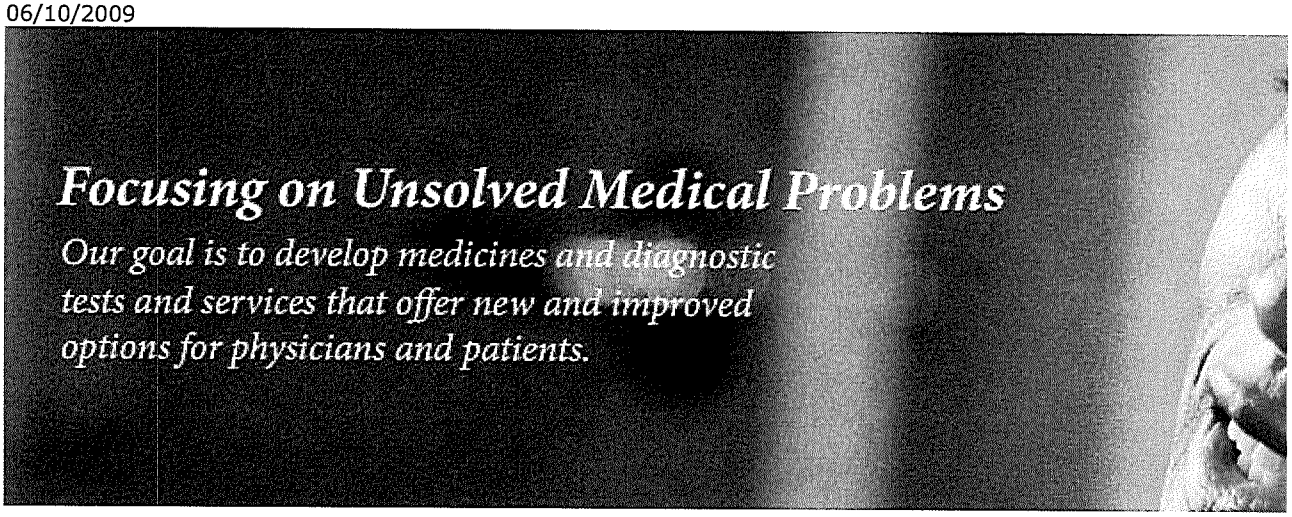

Pharmaceuticals and Diagnostics

The Roche Group, including Genentech and Chugai, has brought many highly effective drugs onto the market and is a world leader in innovative cancer drugs. Other areas include viral infections, metabolic, central nervous system disorders and inflammatory diseases.
More

As the world leader in in-vitro diagnostics, Roche supplies a wide range of rapid, reliable instruments and tests for disease screening and diagnosis in labratories, at the point of care, and for patient self-management.
More

Personalized Healthcare

At Roche, our primary objective is to provide healthcare solutions that deliver superior options for diagnosis and treatment to clinicians and patients. As one of the world's leading healthcare companies with combined strengths in **pharmaceuticals and diagnostics**, no company is better suited for this task.
Roche's personalized healthcare approach uses new molecular insights and molecular diagnostic tests to better tailor medicines and better manage diseases.
More on www.roche.com

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Products > **Solutions for Diagnostics available in the US**

Solutions for Diagnostics available in the US

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Accu-Chek Aviva System
Accu-Chek Compact Plus System
Accu-Chek Lancing Devices for Professionals
Accu-Chek Multiclix Lancet Device
Accu-Chek Smart Pix Device Reader
Accu-Chek Softclix Lancet Device
Accu-Chek Spirit Insulin Pump
Accutrend® Plus System
AmpliChip® CYP450 Test
BenchMark XT
CoaguChek® XS Plus system
CoaguChek® XS system
cobas b 221 Blood Gas system
cobas c 111 analyser
COBAS INTEGRA® Systems
cobas IT solutions
cobas p 512 and 612 pre-analytical systems
cobas s 201 system
cobas® 4000 analyser series
cobas® 6000 analyser series
cobas® 8000 modular analyser series
COBAS® AMPLICOR CT/NG Test
COBAS® AmpliPrep/COBAS® TaqMan® HCV Test
COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0
CONFIRM anti-ER(SP1)
CONFIRM anti-Ki-67 (30-9)
CONFIRM anti-PR(1E2)
DISCOVERY XT
E 170 module for MODULAR ANALYTICS EVO analyser
Elecsys® proBNP
Elecsys® Systems
LightCycler® Carousel-based System
LightCycler® MRSA Advanced Test
MODULAR ANALYTICS EVO solution
MODULAR PRE-ANALYTICS EVO
NexES Special Stains
PATHWAY HER-2/neu
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SYMPHONY
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VIAS

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Exhibit 51

Agilent Technologies

Contact Us | Select Country | Enter search term | Life Sciences & Chemical Analysis | Search

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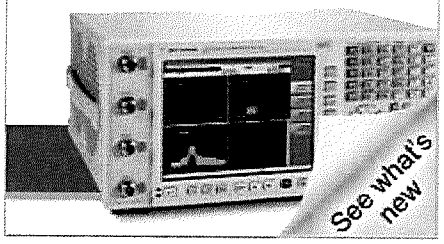
Agilent automation solutions for next-gen sequencing labs

Transform manufacturing test & accelerate wireless devices to market



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- Forensics & Drug Testing
- Genomics
- Geochemistry, Metals & Mining
- Homeland Security

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- Optical & Photonic

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- FAQs
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- Find Support Services
- More...

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- Discussion Forums
- Service Notes
- Parts

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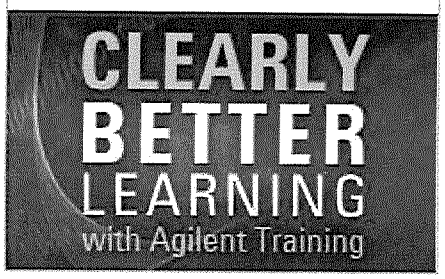

Calibration Services & Instrument Repair

Accelerate next-generation wireless with Power of X app briefs

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- Certificates of Analysis
- More...

Electronic Test & Measurement

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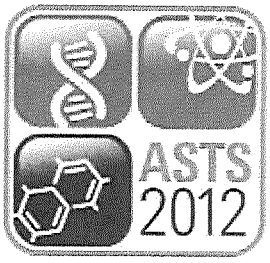
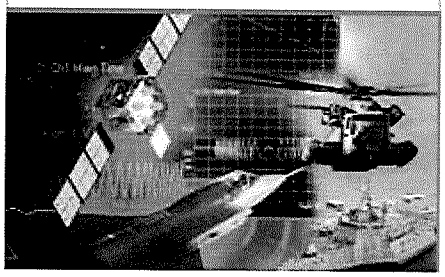
- Agilent Science and Technology Symposium 2012 - Your Access to Innovation



- Characterize emitters using long-duration frequency studies -- webcast



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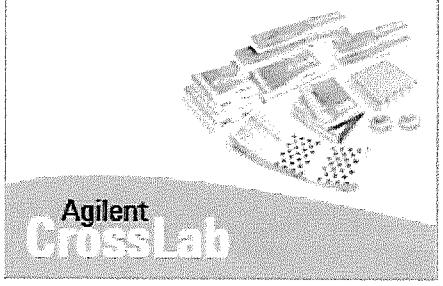
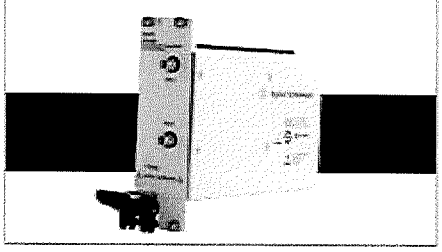


- Enhance measurement precision with PXI step-attenuator module



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Solutions

Agilent Technologies offers you a comprehensive portfolio of innovative solutions – from improving lab productivity for chemical analysis, to vacuum systems, to workflow solutions developed to facilitate unlocking the complexities of living systems. Our experts possess in-depth understanding of issues across a broad spectrum of applications. Agilent's latest technologies are developed for your leading edge research programs. We help you stay current with emerging applications and approved methods, while our integrated workflow solutions deliver accurate, comprehensive data helping you make informed decisions accelerating your time to results. Learn more about the latest solutions and applications for your industry below.

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Clinical Research
Disease Research • Microimaging • Pre-clinical Research • Research Diagnostics

Forensics & Drug Testing
Forensic Toxicology • Postmortem Toxicology • Workplace Drug Testing • Criminalistics • Doping Control • DNA Testing

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Crystal Structure • Metals Analysis • Optical Components & Coatings • Polymers • Raw Materials & Finished Goods • Research & Development • Surface Modifications & Reaction

Genomics
ChIP-on-chip • Cloning • Comparative Genomic Hybridization • Copy Number Variants • DNA Methylation • DNA Sizing & Quantification • Food Testing • Gene Expression • microRNA • Mutagensis • Next-Gen Sequencing • RNA Quality Control

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Water Analysis • Soil, Sludges & Sediments • Air Analysis • Emerging Contaminants

Geochemistry, Mining & Metals
Assay • Geology • Metals Production • Plating • Surveying

Integrated Biology
Genomics • Transcriptomics • Proteomics • Metabolomics • Informatics

Energy & Fuels
Exploration & Production • Refining • Natural Gas • Biofuels & Alternative Energy • Power Generation • Petrochemicals

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Discovery Metabolomics • Targeted Metabolomics

BioPharma
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Proteomics & Protein Sciences
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Semiconductor Analysis
Components Analysis • Contamination Control • Electronics Devices • Environmental Monitoring • Process Chemical Analysis • Silicon Wafer Analysis • Ultra-pure Water Analysis

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Homeland Security
Biological Warfare Agents • Chemical Warfare Agents • Toxic Industrial Chemicals

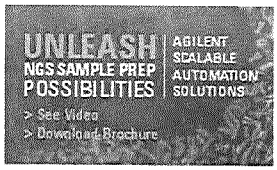
Vacuum Solutions
Industrial Vacuum Processing • Leak Detection • Mass Spectrometry & Analytical Instrumentation • Nanotechnology Instruments • Research & Development

Product Announcements

Vacuum Products Now in Agilent On-Line Store

Upcoming Events

Troubleshooting & Maintenance e-Seminar Series for AA, ICP-OES, and ICP-MS



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Bioreagents, Standards & Kits

- Cloning & Competent Cells
- Instrument Standards & Kits
- MassCode
- Microarrays
- Mutagenesis
- Northern & Southern Blots
- Nucleic Acid Sample Preparation
- PCR & Reverse Transcription

- Protein Int
- Protein Pre
- Real-Time,
- Target Enr
- Total RNA
- Toxicology
- Transfecti

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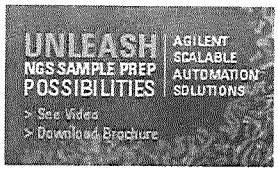
Genomics Solutions



Agilent understands the importance of being your partner in genomic research. By providing a comprehensive suite of high quality genomics arrays, reagents, instruments and software, your work is advanced with flexibility, sensitivity and specificity vital to answering biological questions. Whether you are using gene expression, cloning and quantitative PCR, comparative genomic hybridization to detect copy number variation or selecting for the human exome to sequence regions of interest, Agilent provides the innovation and reliability your research requires.

Segments

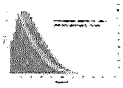
- ChIP-on-chip
- Cloning
- Comparative Genomic Hybridization (CGH)
- Copy Number Variants (CNV)
- DNA Methylation
- DNA Sizing & Quantification
- Food Testing
- Gene Expression
- microRNA
- Mutagenesis
- Next-Gen Sequencing
- RNA Quality Control

Next Generation Sequencing

Harness the Power

Next Generation Sequencing has changed what is possible in the field of genomics. The ability of the researcher to elucidate the underlying genomic mechanisms of biological systems has revolutionized our understanding of biology itself and will only continue to do so. Agilent provides a range of tools that help improve the efficiency of the next generation of sequencing workflow.

Learn More



Literature Highlights

Workflow tools for optimizing the power and efficiency of Next Generation Sequencing

Agilent's SureSelect Target Enrichment System: Bringing Cost and Process Efficiency to Next-Generation

QPCR Designed for You

More

Automation

Bioanalyzer Sample QC & Quantitation

Bioinformatics Software

Cloning & Competent Cells

Microarrays

- Amplification & Labeling
- CGH Microarrays
- CGH+SNP Microarrays
- ChIP-on-chip Microarrays
- CNV Microarrays
- Custom Microarray Design
- DNA Methylation Microarrays
- Gene Expression Microarrays
- Hybridization
- Microarray Data Analysis
- Microarray Hardware
- microRNA Microarrays
- References & Controls
- Wash Solutions

Mutagenesis

Northern & Southern Blots

Nucleic Acid Sample Preparation

PCR & Reverse Transcription

Protein Interaction & Signal Transduction

Real-Time/Quantitative PCR

Target Enrichment

Total RNA & cDNA Libraries

Toxicology

Transfection & Protein Expression

Microarrays

Powered by SurePrint technology

The Agilent DNA microarray sits at the center of an integrated research platform designed to provide you with dynamic tools to perform research on your own terms.

Focused on sensitivity

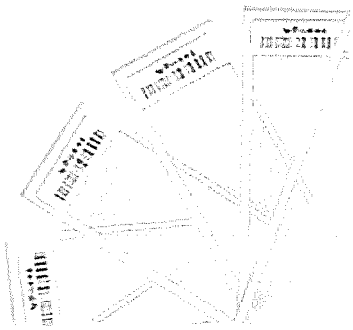
Agilent's microarrays provide superior sensitivity, enabling a clear path from data to decisions. Our 60-mer oligonucleotide probes are synthesized in situ, resulting in highly reproducible features and six times the signal-to-noise ratio of other microarrays.

Engineered for flexibility

Agilent's SurePrint inkjet technology prints oligonucleotides on the fly, enabling total control over array content with no compromise in cost, data quality or scalability. Combined with our web-based portal for custom array design, you now have the capability to move from idea to experiment quickly and easily.

Designed for integration

From sample preparation to data analysis, the Agilent DNA Microarray Platform provides the components you'll need to implement an integrated experimental workflow.



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Nucleic Acid Sample Preparation

High quality results start with the purest samples

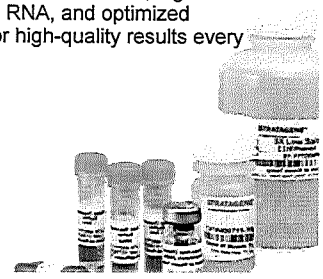
Agilent reagents enable the scientist to quickly and easily purify and analyze a wide range of nucleic acids—DNA, RNA, gDNA, mRNA, miRNA, plasmid DNA—from virtually any kind of sample for downstream analysis. No matter the application, Agilent's portfolio of reagents and analysis tools ensure the highest quality samples for high-confidence results.

A kit for every need

With products like our line of Absolutely RNA Kits, researchers can purify RNA from cells, tissues and archived samples, while our StrataPrep kits enable DNA purification from gels, PCR reaction mixtures and mammalian tissues.

Purity you can count on

Achieve the highest level of purity, free from contamination and degradation, even when working with the most challenging samples, such as FFPE tissue samples. Agilent's portfolio features non-toxic methods, high quality total RNA, and optimized protocols for high-quality results every time.



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Bioinformatics Software

Integrate data and results from multiple applications

Agilent provides a full range of bioinformatics software tools to enable robust visualization and analysis of genomic datasets across a range of applications. GeneSpring software is the industry standard for gene expression analysis, miRNA and splicing analysis, and now includes DNA-Seq and RNA-Seq support for Agilent's SureSelect target enrichment platform. Agilent's bioinformatics solutions help researchers make the most of their data, and get answers they can trust.

Multi-application analysis

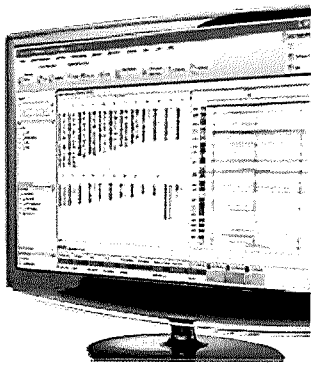
Agilent's bioinformatics tools are designed to enable joint analysis across multiple applications, within—and beyond—genomics.

Data in biological context

Agilent's bioinformatics tools enable researches to put their results into a biological context using pathway analysis, Gene Set Analysis, and GO analysis.

GeneSpring NGS - Optimized for SureSelect Target Enrichment

GeneSpring GX - The Gold Standard for Microarray and qPCR Analysis



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Agendia and Agilent Technologies Announce Plans to Jointly Develop New Diagnostic Tests, Extend Supply Agreement

AMSTERDAM, The Netherlands, and SANTA CLARA, Calif., April 22, 2008

Agendia BV, a world leader in the rapidly evolving field of molecular diagnostics, and Agilent Technologies Inc. (NYSE: A), today announced terms under which they intend to collaborate to develop new in-vitro diagnostic tests. In addition, the two companies announced an agreement in which Agendia products will continue to be supplied on Agilent microarrays through Dec. 31, 2011. Agilent has been manufacturing the components for the Agendia assay since its inception in 2003. The financial terms of the agreement were not disclosed.

"We've established and enjoyed a strong business relationship with Agilent since the incorporation of Agendia," said Dr. Bernhard Sixt, CEO and co-founder of Agendia. "These activities, including joint research and development, have allowed our two companies to forge an even closer strategic alliance focused on Agendia's core competencies, namely the discovery of biomarkers and development of new diagnostic tests for clinical use, which are regulated under the new IVDMIA guidelines of the FDA."

"We're proud that Agendia has selected Agilent to move forward with their critical work in diagnosing breast cancer," said Yvonne Linney, Ph.D., vice president and general manager, Genomics at Agilent. "We eagerly anticipate expanding our role beyond the manufacturing of Agendia products on the Agilent microarrays to helping them build validated diagnostic cancer tests, as well as developing and expanding the company's worldwide distribution channels."

Last year, Agendia's MammaPrint, a molecular diagnostic tool developed to help physicians make informed decisions in treating breast cancer, became the first IVDMIA microarray-based diagnostic test cleared by the United States Food and Drug Administration (FDA). The tests, which are manufactured on Agilent microarrays, are performed at Agendia's laboratory in The Netherlands. Agendia also offers DiscoverPrint, a gene expression-based service for improving the efficacy of clinical trials, and CupPrint, a diagnostic test designed to identify the origin of a metastasis in a cancer type called "Cancer of Unknown Primary." These are also manufactured using Agilent microarrays.

Both companies will share information about their research into genetic biomarkers and jointly assess the commercial potential of each opportunity.

About Agilent Technologies

Agilent is a leading provider of research microarrays used to analyze gene expression, microRNA, comparative genomic hybridization and ChIP-on-chip. More information about Agilent microarrays is available at www.opengenomics.com. Agilent Technologies Inc. (NYSE: A) is the world's premier measurement company and a technology leader in communications, electronics, life sciences and chemical analysis. The company's 19,000 employees serve customers in more than 110 countries. Agilent had net revenue of $5.4 billion in fiscal year 2007. Information about Agilent is available on the Web at www.agilent.com.

About Agendia

Agendia BV, located in Amsterdam, The Netherlands, is a world leader in gene expression analysis-based diagnostics. With three products on the market and an expanding market base, the company focuses on the development and commercialization of diagnostic tests using tumor gene expression profiling. Agendia was the first company to receive FDA clearance for its breast cancer test, MammaPrint, which predicts the risk of breast cancer recurrence. Its second microarray product, CupPrint, is a diagnostic test designed to

identify the origin of the primary tumor in "Cancer of Unknown Primary." Agendia also recently presented data to be developed into its new clinical colon cancer prognosis test, ColoPrint, which is currently undergoing further validation. Agendia maintains close ties with leading academic centers around the world to develop state of the art diagnostic tests for cancer. The company also offers its expertise and its DiscoverPrint service to pharmaceutical and biotech companies focusing on development of highly effective precision therapeutics in oncology. For more information on Agendia, visit www.agendia.com.

# # #

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Agilent Technologies and Integrated Diagnostics Enter Strategic Partnership to Develop Protein-Based Assays for Early Disease Detection

10 January 2012

SANTA CLARA, Calif., and SEATTLE, Wash., – Agilent Technologies Inc. (NYSE: A) and Integrated Diagnostics today announced a strategic partnership to develop assays that detect major human diseases at very early stages to improve treatment outcomes.

Integrated Diagnostics, an emerging leader in molecular diagnostics, is creating large-scale, blood-based molecular diagnostics that leverage advances in proteomics and genomics to detect diseases such as lung cancer and Alzheimer's at their earliest stages. Agilent manufactures a wide range of analytical instrumentation, including liquid chromatography, mass spectrometry and automation systems. Officials of both organizations see an opportunity to rapidly turn medical breakthroughs into diagnostic assays by combining efforts to focus on protein and peptide biomarkers of disease.

"Agilent provides an innovative and forward-looking platform and expertise to enable the development and deployment of next-generation multiplexed proteomic assays," said Paul Kearney, Ph.D., president and chief scientific officer, Integrated Diagnostics. "Integrated Diagnostics will bring expertise in assay development, technology strategy, study design, clinical development, commercialization and systems informatics to leverage the Agilent technology solution. Together, we intend to realize the potential of clinical proteomics today as well as establish the technological platform and strategy for the field. Our partnership with Agilent will allow us to realize the advantages of using a single technology platform from discovery and verification through to commercialization."

"Agilent and Integrated Diagnostics share the vision of harnessing the power of an integrative systems biology approach to solving some of mankind's most vexing health challenges," said Gustavo Salem, vice president and general manager of the Biological Systems Division at Agilent. "It's very exciting to be at the forefront of helping technology migrate from the research lab to the clinic, in this case developing next-generation workflows to improve protein-based LC/MS assays."

As part of the partnership, Integrated Diagnostics purchased two Agilent 1290 Infinity UHPLC systems, two 6490 iFunnel triple quadrupole mass spectrometry systems and two 1260 LCs with ultraviolet detectors for the project.

The partnership will develop high-throughput automated workflows to include sample preparation and LC MS/MS analysis. Participants expect accelerated biomarker discovery to be fueled by novel sample-prep technology, fast liquid chromatography and ultra-sensitive ion funnel triple quadrupole mass spectrometry.

The technology opens the possibility of screening for several hundred biomarkers in a single assay, in a very high-throughput and quantitative fashion, according to Agilent.

About Integrated Diagnostics

Integrated Diagnostics is developing large-scale, blood-based molecular diagnostics that detect important diseases like lung cancer and Alzheimer's at their earliest stages by simultaneously monitoring tens to hundreds of disease markers. The company, founded in October 2009 by systems biology pioneer Dr. Lee Hood, is creating a new generation of personalized medical solutions using sophisticated informatics for biomarker selection and a novel class of synthetically created diagnostic and therapeutic agents with antibody-like properties: protein-catalyzed capture agents. Integrated Diagnostics' mission is to build a new generation of low-cost, large-scale diagnostic assays for early detection of serious diseases. The company is conceptually based on a systems view of disease where pathophysiology arises from disease-perturbed networks of proteins, genes, and other molecules. Investors include InterWest Partners, The Wellcome Trust, and BioTechCube Luxembourg. Foundational intellectual property is exclusively licensed from the Institute of Systems Biology and Caltech. Learn more at www.integrated-diagnostics.com

About Agilent Technologies

Agilent Technologies Inc. (NYSE: A) is the world's premier measurement company and a technology leader in chemical analysis, life sciences, electronics and communications. The company's 18,700 employees serve customers in more than 100 countries. Agilent had net revenues of $6.6 billion in fiscal 2011. Information about Agilent is available at www.agilent.com.

# # #

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Filed Under: Press Releases

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eliot@eliotdobris.com
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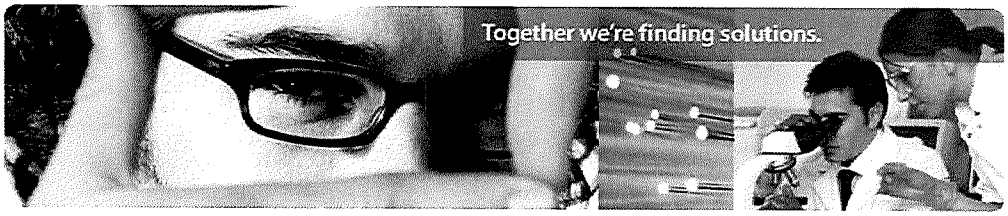
Exhibit 52



Research & Discovery - More ways to discover

Innovative solutions that streamline complex processes

Each step of the Discovery process can be extremely time consuming and costly. Accuracy, quality, efficiency and knowledge are critical to your operations success. When you partner with Beckman Coulter, our team of experts in the field of Life Science work closely with you to understand the intricacies of your everyday operation, as well as your most complex challenges, to offer innovative solutions and ensure your success—our #1 priority.

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- Capillary Electrophoresis
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- Chemiluminescence
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- Flow Cytometry
- Genetic Analysis
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- Nucleic Acid Sample Preparation
- Particle Characterization
- Research Automation
- Spectrophotometers

SPRIworks HT

Liberate your lab from the hassle of manual sample prep! SPRIworks Fragment Library Systems are the only fully automated library construction solutions with built in SPRI- based size selection and enzymatic clean-up. Experience increased productivity and efficiency while decreasing overall cost. Choose from medium to high throughput solutions that include chemistry, automation, and validated methods.

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Agencourt AMPure XP

The Agencourt AMPure XP system is a highly efficient, easily automated PCR purification system that delivers superior quality DNA with no salt carryover. Requiring no centrifugation or filtration, Agencourt AMPure XP can be easily used in manual and automated 96- or 384-well formats.

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Optima XPN

The ultra of ultracentrifuges: no other floor-model preparative ultracentrifuge offers this level of design, functionality, and performance.

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March 29, 2012
Beckman Coulter Life Sciences Acquires Blue Ocean Biomedical

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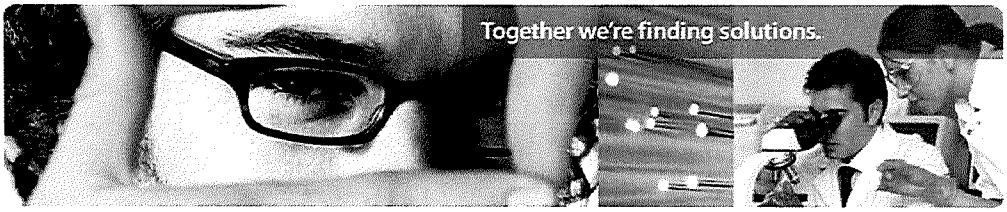

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Genomic Services

Cellular Solutions

Protein Analysis

Drug Discovery & Development

Small Molecule Analysis

Genetic Analysis

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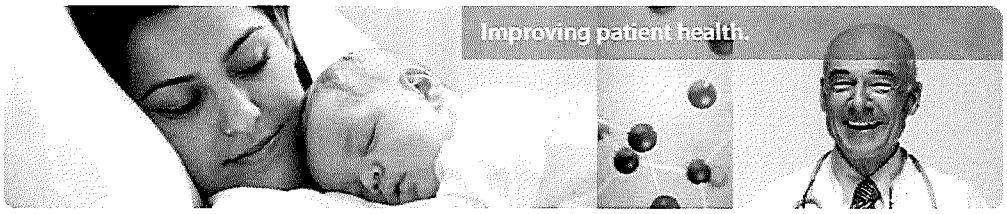


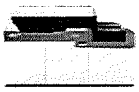
Diagnostics - More solutions to optimize the laboratory

Applications and expertise focused on the diagnostics laboratory
To successfully increase laboratory performance, it's important to choose a partner who offers the breadth of diagnostic tests, instruments and information systems needed to streamline your laboratory. Beckman Coulter brings a proven track record in best-in-class laboratory-solutions design, along with a broad spectrum of diagnostic tests. As a collaborative partner, we work with you to maximize quality, workflow and operational efficiency, meeting your specific needs every step of the way.

Categories

- Automation
- Blood Banking
- Centrifugation
- Chemistry
- Clinical Flow Cytometry
- Hematology
- Hemostasis
- Immunoassay
- Information Systems
- Integrated Systems
- Rapid Diagnostics
- Special Chemistry

Spotlight



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The AU family sets the benchmark in clinical chemistry, with unique reliable technology that provides fast and precise analysis.
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High throughput immunoassay analyzer that processes up to 400 tests per hour with continuous loading of STAT and routine samples.
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UniCel® DxH™ Slidemaker Stainer
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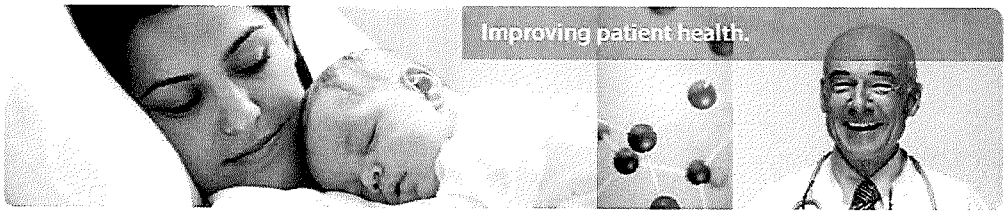

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Clinical Products **Laboratory Types** **Tests by Discipline**

Hospital Laboratory

Physician Office Laboratory

Reference/Private Laboratory

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Exhibit 53

Molecular Diagnostics

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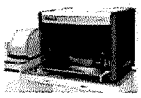
Molecular Diagnostics

Text Size

Siemens molecular testing systems enable early detection of major infectious diseases; monitoring of treatment efficacy; and selection of targeted, individualized treatment options.

Search Products by Name



Systems
Siemens molecular testing systems enable early detection of major infectious diseases.

In Vitro Diagnostics (IVD) Assays

Research Use Only (RUO) Assays

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Research Use Only (RUO) Assays

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Molecular Diagnostics - Research Use Only (RUO) Assays

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Simplify HCV genotyping (Research Use Only).

TRUGENE® HCV Genotyping Assay (RUO)
Provides accurate genotype and subtype determination using direct sequencing analysis (Research Use Only)

TRUGENE® HBV Genotyping Assay (RUO)
Identifies genotype and mutations at the same time using direct sequence analysis (Research Use Only)

VERSANT® HBV bDNA 3.0 Assay (RUO)
The 3rd generation viral load assay for the quantification of Hepatitis B DNA in the serum of HBV-infected individuals (Research Use Only)

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Molecular Diagnostics - In Vitro Diagnostics (IVD) Assays

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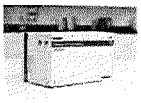
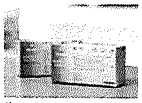
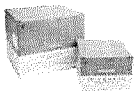


VERSANT® Tissue Preparation Reagents Kit
Use with the Tissue Preparation System for the only fully automated solution for extracting nucleic acids from FFPE samples



TRUGENE® HIV -1 Genotyping Assay
The TRUGENE® HIV-1 Assay guides physicians to the path of least resistance for patient therapy.

VERSANT® HIV-1 RNA 3.0 Assay (bDNA)
The VERSANT HIV-1 RNA 3.0 Assay (bDNA) delivers unmatched precision and reproducibility.



VERSANT® HCV RNA 3.0 Assay (bDNA)
The optimal HCV viral load assay with proven reliability

VERSANT® HCV RNA Qualitative Assay
One of the most sensitive HCV assays for qualitatively confirming the presence of active viral infection

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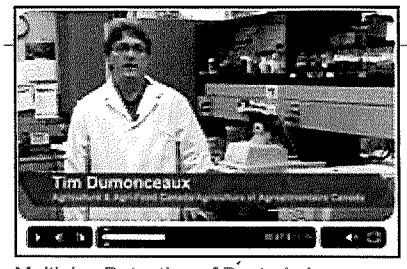
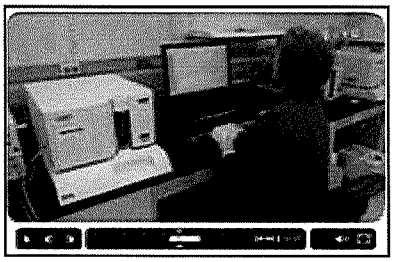


Multiplex Detection of Bacteria in Complex Clinical and Environmental Samples using Oligonucleotide-coupled Fluorescent Microspheres



Multiplexed Fluorometric ImmunoAssay Testing Methodology & Troubleshooting
Videos courtesy of Journal of Visualized Experiments (JoVE).

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- Isotyping
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- Vaccine Testing

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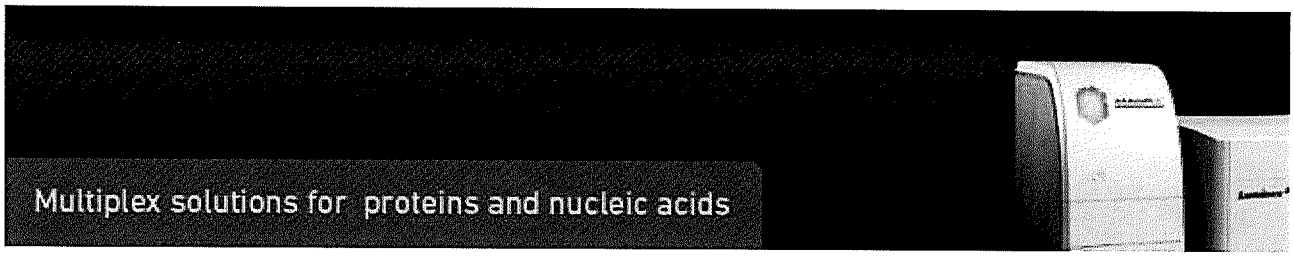

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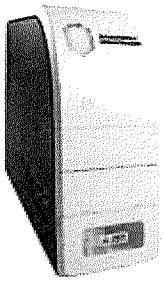
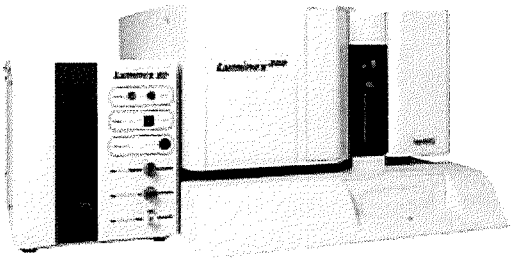
MAGPIX®



The MAGPIX system performs up to 50 tests with a single reaction volume and supports single and multiplex assay analysis.

Luminex 100/200™



The Luminex 100/200 system is designed to meet the multiplexing needs of clinical and research laboratory professionals.

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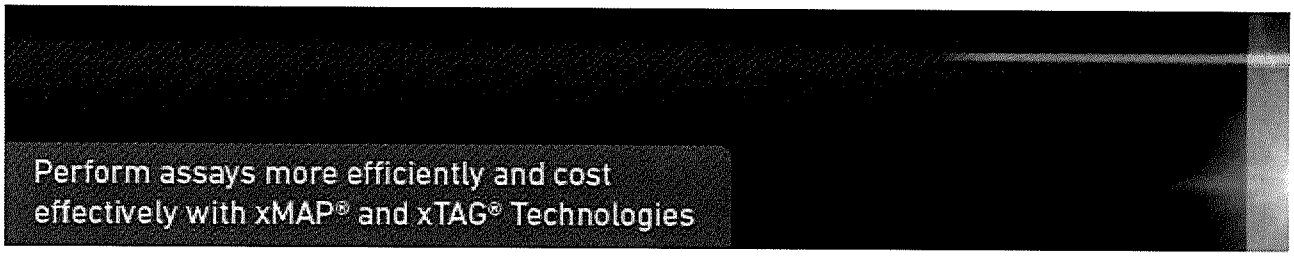

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MedCity News Q&A: Meridian Bioscience CEO Jack Kraeutler

February 1, 2010 by *MedCity News*

Meridian Bioscience CEO Jack Kraeutler on how his diagnostics company is making the big jump into molecular tests for bacteria.

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By Mary Vanac

MEDCITY NEWS

CINCINNATI, Ohio — Over the years, Meridian Bioscience Inc. has gotten good at making diagnostic test kits that detect bugs like *C. difficile* and *H. pylori*, both bacteria that can wreak havoc in the digestive tract — or worse.

The longtime Cincinnati-based life sciences company also knows a lot about testing for upper-respiratory infections, antibodies in the blood, and even for parasites and fungi, as well as supplying big laboratories with the proteins they need to do their work.

Now, the company that sells most of its tests to hospitals, reference laboratories, research centers, doctors' offices and veterinary testing centers is getting into molecular testing — tests that are so sensitive they can spot a single bacterium or virion in a tiny sample.

CEO Jack Kraeutler talked with MedCity News about the promise of this new generation of diagnostic tests — and potential acquisitions – for his company.

MedCity News: In January, Meridian Bioscience started clincial trials of its molecular test for *C. difficile*, which like other future molecular tests will will go by the brand name "Illumigene." What's the development strategy behind this new platform technology?

Jack Kraeutler: As we move into molecular, there are a couple of detection goals that we have. One is, if it's a single-point detection, let's say you're looking for a virus, that uses a simpler technology. Now, if you're looking for a virus or a bacteria that might be resistant to a particular drug, now you need to have multi-analyte capability. With the current generation of illumigene, you could do multi-analyte testing, but it would require two different tests. In the future, we'll be able to do it in the same test.

MedCity News: So what's the market opportunity for Illumigene?

JK: For us, the ability to go into molecular gives us, not necessarily a displacement technology, but a technology that augments all of the immunoassays that we sell. The first platform of *C. difficile* – and that's the one we expect to launch later this year — is capable of producing somewhere in the range of $10 to $20 million annually. The second generation of Illumigene, which will probably start to go into clinical trials later this fiscal year, should be able to do even more than that.

MedCity News: Is Illumigene part of the medical industry's move toward personalized medicine?

JK: Many of the most rapidly growing tests are the ones that can be done in 10 or 15 minutes. Most people tend to think of these tests as doctor-office tests. But when you're dealing with acute diseases, hospitals need to be able to run these tests 24-7 so the right therapies can be selected. That happens to be a type of personalized medicine in the acute-care market.

The interesting thing about Illumigene is that most molecular assays that are in the market are very cumbersome because they're based on a technology called "polymerase chain reaction." With PCR,

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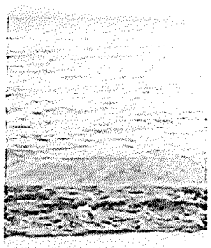

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you have to raise and lower the temperature of the sample several times to amplify it so that it can be tested. It usually requires large pieces of capital equipment to do PCR.

Our product is run at a constant temperature, and it is simple enough to be run one at a time. There's no capital equipment associated with it. So it does have a much greater reach.

MedCity News: Tell me about the amplification technology you licensed from Japan that enables you to run samples without thermo-cycling them.

JK: The technology was developed for oyster farmers who were concerned about norovirus contaminating their oyster beds. So they needed a very sensitive technology that would measure down to a single virion in a water sample. And they came up with the LAMP technology, it's loop-amplification, and we've adapted it to human samples.

MedCity News: Now, sales of your current generation of *C. difficile* test were flat in the fiscal first quarter because of increasing competition. It strikes me that Illumigene could re-energize your test line for this bacteria.

JK: That's actually been our history. We've been detecting *C. difficile* for almost 20 years now. Every 18 to 24 months, we introduce a new product. So, we've had about seven evolutions over the last 20 years. And this, of course, is a major step up. I think Illumigene is a platform that will change the perception of Meridian as being just a little kit company to being a company that is a player with advanced technologies.

MedCity News: What are the major challenges that Meridian Bioscience will face in 2010?

JK: One of the nice things about the business is because it's built around infectious diseases, it doesn't ebb and flow because of economic conditions. And in virtually every case, our products help the patient get better faster with the right therapy. So it's a stable business.

What's impacting us today is so many companies have tried to go for the high-volume diagnostics. They're beginning to open their eyes to see our space, which has largely been populated with Meridian and three or four other companies.

The problem is, the technologies these other companies want to bring into our space are extremely expensive. My worry is these companies are going to do stupid things in terms of pricing their products and other taking market share other ways. And it's going to be disruptive. But this is a fast-moving business, and we can deal with it.

MedCity News: What are the biggest opportunities your company has this year?

JK: One is to increase sales outside the United States. For the first quarter, for example, our international sales were up 17 percent, which was a good number for us. Our international sales increases had been running around 9 or 10 percent. We think we can increase international sales by fine-tuning some of our distributor partnerships. We made some changes in Canada, Australia, Argentina and Israel recently. We're looking at two other markets.

We also have new product lines, like one that tests for the bacteria that causes most stomach ulcers, *H. pylori*. And food-borne infections like *E. coli* and *Campylobacter* are emerging markets for us that are growing very fast.

There is no silver bullet. This year is about balanced opportunities and making sure that we maximize those opportunities.

MedCity News: Your company has zero debt. What gives?

JK: Any time that we've raised debt or borrowed money, we've paid it off quickly. And that makes us a shopper this year. We are looking for opportunities. But because we've got a financially efficient business, we're not going to buy something and make our shareholders wait three years for the payback. It has to be accretive in the first year.

MedCity News: Is there a certain type of company that you're looking for?

JK: We're looking at businesses that we can bolt into our life sciences business. To make it clear, our life science business is one that supplies the biggest automated diagnostic companies like Abbott, Roche, Johnson & Johnson with their essential proteins. So it's a limited customer market, it's a highly technical sale, and the more components and biologics you can offer them, the better that business will grow. So, small, private businesses ... $5, $10, $15 million in revenue are excellent bolt-ons for us.

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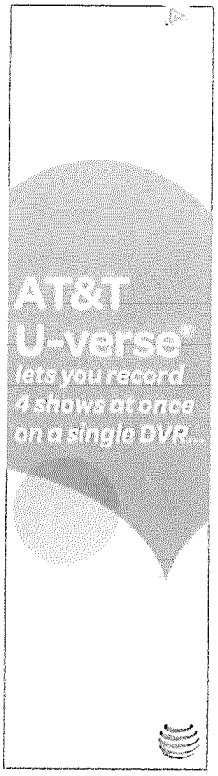

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And then there are a number of diagnostic opportunities, either carve-outs that are non-strategic for bigger businesses, or a number of smaller [firms], both public and private, that have been wandering for years and could use a good home.

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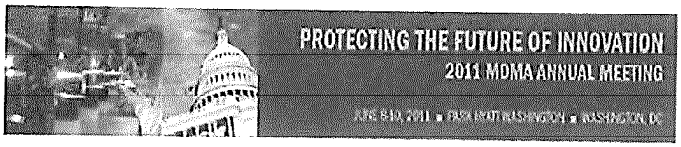

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Exhibit 56

OEM Lecture Series

In these brief presentations, OEM manufacturers present new technologies that may be of interest to industry scientists and R&D executives for inclusion in their new products. Admission is free—but participants must be registered for the Annual Meeting.

Tuesday, July 25, Room 403B, McCormick Place Convention Center

8:00am
The use of nanometer paramagnetic microspheres in immunoassay
Author information: Herb Chow
Organization: Magna BioSciences, LLC

8:20am
Powered by Affymetrix. Developed and Marketed by You
Author information: Cris McReynolds
Organization: Affymetrix, Inc.

8:40am
Mid-IR spectroscopic laser systems for clinical and laboratory applications
Author information: Chad B. Roller
Organization: Ekips Technologies, Inc.

9:00am
Digital Microbead Technology for Multiplex Assays
Author information: Mickie Henshall
Organization: Illumina

9:20am
Customized quality control materials
Author information: Canning, A.
Organization: Randox Laboratories Ltd.

9:40am
Antisera assay components
Author information: Dempsey, C.
Organization: Randox Laboratories Ltd.

10:00am
Innovative technology for rapid development of highly specific monoclonal antibodies
Author information: Joanne Crowe
Organization: Antibodies by Design – A Division of MorphoSys

10:20am
Introducing the LAMPIRE™ Cell Culture Bag, an elegantly simple approach to growing cells
Author information: John Malinowski
Organization: Lampire Biological Laboratories

10:40am
MagaZorb Ò , a "one kit" solution for nucleic acid isolation
Author information: Beverly A. Cormier
Organization: Cortex-Biochem

11:00am
Multiplexed genotyping of CYP450 2C9 and VKORC1 polymorphisms, associated with warfarin dosage using the INFINITI™ Analyzer.
Author information: Michael Phillips, Ram Vairavan

Organization: Genome Quebec / AutoGenomics

Wednesday, July 26, Room 403A, McCormick Place Convention Center

8:00am
ExpressPartsT offers injection molded prototype parts in days not weeks at production quality
Author information: Jenkins, S.M., O'Brien, D.J.
Organization: Metro Mold & Design, Inc.

8:20am
Full OEM manufacturing capability from development to production
Author information: Raymond Tarr
Organization: Idaho Technology

8:40am
Managing Projects From Afar: Building Strong Relationships with OEM Partners
Author information: Mary Wojtas
Organization: Abbott Laboratories

9:00am
Effect of Membrane Thickness on Assay Performance
Author information: Gupta, A., Gupta, N.K.
Organization: Advanced Microdevices Pvt. Ltd.

9:20am
A lateral flow assay format which discriminates between two antigenically similar analytes
Author information: O'Luanaigh, N., Carney, J.
Organization: BBInternational

9:40am
Improving Assay Sensitivity by Sample Preparation
Author information: Kevin Jones
Organization: Whatman Inc

10:00am
Rapid test devices – more portable, more convenient to use, and better results
Author information: Liang, G., Brown, J.V. Fan, J.C.
Organization: Acro Biotech, LLC

10:20am
A new automated QMS Ò Lamotrigine immunoassay
Author information: Melissa Wall
Organization: Seradyn, Inc.

10:40am
Verification of a new photometer system using ReagentStrip™
Author information: Ivars Jaunakais, Ray, Howard
Organization: Industrial Test Systems, Inc.

11:00am
High Volume Manufacturing of Organic Microfluidic Substrates for Point of Care Testing
Author information: Denny G. Aeschliman
Organization: 3M Electronics

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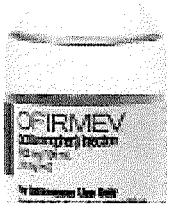

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Local companies dominate the global genetic sequencing market

Illumina beats Wall Street expectations for first

Illumina was among the country's fastest growing technology companies in 2010, according to a new ranking published by Forbes magazine on Thursday.

The San Diego genetic testing company, which ranked 4th, made the annual list for the fourth time in the last five years.

The top spot on the new list went to First Solar, a semiconductor company in Tempe, Ariz., followed by Neutral Tandem, a Chicago telecommunications firm, and Riverbend Technology, a data processing services provider in West Sacramento.

Forbes said it screened more than 5,000 companies for the list, looking for those with at least $25 million in sales and 10-percent sales growth in 2010. The final 25 were ranked on their sales growth rates over the last five years.

Illumina's growth rate over that period was 76 percent, according to Forbes.

The company had sales of $902.7 million in 2010, a 35% jump over the previous year, according to its latest quarterly financial report.

Illumina is a leading global maker of genetic sequencing machines and other genetic testing products and services. The DNA mapping market has growing rapidly in recent years as the cost of the technology plunged and more scientists and doctors used genomics for research and patient care.

Illumina's position in the latest Forbes list was one notch higher than its fifth-place ranking in 2009 but short of the No. 1 ranking it nabbed in 2008 and 2006.

Click here to see the full Forbes list for 2010.

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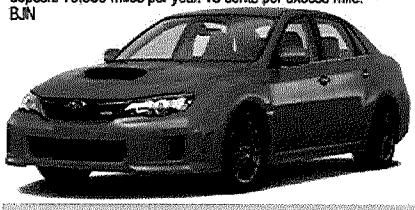

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America's 25 Fastest-Growing Tech Companies

John J. Ray, 02.16.11, 06:00 PM EST

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2010 Forbes Fast Tech List

Rank	Company Name	Business	Closing Price (2/2/2011)	Est. Long-Term EPS Growth (%)	LTM Sales ($Mil)	5-Year Sales Growth (%)
1	**First Solar**	Semiconductors/ Related Devices	$164.40	24	$2,595	182
2	**Neutral Tandem**	Telephone Communications Not Radiotelephone	15.53	12	181	100
3	**Riverbed Technology**	Data Processing And Preparation	35.65	27	552	80
4	**Illumina**	Analytical Instruments	71.31	27	822	76
5	**Cavium Networks**	Semiconductors/ Related Devices	42.29	20	207	56
6	**ViroPharma**	Pharmaceutical Preparations	16.58	10	405	55
7	**Rackspace Hosting**	Internet Software/ Services	35.68	24	735	51
8	**Salesforce.com**	Internet Software/ Services	135.08	27	1,554	50
9	**Celgene**	Pharmaceutical Preparations	53.17	25	3,620	46
10	**SolarWinds**	Prepackaged Software	18.86	18	144	43
11	**Cognizant Technology Solutions**	Computer Programming Services	76.16	20	4,185	43
12	**Equinix**	Information Retrieval Services	90.44	21	1,118	42
13	**LivePerson**	Internet Software/ Services	11.26	26	105	41
14	**Cubist Pharmaceuticals**	Pharmaceutical Preparations	22.24	11	633	41

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15	**Sourcefire**	Security Systems Services	25.46	27	128	40
16	**Apple**	Computer Hardware/ Software	344.32	21	76,283	35
17	**Google**	Internet Software/ Services	612.00	18	29,321	34
18	**Gilead Sciences**	Biological Prod's Not Diagnostic	39.46	15	7,949	31
19	**Red Hat**	Prepackaged Software	42.58	19	860	31
20	**Fortinet**	Security Systems Services	39.01	18	325	31
21	**Luminex**	Hardware, Software and Supplies for Medical Testing	17.34	28	139	30
22	**CommVault Systems**	Prepackaged Software	33.08	18	299	27
23	**Quality Systems**	Prepackaged Software	80.29	18	335	27
24	**NCI**	Computer Integrated Systems Design	21.15	15	535	24
25	**Dolby Laboratories**	Motion Picture Audio Technology	60.26	16	923	23

Prices as of 2/2/11
5-Year sales growth, annualized
Estimated Long-Term EPS Growth: Annualized, projected over next three to five years.

Page: < Previous | 1 | 2

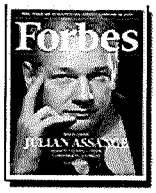

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Exhibit 58

Fast Tech 2009

The 25 Fastest-Growing Technology Companies In America

Paul M. Murdock and John J. Ray 01.29.09, 6:00 PM ET

Buried beneath the downer headlines about massive layoffs and struggling businesses, there is some good news. A select group of tech companies--the 25 Fastest-Growing Technology Stocks in America--is thriving despite the tough economy.

Thirteen companies are making their Fast Tech debuts this year, including Apple, which earned almost $5 billion on $33 billion in sales in its latest 12 months.

The fastest-growing company, based on five-year annualized sales growth, is top-ranked Illumina, which more than doubled its revenues each year for the past five years. Illumina was on our 2007 list, but missed the mark last year because it lost money in 2008. Second-ranked Google has finished first or second in its four years on the Fast Tech 25.

Last year's list of the 25 fastest-growing tech stocks weathered Wall Street's storm somewhat better than the overall market. The class of 2008 Fast Tech stocks fell 30% over the past 12 months, while the Nasdaq 100 Technology Sector Index and the S&P 500 each suffered losses of 39%. This marks the sixth consecutive year--since we first published the Fast Tech list in 2003--that each new group of Fast Tech companies outperformed the market in the subsequent 12 months.

Two of last year's entries, DRS Technologies and LifeCell, were taken over before the end of our one-year performance-measuring period. Both acquisitions came at premiums relative to their January 2008 prices.

Only one 2008 Fast Tech company, Genentech, finished the full 12-month stretch with its stock in positive territory, a 19% gain. The $11 billion (revenues) biotech returns to this year's list with eight other returnees, including Cognizant Technology Solutions, the only company to qualify every year since 2003. More than half of this year's Fast Tech companies are newcomers for 2009. Of the nine repeat performers from last year, five are software developers.

To make the list, companies must have latest 12-month revenues of $25 million or more, annualized sales gains of at least 10% over the past five years and be profitable over the past 12 months. In addition, we require a Thomson IBES long-term consensus profit forecast of at least 10%, annualized. We also exclude companies with significant legal problems or with possible accounting or corporate governance issues, based on scoring data from Audit Integrity of Los Angeles.

Last year, we introduced the Fast 15, a group of companies that may not have met all the stringent requirements of our main list, but appeared to be on track to break into the top ranks at some future date. This year, we tightened our screening criteria for this second group of stocks; these companies must now meet all the criteria of the Fast 25, except we screen them on the basis of three-year sales growth rather than five-year.

On top of our 2009 Fast 15 is OSI Pharmaceuticals,a biotech company specializing in drugs for cancer, diabetes and obesity. Over the last year, the company has registered earnings of $121 million on $365 million in net revenue. On average, OSI has more than doubled its revenues in each of the past three years.

ILLUM-0928

Exhibit 59

Seeking Alpha α

Illumina Dominating the Sequencing Market

by: Dave Westenberg

March 4, 2009 | about: ILMN

While the impact of investor fear on promising biotech developmental stage companies that are moving the entire industry toward the personalized medicine paradigm is unknown, what should be known is that one of those essential road towards that paradigm is paved with cash.

The future of personalized medicine depends on understanding human genome. Over the next decade it will be critical to sequence every piece of organic matter in order to better understand the map of life. Sequencing is profitable and one company dominates the sequencing market: Illumina (ILMN).

While biotech companies' actual sales are more resistant to macroeconomic conditions, the current investment climate calls for cash flow positive picks. The company's Q3 acquisition of Avantome put the company in the red for Q3, but without the acquisition costs, the company still made $.15 EPS. Last quarter, ILMN made $.22 EPS. The company's cash exceeds its debt, and the company can remain strong for the foreseeable future. In a challenging funding environment, the company's cash flow positive standing puts them in position to be acquirers of struggling companies and discounted IP. In the Q4 2008 conference call, management said they would be "opportunistic." With a brilliantly timed secondary offering, the company was able to raise 300 million when the stock price was in the high 80s, right before the huge October and November market slump.

ILMN has shown consistent top growth every quarter. Sequentially they have growth for the last 30 plus quarters. While the basic law of large numbers suggests that the delta will fall, 30 plus percent top line growth still justifies a large multiple. This 30 percent YOY top line growth is on the low end of estimates.

ILMN's product line continues to grow and the company has no shortage of buyers globally. Management believes the demand for sequencing and geno-typing as "insatiable" (quoted from Q3 conference call). Most users and analysts believe that ILMN's products (GA, iScan, and the Beadexpress) are best-in-class and have a strong leg up over its closes competitor Affymetrix (AFFX). In the Q4 call, CEO Flatley said that market share is "60 percent in sequencing and genotyping is 60 percent." Although growing market share in instruments maybe tough when ILMN already has much of the market, consumables will be key going forward.

ILMN's substantial instrument sales in prior quarters put them in a position to grow consumable products. While the typical model of companies is to sell the razor for nothing and charge a premium for the razor blades, ILMN has historically been able to sell both at good margins (consumable still are the higher margin product.) Going forward, they expect instrument sales to plateau and margins in instruments to decline, but consumables to raise considerably more than offsetting instruments. Throughput has also grown considerable. As the instruments continue to get faster more consumables will be used per installed base. The iScan system now produces over $700,000 consumable revenue per installed base.

With its market share and technological advantages, Illumina has the sequencing market for at least near term. Predictable consumable revenue, strong margins going forward, lack of a strong competitor means this company should be a sure bet in the short term. Longer term, Illumina looks promising too as long as it can acquire or produce a diagnostics business. With a strong cash position and progress with the Bead-Express, diagnostics maybe around the corner. In the mean time, I'll take dominating the billion dollar plus sequencing market for a good biotech pick.

Disclosure: Author holds a long position in ILMN

ILLUM-0929

Exhibit 60

Forbes.com

FastTech

Illumina: Shining In Dreary Times

Madalina Iacob 01.29.09, 6:00 PM ET

In a dreary market for life science companies marked by layoffs and drastic research cutbacks, San Diego's Illumina looks positively brilliant. The company, which makes tools that scientists use to analyze the genes of humans, animals and plants, should rack up a 55% increase in revenues in 2008. Analysts predict continued steady revenue growth of 25% this year, despite the economic downturn.

Illumina has grown from just $10 million in revenues in 2002 to an estimated $567 million in 2008. Along the way it has captured as much as 70% market share in certain fields of genetic analysis, says Tycho Peterson, an analyst at J.P. Morgan. Net income is projected to hit $79 million in 2008, up from $51 million the previous year, according to Deutsche Bank Securities.

Though Illumina's stock price, at a recent $28.45, is 40% off last year's high, it's rebounded from the $19 range it traded in December, and the company boasts a $3.5 billion market capitalization.

Illumina's strong performance helped it land the No. 1 spot on Forbes' 2009 list of the 25 fastest-growing tech companies. Illumina grabbed the top spot on Forbes' 2007 fast tech list but dropped off the list in 2008.

A group of scientists and physicians created Illumina in 1998 to pursue what was then a new technology to characterize the genome of humans, bacteria and animals. Its biggest competitor has been Affymetrix. The two companies settled a heated patent dispute early last year. In 2007, Illumina bought Solexa, a Hayward, Calif., gene analysis company, for $600 million in stock.

Illumina Chief Executive Jay Flatley knows that his company cannot relax for a moment. "The challenge in sequencing is the competition, because such a big market attracts smaller start-ups," Flatley says. "We will continue to innovate and make sure we develop technology faster than the competition."

The company has generated some dedicated customers. Edward Rubin, director at the U.S. Department of Energy Joint Genome Institute in Walnut Creek, Calif., has 12 Illumina gene-analysis machines at his lab. "What the company offers us now is the state of the art," says Rubin. The institute uses the Illumina-Solexa sequencer to examine genetic information in plants and microbes in order to further biofuels research.

"They do a phenomenal job at coming out with great products," says Ross Muken, analyst at Deutche Bank Securities.

Illumina sells its genetic analysis products to both the high-end sequencing market--large genomic centers and labs like the Trust Beijing Genome Institute, the Sanger Institute in the U.K. and the National Institutes of Health--as well as to small labs like the Bovine SNP Consortium, which uses Illumina's products to diagnose specific diseases in cows, says Muken.

"Their genotyping products are spectacular," says Dr. Stephen Scherer, director of the Centre for Applied Genomics at the Hospital for Sick Children in Toronto. The Center uses a new Illumina product, the Human 660W-Quad BeadChip, launched last November, to target regions in the human genome that may play a role in common diseases, like autism, diabetes or heart disease.

"This experiment was not possible before because of technical challenges, but with the new 660W it is. Throughout 2009 we will see a lot of data," Scherer said.

Despite its terrific growth, technical obsolescence remains a concern. "Ilumina might get leap-frogged by a novel technology," says Alastair Mackay of Garp Research and Securities. The market for genetic analysis is giant, estimated at $2.6 billion in

2008 and projected to grow to $3.6 billion by 2011. Illumina aims to lower the price of its products drastically so that one day everyone might benefit from DNA sequencing.

"One thing people don't realize is how important this technology is to the consumer market. This will become commonplace to the consumer in five, 10 years," says Illumina's Flatley. "That's what gives us confidence. It's an enormous market."

See Also:

Illumina Helps Shed Light On Cancer

Illumina's Capital Idea

Google's Genetic Start-Up

ILLUM-0931

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Exhibit 67

July 2010

C. *difficile* detection

The article "Smart choice for *C. diff* detection—PCR" (May 2010, page 1) provided an excellent review of new PCR assays available for detecting toxigenic *Clostridium difficile* in stool. The commercialization of these PCR assays has made it possible for more laboratories to do molecular testing for *C. diff.*

While the article provided a comprehensive review of PCR options available, it did not discuss other molecular methods that will soon be available for *C. difficile* testing. I recently had the opportunity to conduct a *C. diff* detection study using Illumigene *C. difficile* (Meridian Bioscience), a molecular *C. diff* test based on LAMP (Loop Mediated Isothermal Amplification) technology that is currently awaiting Food and Drug Administration approval.

In the course of our study, we compared the LAMP-based *C. difficile* assay to both culture and cytotoxin assays. We found that the LAMP-based assay performed very well, with a sensitivity and specificity extremely comparable to that of a number of other PCR-based assays we have seen. Additionally, we observed a poster from D. J. Mayne and M. Tubban of Sacred Heart Hospital in Pensacola, Fla., at the recent American Society for Microbiology meeting that compared three molecular *C. difficile* assays (one LAMP-based and two PCR-based) for performance. In this study, all three assays correlated very well. The conclusion was that all three molecular assays are acceptable alternatives to toxigenic culture for detecting toxigenic *C. difficile* directly from stool specimens.

Additionally, as we used the LAMP-based assay in our laboratory, we found that it was a very good alternative for the needs of our laboratory. The assay process was extremely simple, the hands-on time was two to three minutes per sample, the total turnaround time of the assay was less than 60 minutes on average, and with no capital equipment cost, the system is very cost-effective. Overall, this LAMP-based assay is a very accurate, easy-to-use and a viable alternative for molecular *C. difficile* testing.

I would hope that CAP TODAY, with its reputation for comprehensive coverage of all viable assay alternatives, would present this as an alternative in its discussion of molecular *C. difficile* assays. While PCR is a solid technology and is the basis for a number of high-quality assays, our facility as well as others have demonstrated that there are clearly other easy, fast and affordable molecular alternatives.

Judy A. Daly, PhD
Professor of Pathology
University of Utah
Director, Microbiology Laboratories
Primary Children's Medical Center

Exhibit 68

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C. diff molecular testing with less time and effort

CAP TODAY

November 2010
Feature Story

Michelle Merrigan

C. difficile testing is in a tough spot—caught between the convenient but relatively insensitive immunoassays and the sensitive but more expensive and labor-intensive real-time PCR assays. Now, a new assay approved by the FDA in July has the potential to bridge the gap.

The Illumigene assay from Meridian Bioscience uses a new type of gene amplification that makes it possible for any lab to perform the test, in less than one hour, without investing in RT-PCR instrumentation.

C. difficile culturing and subsequent cytotoxin testing is highly sensitive and the gold standard against which all new tests are judged. But the 24- to 48-hour turnaround time makes it antiquated in today's hospital setting. The more manageable stool cytotoxin assay, or CTA, is sensitive, but slightly less so than culture.

More than 90 percent of laboratories in the U.S. use immunoassays for *C. difficile* detection. Their low sensitivity makes the positive predictive value about 0.75, and it only worsens with repetition (Peterson LR, Robicsek A. *Ann Intern Med.* 2009;151: 176 –179). Many labs employ multistep algorithms, usually for the surface antigen glutamate dehydrogenase, or GDH, followed by further screening with CTA or culture (Fenner L, et al. *J Clin Microbiol*. 2008;46:328–330). But few like the algorithms because GDH is not a sensitive screen. Christine Biggs, SM(ASCP), microbiology supervisor at Chester County Hospital in West Chester, Pa., says she and her colleagues had consistent problems with their GDH screen before abandoning it.

"The GI physicians would call me and say, 'This patient clinically has *C. diff*. Can you run the ToxAB assay anyway?' And we would do that, and we had a number of consecutive positives on their patients." That was enough for the physicians to say, "We don't want you to use the [GDH] antigen anymore."

Further, hospital regulations often stipulate that labs can't offer only a bundled choice without offering the tests singly. Paul Lephart, PhD, associate technical director of microbiology at Detroit Medical Center University Laboratories, says: "It just makes it confusing for the physicians. It would be better for the physicians to have one choice rather than offering a good choice and a not so good choice and hoping they know the difference."

Given these limitations, the industry has been moving toward single molecular tests for all samples that detect the presence of *C. difficile* DNA in stool (see CAP TODAY, May 2010). "Nationwide, people are going to some form of molecular testing for *C. diff*," Biggs says. Real time-PCR tests for *C. difficile* are on the market now: BD's GeneOhm, Gen-Probe Prodesse's ProGastro Cd assay, and Cepheid's Xpert.

The Illumigene *C. difficile* assay employs a different type of nucleic acid amplification from standard PCR called loop-mediated isothermal DNA amplification, or LAMP. In standard PCR, primers anneal to the single strands of a melted DNA template, and polymerase replicates the strands. Thermal cycling repeatedly melts the DNA and allows for exponential amplification. LAMP is performed at a constant temperature of about 63°C and relies on the strand-displacing activity of the DNA polymerase. In PCR, one product is made. In LAMP, the action of multiple primers results in the amplification of a "cauliflower" structure, a complex of stem-loops. The amplification is rapid—it can be done in under an hour.

The Illumigene *C. difficile* assay uses a set of six primers directed to the highly conserved toxin A gene sequence in the PaLoc pathogenicity locus of *C. difficile*. *C. diff* strains that produce toxin B and not toxin A (A–B+) have been reported and caused complications previously in *C. difficile* testing. However, A–B+ strains still carry a target sequence for the Illumigene assay that can be detected in all the A–B+ strains described so far.

In standard PCR, the amplified product is detected by gel electrophoresis, or by the interaction of fluorescent probes or dyes in the case of real-time PCR. In the LAMP amplification reaction, there is a buildup of magnesium pyrophosphate, which forms a white precipitate, leading to a turbid reaction solution. The accumulation of turbidity indicates a positive amplification.

Sample preparation takes 15 to 20 minutes and does not require a centrifugation step. The stool sample is collected on a brush, which is placed in a filtering apparatus with diluent and then vortexed. The sample is filtered through the apparatus cap by squeezing the tube, and the filtrate is transferred to a new extraction tube with reaction

reagents. After heating at 95°C for 10 minutes, the sample is ready for application to two conjoined reaction tubes, one containing *C. difficile*-specific primers (the test chamber), the other containing *S. aureus* primers (the control chamber). Says Biggs: "I liked the internal control with each patient. I think that was a smart design."

In the Illumigene assay, the amplification and detection are performed in one instrument, the Illumipro-10 incubator/reader. The machine allows up to 10 samples per run. Time to completion is about 40 minutes. Each kit contains 50 tests and comes with positive and negative controls.

Kirk Doing, PhD, director of clinical and molecular microbiology at Eastern Maine Healthcare Systems, says his laboratory did a 450-sample head-to-head comparison of Illumigene and the Prodesse ProGastro Cd assay, which it has used for more than a year. It found 98 percent agreement using toxigenic culture as the gold standard.

At Chester County Hospital, Biggs' lab did a validation test with 68 specimens, comparing Illumigene with the lab's algorithm of a GDH screen followed by the ToxAB immunoassay. The result, Biggs says: "A false-negative rate of approximately 10 percent in our current *C. difficile* testing algorithm."

The Illumigene trial in Dr. Lephart's laboratory found the same: The immunoassay was missing true positives in about 10 percent of the total samples.

With Illumigene, Dr. Doing says, the laboratory has to run an external extraction control with every run, so Illumigene cannot be used to test samples as they arrive in the lab. "It's still a batch test. Only Cepheid offers true random testing capability," he notes, adding that the need for the extraction control "adds significantly to the cost per reportable."

One of the main advantages of Illumigene is not having to spare time and personnel from the molecular lab to do *C. difficile* testing. "It's definitely advantageous to keep it in the main lab," Biggs says. "My molecular room is in a different building. I don't always have someone to pull out to do that testing," particularly on second or third shift. Dr. Lephart, who says the molecular technologists at Detroit Medical Center University Laboratories "are busy doing HIV tests," says *C. diff* testing with Illumigene is done in the virology lab, "because traditionally that's where cytotoxicity testing was done." There is also the time savings that comes with a more efficient workflow. Detroit Medical was using the ToxAB immunoassay with molecular confirmation, if necessary, and planning at CAP TODAY press time to transition all such testing to Illumigene.

Now that there are new options, hospitals may face a culture change over when *C. difficile* tests are ordered and how physicians respond. One disadvantage to low-sensitivity tests is that when doctors doubt results, they treat empirically. Alternatively, they order multiple tests, a practice many labs have rules against. To avoid the problem of physicians ordering repeats of tests for which the results are negative, Dr. Lephart says, "We are going to battle that with lots of physician education." Dr. Doing says the laboratory at Eastern Maine accepts only one sample in a seven-day period: "We showed that with PCR testing, testing multiple samples is of no value to the patient."

Molecular tests also face a change in how they are interpreted. A positive molecular test indicates only the presence of the organism, not of disease. "It's a paradigm shift," says Dr. Lephart. He and Dr. Doing say this will be dealt with on the leading end by implementing careful pre-test criteria.

Despite having difficulties with CPT codes and getting some payers to cover the Illumigene assay, Biggs says the Chester County Hospital laboratory will probably bring the test in, saying "It's the right thing to do."

"The pathologists are in favor of this method, and the GI doctors are salivating over this—to finally get good information."

Michelle Merrigan is a writer in Chicago.

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Exhibit 69

JUL 0 9 2010

510(k) number: **K100818** **Date of preparation:** June 21, 2010

Submitter: **Meridian Bioscience, Inc.**
Submitter's address: 3471 River Hills Drive
Cincinnati, Ohio 45244
Contact: Michelle Smith
Contact number: (513) 271-3700

Device name: ***illumigene*™ *C. difficile***
***illumipro-10*™ Automated Isothermal Amplification and Detection System**
***illumigene*™ *C. difficile* External Control Kit**

Common name: *C. difficile* DNA Amplification Assay
Classification name: *C. difficile* Nucleic Acids
OMN, CFR Section 866.2660

Predicate device: K091109: Cepheid® Xpert® *C. difficile*
Model GXVDIFFICILE-10,900-0423, 900-0065, 900-0144, 900-0145, 900-0146, 900-0381, 900-0391, 900-0392, 900-393, 950-0151

Reference comparator: Cytotoxic bacterial culture

Description of the device:

The ***illumigene*** Molecular Diagnostic Test System is comprised of the ***illumigene C. difficile*** DNA Amplification Test Kit, the ***illumigene C. difficile*** External Control Kit and the *illumipro-10* Automated Isothermal Amplification and Detection System. The ***illumigene C. difficile*** DNA amplification assay utilizes loop-mediated isothermal amplification (LAMP) technology to detect the presence of toxigenic *C. difficile* in patients suspected of having *C. difficile* associated disease (CDAD). Each ***illumigene C. difficile*** assay is completed using an ***illumigene*** Sample Preparation Apparatus, ***illumigene*** Reaction Buffer, ***illumigene C. difficile*** Test Device, Sample Collection Brush, and ***illumigene*** Extraction Tube. Samples are prepared using the Sample Collection Brush and the ***illumigene*** Sample Collection Apparatus, target DNA is heat extracted in the Extraction Tube and DNA amplification occurs in the ***illumigene C. difficile*** Test Device.

The ***illumipro-10*** heats each ***illumigene C. difficile*** Test Device containing prepared samples, facilitating amplification of target DNA. When toxigenic *C. difficile* is present in the patient sample, a cytotoxin specific sequence is amplified and Magnesium pyrophosphate is formed. Magnesium pyrophosphate forms a precipitate in the reaction mixture. The ***illumipro-10*** detects the change in light transmission through the reaction mixture created by the precipitating Magnesium pyrophosphate. Sample results are reported as Positive or Negative based on the detected change in transmission.

The ***illumigene C. difficile*** External Control Kit consists of a Positive Control Reagent and a Negative Control Reagent. External Control reagents are provided to aid the user in detection of reagent deterioration, adverse environmental or test conditions, or variance in operator performance that may lead to test errors. The ***illumigene C. difficile*** External Control Kit is required for routine Quality Control.

Intended Use:

The ***illumigene*** *C. difficile* DNA amplification assay, performed on the ***illumipro-10,*** is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from patients suspected of having *Clostridium difficile*-associated disease (CDAD).

The ***illumigene*** *C. difficile* assay utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile*. The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (*tcdA*) and the Toxin B gene (*tcdB*), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains[3]. The ***illumigene*** *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The *tcdA* target region was selected as an intact region remaining in all known A+B+ and A-B+ toxinotypes.

illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

Comparison to predicated device:

Characteristic	*Illumigene*™ C. *difficile*	Cepheid® Xpert® *C. difficile*
Test Format	DNA Amplification Assay	DNA Amplification Assay
Intended Use		
DNA Amplification Technology	Loop-Mediated Isothermal Amplification (LAMP)	Real-Time Polymerase Chain Reaction (PCR)
Target Sequences Detected	Partial DNA fragment on the Toxin A gene of the pathogenicity locus (PaLoc) found in all known strains for toxigenic *C. difficile*.	Toxin B sequences
Qualitative/Quantitative	Qualitative	Qualitative
Screening, Diagnostic or Identification Test	Diagnostic	Diagnostic
Specimen Types		
Unformed Human Stool	Yes	Yes
Human Stool in Cary-Blair-based Media	Yes	No
Reagents/Components	***illumigene*** Sample Preparation Apparatus ***illumigene*** Reaction Buffer ***illumigene*** *C. difficile* Assay Device ***illumigene*** Extraction Tubes Sample Collection Brushes	Xpert *C. difficile* Assay cartridges Sample Reagent Reagent 1 Reagent 2
Extraction	Manual	Self-contained and automated
Amplification	Self-contained and automated	Self-contained and automated
Detection	Self-contained and automated	Self-contained and automated
Testing Time	Approximately 60 minutes	Approximately 45 minutes

Calibration	Not required	Not required

Comparison to predicated device:

Characteristic	***Illumigene*™ C. difficile**	**Cepheid® Xpert® *C. difficile***
Controls		
Inhibition, Assay	Provided *illumigene* Sample Preparation Apparatus: *Staphylococcus aureus* *illumigene C. difficile* Assay Device: *Staphylococcus aureus* LAMP Primers	Provided Sample Processing Control (SPC): *Bacillus globigii* Probe Check Control (PCC): Fluorescence emitting probes
External	Adjunct Reagents *illumigene C. difficile* External Control Kit Catalog 279920	User Supplied KWIK-STIK™ from MicroBioLogics catalog 0329 (toxigenic C. *difficile*) as positive control KWIK-STIK™ from MicroBioLogics catalog 0331 (*C. sordelli*) as negative control
Extraction	User Supplied	User Supplied
Equipment		
Instrumentation	*illumipro-10*™ Automated Isothermal Amplification and Detection System	GeneXpert® Dx System
General Laboratory Equipment	Micropipette 50 µL, 200 µL Dry-bath with 12mm heat block, 95 C Interval Timer Vortex Mixer	Vortex Mixer
Reading Method	Visible Light Transmission	Fluorescence
Results		
***C. difficile* ToxinotypesTested**	**0 (A+/B+)** **III (A+/B+)** **V (A+/B+)** **VIII (A-/B+)** **X (A-/B+)** **XII (A+/B+)** **IX/XXIII (A+/B+)**	**0 (A+/B+)** **III (A+/B+)** **V (A+/B+)** **VIII (A-/B+)** **XII (A+/B+)**
Results Interpretation	INVALID POSITIVE NEGATIVE	Toxigenic *C. difficile* POSITIVE Toxigenic *C. difficile* NEGATIVE INVALID ERROR NO RESULT

Performance Comparison, Non-clinical Tests:

Interference Testing

Selected drugs and other non-microbial substances that might be present in stool samples from healthy persons or patients suspected of having *C. difficile* associated disease were added to a natural negative and a contrived positive sample. The natural negative and contrived positive samples were prepared from donor samples and were confirmed negative by cytotoxic bacterial culture. The contrived positive sample was prepared by spiking a confirmed negative sample with toxinogenic *C. difficile* strain VPI 10463 to 18 CFU/test, slightly above the 16 CFU assay limit of detection for this organism. Potentially interfering substances were added at final concentrations of 5% V/V or greater. Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially interfering substance. Each sample was tested in triplicate.

The following substances, at the specified saturated solvent/diluents concentrations, do not interfere with ***illumi**gene C. difficile* test results in the final concentrations listed: Barium sulfate (5 mg/mL), fecal fat (equivalent to 2.65 mg stearic plus 1.3 mg palmitic acids per mL), hemoglobin (as methemoglobin) (3.2 mg/mL), IgA (5 mg/mL), Imodium AD® (0.00667 mg/mL), Kaopectate® (0.87 mg/mL), Metronidazole (12.5 mg/mL), mucin (3.33 mg/mL) Mylanta® (4.2 mg/mL), Pepto-Bismol® (0.87 mg/mL), Prilosec® (0.5 mg/mL), Tagamet® (0.5 mg/mL), TUMS® (0.5 mg/mL), Vancomycin (12.5 mg/mL), white blood cells (5%V/V), whole blood (5% V/V).

Cross-reactivity Study

Potentially cross-reactive microorganisms that might be present in stool samples from healthy persons or patients suspected of having *C. difficile* associated disease were added to a natural negative and a contrived positive sample. The natural negative and contrived positive samples were prepared from donor samples and were confirmed negative by cytotoxic bacterial culture. The contrived positive sample was prepared by spiking a confirmed negative sample with toxinogenic *C. difficile* strain VPI 10463 to 18 CFU/test, slightly above the 16 CFU assay limit of detection for this organism. Potentially cross-reactive microorganisms were added at concentrations of 1.2×10^{8}/mL (bacteria and fungi) or $1 \times 10^{5.29}$/mL $TCID_{50}$/mL (viruses). Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially cross-reactive microorganisms. Each sample was tested in triplicate.

The following microorganisms, at the indicated concentrations, do not interfere with ***illumi**gene C. difficile* test results: *Aeromonas hydrophila, Bacteroides fragilis, Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Candida albicans, Citrobacter frendii, Clostridium sordellii, Clostridium perfringens, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Escherichia coli O157:H7, Escherichia fergusonii, Escherichia hermannii, Helicobacter pylori, Klebsiella pneumoniae, Lactococcus lactis, Listeria monocytogenes, Peptostreptococcus anaerobius, Plesiomonas shigelloides, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella* Groups B-E, *Serratia liquefaciens, Serratia marcescens, Shigella boydii, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Vibrio parahaemolyticus, Yersinia enterocolitica,* Adenovirus Types 40 and 41, Coxsackievirus, Echovirus, Rotavirus.

Performance Comparison, Clinical Tests:

Clinical trials for the ***illumi**gene C. difficile* assay, including the ***illumi**pro-10* Automated Isothermal amplification and detection system, were conducted in 2010. Performance characteristics of the ***illumi**gene C. difficile* assay were determined by comparison to cytotoxic bacterial culture. Four independent clinical test sites located in the Midwestern and Southern regions of the United States and the manufacturer evaluated a total of 697 qualified patient samples. Samples were collected from 274 (39.3%) males and 419 (60.1%) females. In the case of 4 (0.6%) of the patients, sex was not known. The age groups of patients range from 2 years of age to 96 years. No differences in test performance were observed based on patient age, sex, or geographical location. Overall Sensitivity was determined to be 95.2% (95% CI: 89.2% - 97.9%). Overall Specificity was determined to be 95.3% (95% CI: 93.2% - 96.7%). Subsequent tables show overall assay performance as well as performance by clinical site and patient age.

Table 1. Overall performance data

Cytotoxic bacterial culture	*illumigene C. difficile*		
	Positive	**Negative**	**Total**
Positive	99	5**	104
Negative	27*	546	573
Total	126	551	677
			95% CI
Sensitivity	99/104	**95.2%**	89.2 - 97.9%
Specificity	546/573	**95.3%**	93.2 - 96.7%
Correlation	645/677	**95.3%**	93.4 - 96.6%

* *15/27 false positive results were positive by another FDA cleared molecular assay. Of the remaining 12 false positive results, 8 were positive by an FDA cleared assay for the detection of GDH.*

** *2/5 false negative results were negative by another FDA cleared molecular assay*

Table 2. Performance characteristics by site

Site	Positive Samples			Negative Samples		
	illumigene/ Cytotoxic bacterial culture	Sensitivity %	95% CI	*illumigene*/ Cytotoxic bacterial culture	Specificity %	95% CI
Total	**99/104**	**95.2%**	**89.2 – 97.9%**	**546/573**	**95.3%**	**93.2 – 96.7%**
Site 1	4/5	80.0%	37.6 – 96.4%	58/60	97.6%	88.6 – 99.1%
Site 2	12/12	100%	75.7 – 100%	62/67	92.5%	83.7 – 96.8%
Site 3	20/20	100%	83.9 – 100%	87/92	94.6%	87.9 – 97.7%
Site 4	8/8	100%	67.6 – 100%	36/39	92.3%	79.7 – 97.3%
Site 5	55/59	93.2%	83.8 – 97.3%	303/315	96.2%	93.5 – 97.8%

Table 3. Invalid rates by site

Site	Clinical Site Evaluation			
	Total Invalids	Assay Invalids	Instrument Invalids	Invalid Rate
Site 1	3	3	0	3/68 (4.4%)
Site 2	1	0	1	1/80 (1.3%)
Site 3	8	1	7	8/120 (6.7%)
Site 4	1	1	0	1/48 (2.1%)
Site 5	7	6	1	7/381 (1.8%)
Total	**20**	**11/697 (1.6%)*****	**9/697 (1.3%)**	**20/697 (2.9%)**

*** *1 Specimen remained invalid after repeat testing from the original sample.*

Table 4. Results by patient age

Patient age	Positive Samples			Negative Samples		
	illumigene/ Toxigenic culture	Sensitivity %	95% CI	*illumigene*/ Toxigenic culture	Specificity %	95% CI
≥ 2 - 12 years	10/11	90.9%	62.3 – 98.4%	75/79	94.9%	87.7 – 98.0%
> 12 to 21 years	5/5	100%	56.6 – 100%	53/56	94.6%	85.4 – 98.2%
> 21 years	83/87	95.4%	88.8 – 98.2%	417/437	95.4%	93.0 – 97.0%
Age Unknown	1/1	100%	20.7 – 100%	1/1	100%	20.7 – 100%

Analytical Sensitivity

The analytical sensitivity of this assay for *C. difficile* was based on 20 replicates for each measurand and with a stated probability (e.g., 95% or 19/20 positive replicates) of obtaining positive responses at the following levels of the measurands:

Strain ID	Toxinotype	Phenotype	LoD/Test
VPI 10463	0	A+/B+	4 CFU/test
2007431	III (NAP1)	A+/B+	32 CFU/test
CF1	VIII	A-/B+	64 CFU/test
2006240	V (NAP7)	A+/B+	32 CFU/test
BI8	III	A+/B+	64 CFU/test
2007858	IX/XXIII	A+/B+	32 CFU/test
8864	X	A-/B+	64 CFU/test

Additional *C. difficile* stock cultures from different sources were tested and produced positive reactions at 64 CFU/test with ***illumigene C. difficile***. Strains and toxinotypes tested were as follows: **Type 0 Strains:** 10463, 2004111, 2004205, 2005070, 2005257, 2008029, 2008162, 2008341, 2008351, 2009066, 2009099, B1, G1, J7, K12, Y1; **Type III Strains:** 2004052, 2004118, 2007431, BI17, BI8; **Type V Strains:** 2005325, 2006240, 2008188, 2009018, 2009065, BK6; **Type VIII Strains:** 43598, 2008016, CF1; **Type X Strains:** 8864; **Type XII Strains:** 2007435; **Type IX/XXIII Strains:** 2007858; **Unknown Strains:** 2009132, 2009155, 2009277.

Reproducibility

Blind coded panels of 10 samples were supplied to three independent laboratories for precision studies. Samples were randomly sorted within each panel to mask sample identities. The panels included contrived samples manufactured at the assay limit of detection (n = 3) and just below the limit of blank (i.e., high negative sample, n = 3). The panels also included uncharacterized positive (n = 2) and negative (n = 2) samples. Testing was performed by different operators at each site on the same day (intra-assay variability) for five days (inter-assay variability). Three lots of ***illumigene C difficile*** were used in this study. The results are given in the table below:

	Site 1		Site 2		Site 3		Total	
Sample Type	**Percent agreement**		**Percent agreement**		**Percent agreement**		**Percent agreement**	
Negative	20/20	100%	20/20	100%	19/19****	100%	59/59	100%
High Negative	25/30	83%	29/30	97%	28/30	93%	82/90	91%
Low Positive	30/30	100%	30/30	100%	30/30	100%	90/90	100%
Positive	20/20	100%	20/20	100%	20/20	100%	60/60	100%

***** 1 specimen generated an instrument invalid test result.*

Conclusions

The ***illumigene C. difficile*** assay used in conjunction with the ***illumipro-10*** can be used to detect toxigenic *C. difficile* in human stool samples. The test is diagnostic for toxigenic *C. difficile* infection.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
10903 New Hampshire Avenue
Document Mail Center – WO66-0609
Silver Spring, MD 20993-0002

JUL 9 2010

Meridian Bioscience, Inc.
c/o Michelle L. Smith
Senior Director, Quality Systems
3471 River Hills Dr.
Cincinnati, OH 45244

Re: K100818

Trade/Device Name:	Illumigene C. *difficile* Assay
Regulation Number:	21 CFR §866.2660
Regulation Name:	Microorganism differentiation and identification device
Regulatory Class:	Class I
Product Code:	OMN
Dated:	June 21, 2010
Received:	June 22, 2010

Dear Ms. Smith:

We have reviewed your Section 510(k) premarket notification of intent to market the device referenced above and have determined the device is substantially equivalent (for the indications for use stated in the enclosure) to legally marketed predicate devices marketed in interstate commerce prior to May 28, 1976, the enactment date of the Medical Device Amendments, or to devices that have been reclassified in accordance with the provisions of the Federal Food, Drug, and Cosmetic Act (Act) that do not require approval of a premarket approval application (PMA). You may, therefore, market the device, subject to the general controls provisions of the Act. The general controls provisions of the Act include requirements for annual registration, listing of devices, good manufacturing practice, labeling, and prohibitions against misbranding and adulteration.

If your device is classified (see above) into either class II (Special Controls) or class III (PMA), it may be subject to additional controls. Existing major regulations affecting your device can be found in the Code of Federal Regulations, Title 21, Parts 800 to 898. In addition, FDA may publish further announcements concerning your device in the Federal Register.

Please be advised that FDA's issuance of a substantial equivalence determination does not mean that FDA has made a determination that your device complies with other requirements of the Act or any Federal statutes and regulations administered by other Federal agencies. You must comply with all the Act's requirements, including, but not limited to: registration and listing (21 CFR Part 807); labeling (21 CFR Part 801); medical device reporting (reporting of medical device-related adverse events) (21 CFR 803); good manufacturing practice requirements as set forth in the quality systems (QS) regulation (21 CFR Part 820); and if applicable, the electronic product radiation control provisions (Sections 531-542 of the Act); 21 CFR 1000-1050.

If you desire specific advice for your device on our labeling regulation (21 CFR Part 801), please go to http://www.fda.gov/AboutFDA/CentersOffices/CDRH/CDRHOffices/ucm115809.htm for the Center for Devices and Radiological Health's (CDRH's) Office of Compliance. Also, please note the regulation entitled, "Misbranding by reference to premarket notification" (21CFR Part 807.97). For questions regarding the reporting of adverse events under the MDR regulation (21 CFR Part 803), please go to http://www.fda.gov/MedicalDevices/Safety/ReportaProblem/default.htm for the CDRH's Office of Surveillance and Biometrics/Division of Postmarket Surveillance.

You may obtain other general information on your responsibilities under the Act from the Division of Small Manufacturers, International and Consumer Assistance at its toll-free number (800) 638-2041 or (301) 796-7100 or at its Internet address http://www.fda.gov/MedicalDevices/ResourcesforYou/Industry/default.htm.

Sincerely yours,

Sally A. Hojvat, M.Sc., Ph.D.
Director
Division of Microbiology Devices
Office of *In Vitro* Diagnostic Device Evaluation and Safety
Center for Devices and Radiological Health

Enclosure

Indication(s) for Use Form

510(k) Number (if known): K100818

Device Name: *illumigene* Molecular Diagnostic Test System (***illumigene*** *C. difficile* DNA Amplification Assay, ***illumipro-10***)

Indications for Use:

The ***illumigene*** *C. difficile* DNA amplification assay, performed on the ***illumipro-10,*** is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from patients suspected of having *Clostridium difficile*-associated disease (CDAD).

The ***illumigene*** *C. difficile* assay utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile*. The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (*tcdA*) and the Toxin B gene (*tcdB*), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains[3]. The ***illumigene*** *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The *tcdA* target region was selected as an intact region remaining in all known A+B+ and A-B+ toxinotypes.

illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

Prescription Use ___X____ (Part 21 CFR 801 Subpart D) AND/OR Over-The-Counter Use _____ (21 CFR 801 Subpart C)

(PLEASE DO NOT WRITE BELOW THIS LINE-CONTINUE ON ANOTHER PAGE OF NEEDED)

Concurrence of CDRH, Office of In Vitro Diagnostic Devices (OIVD)

Division Sign-Off
Office of In Vitro Diagnostic Device
Evaluation and Safety

510(k) K10 0818

Exhibit 70

510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY
ASSAY AND INSTRUMENT COMBINATION TEMPLATE

A. 510(k) Number:

K100818

B. Purpose for Submission:

To obtain substantial equivalence for the Illumigene *C. difficile* assay

C. Measurand:

C. difficile toxin A gene

D. Type of Test:

Loop mediated isothermal amplification

E. Applicant:

Meridian Bioscience Inc.

F. Proprietary and Established Names:

Illumigene *C. difficile* Assay

G. Regulatory Information:

1. Regulation section:

   21 CFR 866.2660 – Microorganism differentiation and identification device

2. Classification:

   Class 1

3. Product code:

   OMN – *C. difficile* nucleic acids

4. Panel:

   83 - Microbiology

H. Intended Use:

1. Intended use:

   The illumigene *C. difficile* DNA amplification assay, performed on the *illumipro-10,* is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from patients suspected of having *Clostridium difficile*-associated disease (CDAD).

   The illumigene *C. difficile assay* utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile.* The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (tcdA) and the Toxin B gene (tcdB), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains. The illumigene *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The tcdA target region was selected as an intact region remaining in all known A+/B+ and A-/B+ toxinotypes.

   illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

2. Indication(s) for use:

   The illumigene *C. difficile* DNA amplification assay, performed on the *illumipro-10,* is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from patients suspected of having *Clostridium difficile*-associated disease (CDAD).

   The illumigene *C. difficile* assay utilizes loop-mediated isothermal amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile.* The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (tcdA) and the Toxin B gene (tcdB), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains. The illumigene *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The tcdA target region was selected as an intact region remaining in all known A+/B+ and A-/B+ toxinotypes.

   illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

3. Special conditions for use statement:

   For professional use

4. Special instrument requirements:

   Illumipro -10 Automated Isothermal Amplification and Detection System

I. Device Description:

The ***illumi****gene* Molecular Diagnostic Test System is comprised of the ***illumi****gene C. difficile* DNA Amplification Test Kit, the ***illumi****gene C. difficile* External Control Kit and the ***illumi****pro-10* Automated Isothermal Amplification and Detection System. Additional specialized laboratory equipment is not required for execution of ***illumi****gene* Molecular Diagnostic Test System assays.

The ***illumi****gene C. difficile* DNA amplification assay utilizes loop-mediated isothermal amplification (LAMP) technology to detect the presence of toxigenic *C. difficile* in patients suspected of having *C. difficile* associated disease (CDAD). Each ***illumi****gene C. difficile* assay is completed using materials provided: ***illumi****gene* Sample Preparation Apparatus, ***illumi****gene* Reaction Buffer, ***illumi****gene C. difficile* Test Device, Sample Collection Brush, and ***illumi*** *gene* Extraction Tube. Samples are prepared using the Sample Collection Brush and the ***illumi****gene* Sample Collection Apparatus, target DNA is heat extracted in the Extraction Tube and DNA amplification occurs in the ***illumi****gene C. difficile* Test Device.

The ***illumi****pro-10* heats each ***illumi*** *gene C. difficile* Test Device containing prepared samples, facilitating amplification of target DNA. When toxigenic *C. difficile* is present in the patient sample, a cytotoxin specific sequence is amplified and magnesium pyrophosphate is formed. As the LAMP reaction progresses, magnesium pyrophosphate forms a precipitate in the reaction mixture. The optics system of the ***illumi****pro-10* detects the change in light transmission through the reaction mixture created by the precipitating Magnesium pyrophosphate. Sample results are reported as Positive or Negative based on the detected change in transmission.

The ***illumi****gene C. difficile* External Control Kit consists of a Positive Control Reagent and a Negative Control Reagent. External Control reagents are provided to aid the user in detection of reagent deterioration, adverse environmental or test conditions, or variance in operator performance that may lead to test errors. The ***illumi****gene C. difficile* External Control Kit is required for routine Quality Control.

Final Component Summary

illumi*gene C. difficile DNA* Amplification Assay

·***illumi**gene* ***C. difficile*** **Sample Preparation Apparatus:** Sampling unit consisting of sample preparation chamber, dropper tip, cap and Sample Dilution Buffer (Phosphate Buffered Saline and formalin treated *Staphylococcus aureus* , with sodium azide (0.09%) as a preservative)
·***illumi**gene* **Reaction Buffer:** Tris-buffered solution containing sodium azide (0.09%)as a preservative
·***illumi**gene* ***C. difficile*** **Test Device:** Two separate chambers containing dry reagent lyospheres comprised of DNA polymerase, Deoxyribonucleoside Triphsophate (dNTPs), and either *C. difficile* specific primers (TEST Chamber) or *S. aureus* primers (CONTROL Chamber). The Control lyosphere contains quinoline yellow dye to differentiate it from the Test lyosphere
·**Sample Collection Brushes:** Tube cap fitted with stainless steel rod and nylon bristles
·***illumi**gene* **Extraction Tubes:** 1.5 mL polypropylene microcentrifuge tubes with caps; RNase, DNase and pyrogen-free
***illumi**gene C. difficile* External Control Kit
·***illumi**gene* **Positive Control:** Tris-buffered solution containing non-infectious Plasmid DNA (*S. aureus* and *C. difficile inserts)* with azide (0.09%) as a preservative
·***illumi**gene* **Negative Control:** Tris-buffered solution containing non-infectious Plasmid DNA (*S. aureus insert)* with azide (0.09%) as a preservative

J. Substantial Equivalence Information:

1. Predicate device name:
   Cepheid Xpert C.difficile

2. Predicate 510(k) number:
   K091109

3. Comparison with predicate:

Similarities		
Item	Device	Predicate
Sample matrix	Uniformed unpreserved stool samples	Same
Assay type	DNA amplification	Same

Differences		
Item	Device	Predicate
Intended Use	Assay targets a conserved DNA fragment on the toxin A (tcdA) gene in the pathogenicity locus of toxigenic *C. difficile.*	Assay targets the toxin B (tcdB) gene
Instrument	Assay uses the Illumipro-10 instrument	Assay uses the Cepheid GeneXpert Dx System

Differences		
Item	Device	Predicate
Detection method	Assay uses loop mediated isothermal amplification (LAMP) technology and detects changes in turbidity by visible light transmission	Assay uses automated real time polymerase chain reaction (PCR) and detects fluorescence
Transport media	Sample in Cary Blair media is acceptable	Not for use with Cary Blair media
Extraction method	Manual	Self contained, automated

K. Standard/Guidance Document Referenced (if applicable):

User Protocol for Evaluation of Qualitative Test Performance (EP 12-A2)
User Verification of Performance for Precision and Trueness (IVD) (EP 15-A2)
Guidance for Industry and FDA Staff: Class II Special Controls Guidance Document: Nucleic Acid Amplification Assay for the Detection of Enterovirus RNA
Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices - Guidance for Industry and FDA Staff
Draft Guidance for Industry and FDA Staff: Establishing the Performance Characteristics of In Vitro Diagnostic Devices for the Detection or Detection and Differentiation of Influenza Viruses
General Principles of Software Validation; Final Guidance for Industry and FDA Staff

L. Test Principle:

The ***illumi****gene C. difficile* Assay is based on loop-mediated isothermal amplification technology (LAMP). Loop-mediated amplification is accomplished by the use of specially designed primers that provide specific and continuous isothermal DNA amplification. Magnesium-pyrophosphate is produced as a by-product of LAMP amplification. The magnesium-pyrophosphate forms a white precipitate in the reaction solution, giving the reaction solution a turbid appearance. The presence of turbidity signifies a positive reaction while the absence of turbidity represents a negative reaction.

The Toxigenic *C. difficile* Pathogen Locus (PaLoc) and *illumigene C. difficile* Primer Regions:

The ***illumi****gene C. difficile* assay contains primers that specifically amplify an ~204 base pair (bp) sequence within the 5' region of the toxin A (*tcd*A) gene that is part of the cytotoxin pathogen locus found in toxigenic *C. difficile*. This region is found in all cytotoxin positive (A+/B+ and A-/B+) strains characterized and is not present in cytotoxin negative strains (A-/B-).

The ***illumi****gene C. difficile* assay detects the presence of toxigenic *C. difficile* in stool samples from patients suspected of having *Clostridium difficile* associated disease in the following manner:

Patient stool sample is collected using the sample brush portion of the ***illumi****gene* Sample Collection Apparatus. The sample is added to the Sample Collection Apparatus containing Sample Dilution Buffer and formalin treated *Staphylococcus aureus*. *S. aureus* serves as an internal control for inhibition and system suitability.

The diluted sample is mixed, filtered through the Sample Collection Apparatus and dispensed drop-wise into an ***illumi*** *gene* Extraction Tube. The Extraction Tube is heated to facilitate DNA extraction of the sample and the internal control material.

Extracted DNA is added to an ***illumi****gene* Reaction Buffer tube, mixed and added to the ***illumi****gene C. difficile* Test Device. The ***illumi*** *gene* Test Device consists of two chambers, one for the sample (TEST) and one for the internal assay control (CONTROL). The TEST chamber contains a lyosphere comprised of DisplaceAce™ DNA polymerase, dNTPs, Bovine Serum Albumin, and ***illumi****gene C. difficile* primers. The CONTROL chamber contains a lyosphere comprised of DisplaceAce™ DNA polymerase, dNTPs, Bovine Serum Albumin, yellow dye and ***illumi****gene Staphylococcus aureus* internal control primers.

The Test Device is placed in the ***illumi****pro-10*™ for amplification and detection. Amplification occurs when the ***illumi****gene C. difficile* Test Device is incubated at 63 C. When toxigenic *C. difficile* is present in the patient sample, the cytotoxin specific sequence is amplified. Magnesium pyrophosphate, a by-product of DNA amplification, will reach saturation and precipitate producing a turbid solution in the TEST chamber of the Test Device. When sample preparation and the assay procedure have has been performed successfully and inhibition has not occurred, DNA from the internal control will amplify. Magnesium pyrophosphate will precipitate and form a turbid solution in the CONTROL chamber of the test device. Assay reactions take place in approximately 40 minutes.

Light transmission (650 ± 20 nm) through the TEST and CONTROL solutions is measured by the ***illumi****pro-10* at the on-set of the assay run and at completion. The ***illumi****pro-10* calculates the ratio between the initial absorbance reading and the final absorbance reading and compares the ratio to an established cut-off value. Valid test results will be reported for a change in absorbance above 90% in the CONTROL chamber. Positive test results will be reported for a change in absorbance above 90% in the TEST chamber and the CONTROL

chamber.

M. Performance Characteristics (if/when applicable):

1. Analytical performance:

   a. *Precision/Reproducibility:*

   Reproducibility studies were performed by three clinical sites using coded panels. The panels consisted of moderately positive (n=2), low positive (n=3), high negative (n=3) and negative (n=2) samples. The moderately positive and low positive samples were contrived from negative specimens spiked with toxinogenic *C. difficile* VPI 10463 to just above the LoD for the strain (weak positive) or below the limit of blank (high negative sample), as determined by replicate testing during manufacture of the samples. Negative samples were natural samples. Three lots of ***illumi****gene C. difficile* and nine ***illumi*** *pro-10* instruments were used in reproducibility studies. Positive and Negative Controls were tested with each panel. Each clinical site tested two panels each day for five days. At least two operators each day at each facility performed the testing to demonstrate reproducibility. Reproducibility of the assay is 100% for moderate positive, low positive and negative samples. Reproducibility is 91% for high negative samples.

   Positive samples were contrived from negative specimens. The moderately positive and low positive samples were contrived from negative specimens spiked with toxinogenic C. difficile VPI 10463 to just above the LoD for the strain (weak positive) or below the limit of blank (high negative sample), as determined by replicate testing during manufacture of the samples. Negative samples were natural samples. **Final concentrations of C. difficile in reproducibility panel samples were as follows:** Moderate Positive: 200 CFU/test (Positive) Low Positive: 64 CFU/test (Positive) High Negative: 0.5 CFU/test (Negative)

   The sources of variability examined in the study included operators (minimum 2 at each site), laboratories (3 sites), runs (Panels tested twice per day), test date (panels tested for five days), and ***illumi*** pro-10 instruments (9 instruments).

   Precision (within-run and within-laboratory) was calculated following CLSI EP 15-A2. 95% confidence intervals were calculated using the Wilson method.

   Reproducibility of the assay is 100% for moderate positive, low positive and negative samples. Reproducibility is 91% for high negative samples. Sample type and replicate data is as follows: Percent Agreement Positive Samples: 60/60 Replicates or 100% Percent Agreement Low Positive: 90/90 Replicates or 100% Percent Agreement High Negative: 82/90 Replicates or 91% Percent Agreement Negative: 59/59 Replicates or 100% *(One negative sample produced an invalid result during testing. This value was not included in the calculations.)*

b. Linearity/assay reportable range:

N/A

c. Traceability, Stability, Expected values (controls, calibrators, or methods):

Internal control is provided in the Sample Preparation Apparatus and the Assay Device. The Sample Preparation Apparatus contains Sample Dilution Buffer with formalin treated *Staphylococcus aureus*. The CONTROL Chamber of the Assay Device contains a lyosphere with *S. aureus* primers. The internal control has the same reactants to that of the test chamber except for the primer composition and a yellow dye. Internal Control components are intended to monitor sample preparation, polymerase inhibition, system suitability and reagent integrity.

illumi*gene* External Control Reagents will be sold separately as adjunct reagents to the ***illumi****gene C. difficile* Assay. Each External Control kit contains a Negative Control reagent and a Positive Control reagent. External Controls aid the user in detection of reagent deterioration, adverse environmental or test conditions or variance in operator performance that can cause test errors. External Controls are required for routine Quality Control.

Positive Control: Tris-buffered solution containing non-infectious Plasmid DNA (S. aureus and C. difficile inserts) at 200.0 fg/µL with sodium azide (0.09%) as a preservative.

Negative Control: Tris-buffered solution containing non-infectious Plasmid DNA (*S. aureus insert)* at 200.0 fg/µL with sodium azide (0.09%) as a preservative.

d. Detection limit:

Sensitivity studies were designed to determine, within 95% confidence intervals, the analytical limit of detection (LoD) of *C. difficile* diluted in a human stool matrix. The LoD is the lowest number of colony-forming units (CFUs) per test aliquot that can be distinguished from Native samples with a high degree of probability.

Seven different strains representing five toxinotypes of *C. difficile* were evaluated. Each bacterial strain was spiked into negative stool and then diluted serially. Twenty replicates of each dilution were individually processed and tested to give a stated probability of 95% for the LoD dilution. The results obtained with each individual strain are given in Tables 2 – 5 on the following pages. The LoD point estimates and 95% upper and lower confidence intervals for each toxinotype are summarized in Table 1 below.

Table 1. 95% confidence intervals for *illumigene C. difficile* analytical LoD.

Strain ID	Toxinotype	Phenotype	LoD/Test	Lower Limit (95% CI)	Upper Limit (95% CI)
VPI 10463	0	A+/B+	4 CFU/test	83.9%	100%
2007431	III (NAP1)	A+/B+	32 CFU/test	83.9%	100%
BI8	III	A+/B+	64 CFU/test	76.4%	99.1%
2006240	V (NAP7)	A+/B+	32 CFU/test	83.9%	100%
CF1	VIII	A-/B+	64 CFU/test	83.9%	100%
2007858	IX/XXIII	A+/B+	32 CFU/test	83.9%	100%
8864	X	A-/B+	64 CFU/test	83.9%	100%

e. Analytical specificity:

Interference Studies:

Potentially interfering substances were added to a natural negative and a contrived positive sample at final concentrations of 5% v/v or greater. The contrived positive samples were prepared from a pool of donor stools that were confirmed negative by toxinogenic culture. The sample was inoculated with *C. difficile* VPI 10463 to just above the limit of detection (18 CFU/test) for this organism.

Potentially interfering substances were added at final concentrations of 5% V/V or greater. Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially interfering substance. Each sample was tested in triplicate. The following substances, at the specified saturated solvent/diluents concentrations, were included in the study: Barium sulfate (5 mg/mL), fecal fat (equivalent to 2.65 mg stearic plus 1.3 mg palmitic acids per mL), hemoglobin (as methemoglobin) (3.2 mg/mL), IgA (5 mg/mL), Imodium AD® (0.00667 mg/mL), Kaopectate® (0.87 mg/mL), Metronidazole (12.5 mg/mL), mucin (3.33 mg/mL) Mylanta® (4.2 mg/mL), Pepto-Bismol® (0.87 mg/mL), Prilosec® (0.5 mg/mL), Tagamet® (0.5 mg/mL), TUMS® (0.5 mg/mL), Vancomycin (12.5 mg/mL), white blood cells (5%V/V), whole blood (5% V/V).

Crossreactivity Studies:

Potentially crossreactive or interfering microorganisms were added to a natural negative and a contrived positive sample at concentrations of 1.2×10^{8}/mL (bacteria and fungi) or at a minimum of $1 \times 10^{5.06}$ $TCID_{50}$/mL (viruses). The contrived positive sample was prepared from a pool of donor stools that were confirmed negative by toxinogenic culture. The sample was inoculated with toxinogenic *C. difficile* VPI 10463 to just above the limit of detection (18 CFU/test) for this strain.

Potentially cross-reactive microorganisms were added at concentrations of 1.2 x 10^8/mL (bacteria and fungi) or at a minimum of 1 x $10^{5.06}$ /mL $TCID_{50}$/mL (viruses). Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially cross-reactive microorganisms. Each potential cross-reactive microorganism was tested in replicates of three. The following microorganisms were included in the study: *Aeromonas hydrophila, Bacteroides fragilis, Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Candida albicans, Citrobacter freundii, Clostridium sordellii, Clostridium perfringens, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Escherichia coli O157:H7, Escherichia fergusonii, Escherichia hermannii, Helicobacter pylori, Klebsiella pneumoniae, Lactococcus lactis, Listeria monocytogenes, Peptostreptococcus anaerobius, Plesiomonas shigelloides, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella* Groups B-E, *Serratia liquefaciens, Serratia marcescens, Shigella boydii, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Vibrio parahaemolyticus, Yersinia enterocolitica,* Adenovirus Types 40 and 41, Coxsackievirus, Echovirus, Rotavirus.

Strain Reactivity Studies:

Aliquots from culture filtrates of each organism were first adjusted to MacFarland Standard 4 (approximately 1.2 X 10^9), then spiked into a natural negative stool matrix to a final concentration of (1) 64 CFU/test (the LoD at 95% confidence as determined in the LoD study, and (2) at 100 times the LoD (to represent heavily infected samples).

Strain reactivity studies were performed by testing *C. difficile* stock cultures from different sources at 64 CFU/test and 640 CFU/test with ***illumi****gene C. difficile.* Each sample was testing in triplicate. Strains and toxinotypes tested were as follows: **Type 0 Strains:** 10463, 2004111, 2004205, 2005070, 2005257, 2008029, 2008162, 2008341, 2008351, 2009066, 2009099, B1, G1, J7, K12, Y1; **Type III Strains:** 2004052, 2004118, 2007431, BI17, BI8; **Type V Strains:** 2005325, 2006240, 2008188, 2009018, 2009065, BK6; **Type VIII Strains:** 43598, 2008016, CF1; **Type X Strains:** 8864; **Type XII Strains:** 2007435; **Type IX/XXIII Strains:** 2007858; **Unknown Strains:** 2009132, 2009155, 2009277.

f. Assay cut-off:

The assay has a fixed cut-off based on the measured change in light transmission at the assay endpoint. There are no range values for the cut-off. The ***illumi****pro-10* measures the transmission of light through the Test Solution and the Control Solution at the start of the Assay Run and at the end point of the Assay Run, approximately 40 minutes for the ***illumi****gene C. difficile* assay. The ***illumi****pro-10* calculates the ratio of the Run End (Signal final or S_f) reads with the Run Start (Signal Initial or S_i) reads. Calculated S_f : S_i ratios less than 90% are reported as positive; Calculated S_f : S_i ratios greater than or equal to 90% are reported as negative. Fixed cut-off values

were based on well characterized clinical specimens.

2. Comparison studies:

   a. *Method comparison with predicate device:*

   Only discrepant samples were tested with the predicate device, Cepheid® Xpert® *C. difficile,* and TECHLAB® C. DIFF CHEK™ - 60.

   Of 5 false-negative samples, 2 were negative by an FDA-cleared molecular assay.

   Of 27 false-positive samples, 15 were positive by an FDA-cleared molecular assay

   Of the 12 remaining false-positive samples, 8 were positive by an FDA-cleared assay for C. difficile Common Antigen.

   b. *Matrix comparison:*

   N/A

3. Clinical studies:

   a. *Clinical Sensitivity:*

   Samples utilized in this study were stool specimens from patients suspected of having C. *difficile* associated disease. No restrictions with regard to age, gender or geographical location were included. All samples were left-over samples. The consistency of each sample was categorized as solid, semisolid, bloody, and watery with no solids or in transport medium. A total of 697 qualified patient samples were used in the study. One sample was obtained per patient. Two tests were performed with each sample (cytotoxic bacterial culture and illumigene C. difficile assay). Performance characteristics of the illumigene C. difficile assay were determined by comparison to cytotoxic bacterial culture. Four independent clinical test sites located in the Midwestern and Southern regions of the United States and the manufacturer evaluated a total of 697 qualified patient samples. Samples were collected from 274 (39.3%) males and 419 (60.1%) females. In the case of 4 (0.6%) of the patients, sex was not known. The age groups of patients range from 2 years of age to 96 years. No differences in test performance were observed based on patient age, sex, or geographical location.

   Overall sensitivity was determined to be 95.2% (95% CI: 89.2% - 97.9%); overall specificity was determined to be 95.3% (95% CI: 93.2% - 96.7%).

Overall performance data

Cytotoxic bacterial culture		*illumigene C. difficile*	
	Positive	**Negative**	**Total**
Positive	99	5	104
Negative	27	546	573
Total	126	551	677
			95% CI
Sensitivity	99/104	**95.2%**	89.2 - 97.9%
Specificity	546/573	**95.3%**	93.2 - 96.7%
Correlation	645/677	**95.3%**	93.4 - 96.6%

*b. Clinical specificity**:***

See 3(a) above

c. Other clinical supportive data (when a. and b. are not applicable):

N/A

4. Clinical cut-off**:**

N/A

5. Expected values/Reference range:

Samples used in this study were left-over samples for which a physician had ordered *C. difficile* testing. Observed *C. difficile* prevalence rate was approximately 15%.

N. Instrument Name:

The ***illumipro***-10

O. System Descriptions:

1. Modes of Operation:
The ***illumi****pro-10* is a menu driven laboratory instrument with two independent sample processing blocks: Block A and Block B. Each ***illumi****pro-10* Block is capable of batch processing up to five closed ***illumi****gene* test devices.
The ***illumi****pro*-10 operates in four basic modes: ASSAY, RESULTS, SERVICE, and SYSTEM. Assay Selection and Sample Amplification occur in the ASSAY mode; Test Results are managed in the RESULTS mode; Basic instrument set-up is performed in the SYSTEM mode; and Optical performance verification is completed in the SERVICE

mode.

2. Software:

   FDA has reviewed applicant's Hazard Analysis and software development processes for this line of product types:

   Yes ____X____ or No _____

3. Specimen Identification:

   Samples are identified by position. Default Sample Identification is based on Block and Well position (e.g. Block A, Well 1). The user may input Sample Identification information using the keypad, the barcode scanner or the optional external keyboard.

4. Specimen Sampling and Handling:

   Specimen Sampling and Handling is performed external to the ***illumi*** *pro-10*. Prepared Samples in closed ***illumi****gene* Test Devices are placed in the ***illumi****pro-10* for amplification and detection. The ***illumi****pro-10* has no direct contact with samples. Closed ***illumi****gene* Test Devices are discarded at the end of the assay to reduce the likelihood of contamination of the ***illumi****pro-10* or the workspace.

5. Calibration:

   The ***illumi****pro-10* was designed to be a self-monitoring instrument. Calibration by the end user is not required.

6. Quality Control:

   Quality Control requirements for the ***illumi****pro-10* are limited to verification of optical performance and routine surface cleaning/decontamination. Optics system verification is performed by the user at installation and at 30 day intervals thereafter. Optics Verification Standards consist of ten red acrylic pieces molded and polished to replicate the dimensions of each ***illumi****gene* Test Device chamber. The verification standards act like a high pass optical filter with the pass wavelength of 650 nm extending into the infrared range. Light transmission through the verification standard is used to confirm proper performance of the optics system. Failed optics verification testing for an instrument block will disable the block until the error is resolved. The ***illumi****pro-10* completes a Power-On Self Test (POST) at each power-on. POST testing confirms that the Hardware and Software elements of the system are performing as expected. The ***illumi****pro-10* reports an error if POST failures are obtained; the instrument is disabled until the error is resolved.

P. Other Supportive Instrument Performance Characteristics Data Not Covered In The "Performance Characteristics" Section above:

N/A

Q. Proposed Labeling:

The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.

R. Conclusion:

1. The submitted information in this premarket notification is complete and supports a substantial equivalence decision.

Exhibit 71

510(k) number: K110012 **Date of preparation:** December 31, 2010

Submitter: **Meridian Bioscience, Inc.**
Submitter's address: 3471 River Hills Drive
Cincinnati, Ohio 45244
Contact: Michelle Smith
Contact number: (513) 271-3700

FEB 2 4 2011

Device name: *illumigene® C. difficile*

Common name: *C. difficile* DNA Amplification Assay

Classification name: *C. difficile* Nucleic Acids
OMN, CFR Section 866.2660

Predicate device: K100818: ***illumigene®*** Molecular Diagnostic Test System (***illumigene C. difficile*** DNA Amplification Assay, ***illumipro-10***)

Model 280050, 610172

Reference comparator: Cytotoxic bacterial culture

Description of the device:
The ***illumigene*** Molecular Diagnostic Test System is comprised of the ***illumigene C. difficile*** DNA Amplification Test Kit, the ***illumigene C. difficile*** External Control Kit and the *illumipro-10* Automated Isothermal Amplification and Detection System. The ***illumigene C. difficile*** DNA amplification assay utilizes loop-mediated isothermal amplification (LAMP) technology to detect the presence of toxigenic *C. difficile* in patients suspected of having *C. difficile* associated disease (CDAD). Each ***illumigene C. difficile*** assay is completed using an ***illumigene*** Sample Preparation Apparatus, ***illumigene*** Reaction Buffer, ***illumigene C. difficile*** Test Device, Sample Collection Brush, and ***illumigene*** Extraction Tube. Samples are prepared using the Sample Collection Brush and the ***illumigene*** Sample Collection Apparatus, target DNA is heat extracted in the Extraction Tube and DNA amplification occurs in the ***illumigene C. difficile*** Test Device.

The ***illumipro-10*** heats each ***illumigene C. difficile*** Test Device containing prepared samples, facilitating amplification of target DNA. When toxigenic *C. difficile* is present in the patient sample, a cytotoxin specific sequence is amplified and Magnesium pyrophosphate is formed. Magnesium pyrophosphate forms a precipitate in the reaction mixture. The ***illumipro-10*** detects the change in light transmission through the reaction mixture created by the precipitating Magnesium pyrophosphate. Sample results are reported as Positive or Negative based on the detected change in transmission.

The ***illumigene C. difficile*** External Control Kit consists of a Positive Control Reagent and a Negative Control Reagent. External Control reagents are provided to aid the user in detection of reagent deterioration, adverse environmental or test conditions, or variance in operator performance that may lead to test errors. The ***illumigene C. difficile*** External Control Kit is required for routine Quality Control.

Intended Use:

The ***illumigene*** *C. difficile* DNA amplification assay, performed on the ***illumipro-10,*** is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from pediatric and adult patients suspected of having *Clostridium difficile*-associated disease (CDAD).

The ***illumigene*** *C. difficile* assay utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile*. The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (*tcdA*) and the Toxin B gene (*tcdB*), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains. The ***illumigene*** *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The *tcdA* target region was selected as an intact region remaining in all known A+B+ and A-B+ toxinotypes.

illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

Comparison to predicated device:

Characteristic	***illumigene*™ C. difficile, Revised**	***illumigene*™ C. difficile, K100818**
Test Format	No Change	DNA Amplification Assay
Intended Use		
DNA Amplification Technology	No Change	Loop-Mediated Isothermal Amplification (LAMP)
Target Sequences Detected	No Change	Partial DNA fragment on the Toxin A gene of the pathogenicity locus (PaLoc) found in all known strains for toxigenic *C. difficile*.
Qualitative/Quantitative	No Change	Qualitative
Screening, Diagnostic or Identification Test	No Change	Diagnostic
Specimen Types		
Unformed Human Stool	No Change	Yes
Human Stool in Cary-Blair-based Media	No Change	Yes
Reagents/Components	***illumigene*** Sample Preparation Apparatus ***illumigene*** Reaction Buffer ***illumigene*** *C. difficile* Assay Device ***illumigene*** Heat Treatment Tubes Sample Collection Brushes	***illumigene*** Sample Preparation Apparatus ***illumigene*** Reaction Buffer ***illumigene*** *C. difficile* Assay Device ***illumigene*** Extraction Tubes Sample Collection Brushes
Extraction	Not Applicable. Sample preparation by heat treatment. DNA Extraction and purification not required.	Manual
Amplification	No Change	Self-contained and automated

Comparison to predicated device:

Characteristic	***illumigene™* C. difficile, Revised**	***illumigene™* C. difficile**
Detection	No Change	Self-contained and automated
Testing Time	No Change	Approximately 60 minutes
Calibration	No Change	Not required
Controls		
Inhibition, Assay	No Change	Provided ***illumigene*** Sample Preparation Apparatus: *Staphylococcus aureus* ***illumigene C. difficile*** Assay Device: *Staphylococcus aureus* LAMP Primers
External	No Change	Adjunct Reagents ***illlumigene C. difficile*** External Control Kit Catalog 279920
Extraction	Not Applicable. Sample preparation, including heat treatment monitored by external thermometer and interval timer. Equipment is user supplied.	User Supplied
Equipment		
Instrumentation	No Change	***illumipro-10™*** Automated Isothermal Amplification and Detection System
General Laboratory Equipment	Micropipette 50 µL, 200 µL Dry-bath with 12mm Heat Block, 95 C Interval Timer Vortex Mixer Digital Thermometer with Max/Min Temperature Memory	Micropipette 50 µL, 200 µL Dry-bath with 12mm Heat Block, 95 C Interval Timer Vortex Mixer
Reading Method	No Change	Visible Light Transmission
Results		
***C. difficile* Toxinotypes Tested**	No Change	**0 (A+/B+)** **III (A+/B+)** **V (A+/B+)** **VIII (A-/B+)** **X (A-/B+)** **XII (A+/B+)** **IX/XXIII (A+/B+)**
Results Interpretation	No Change	INVALID POSITIVE NEGATIVE

Performance Comparison, Non-clinical Tests:

Interference Testing *(Reference K100818)*

Selected drugs and other non-microbial substances that might be present in stool samples from healthy persons or patients suspected of having *C. difficile* associated disease were added to a natural negative and a contrived positive sample. The natural negative and contrived positive samples were prepared from donor samples and were confirmed negative by cytotoxic bacterial culture. The contrived positive sample was prepared by spiking a confirmed negative sample with toxinogenic *C. difficile* strain VPI 10463 to 18 CFU/test, slightly above the 16 CFU assay limit of detection for this organism. Potentially interfering substances were added at final concentrations of 5% V/V or greater. Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially interfering substance. Each sample was tested in triplicate.

The following substances, at the specified saturated solvent/diluents concentrations, do not interfere with ***illumigene** C. difficile* test results in the final concentrations listed: Barium sulfate (5 mg/mL), fecal fat (equivalent to 2.65 mg stearic plus 1.3 mg palmitic acids per mL), hemoglobin (as methemoglobin) (3.2 mg/mL), IgA (5 mg/mL), Imodium AD® (0.00667 mg/mL), Kaopectate® (0.87 mg/mL), Metronidazole (12.5 mg/mL), mucin (3.33 mg/mL) Mylanta® (4.2 mg/mL), Pepto-Bismol® (0.87 mg/mL), Prilosec® (0.5 mg/mL), Tagamet® (0.5 mg/mL), TUMS® (0.5 mg/mL), Vancomycin (12.5 mg/mL), white blood cells (5%V/V), whole blood (5% V/V).

Cross-reactivity Study *(Reference K100818)*

Potentially cross-reactive microorganisms that might be present in stool samples from healthy persons or patients suspected of having *C. difficile* associated disease were added to a natural negative and a contrived positive sample. The natural negative and contrived positive samples were prepared from donor samples and were confirmed negative by cytotoxic bacterial culture. The contrived positive sample was prepared by spiking a confirmed negative sample with toxinogenic *C. difficile* strain VPI 10463 to 18 CFU/test, slightly above the 16 CFU assay limit of detection for this organism. Potentially cross-reactive microorganisms were added at concentrations of 1.2×10^{8}/mL (bacteria and fungi) or $1 \times 10^{5.29}$/mL $TCID_{50}$/mL (viruses). Dilution Controls for each sample were prepared by adding a phosphate-buffered saline solution in place of the potentially cross-reactive microorganisms. Each sample was tested in triplicate.

The following microorganisms, at the indicated concentrations, do not interfere with ***illumigene** C. difficile* test results:
Aeromonas hydrophila, Bacteroides fragilis, Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Candida albicans, Citrobacter frendii, Clostridium sordellii, Clostridium perfringens, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Escherichia coli O157:H7, Escherichia fergusonii, Escherichia hermannii, Helicobacter pylori, Klebsiella pneumoniae, Lactococcus lactis, Listeria monocytogenes, Peptostreptococcus anaerobius, Plesiomonas shigelloides, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella Groups B-E, *Serratia liquefaciens, Serratia marcescens, Shigella boydii, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Vibrio parahaemolyticus, Yersinia enterocolitica,* Adenovirus Types 40 and 41, Coxsackievirus, Echovirus, Rotavirus.

Performance Comparison, Clinical Tests:

Clinical trials for the ***illumigene** C. difficile* assay, including the ***illumipro-10*** Automated Isothermal amplification and detection system, were conducted in 2010. Performance characteristics of the ***illumigene** C. difficile* assay were determined by comparison to cytotoxic bacterial culture in two separate studies: (1) Patients 2 years of age and above and (2) Patients less than 2 years of age.

(1) Patients 2 years of age and above: Independent clinical test sites located in the Midwestern and Southern regions of the United States and the manufacturer evaluated a total of 697 qualified patient samples. Samples were collected from 274 (39.3%) males and 419 (60.1%) females. In the case of 4 (0.6%) of the patients, sex was not known. The age groups of patients range from 2 years of age to 96 years. No differences in test performance were observed based on patient age, gender or geographical location. Overall Sensitivity was determined to be 95.2% (95% CI: 89.2% - 97.9%). Overall Specificity was determined to be 95.3% (95% CI: 93.2% - 96.7%). Subsequent tables show overall assay performance as well as performance by clinical site and patient age.

Table 1. Performance data (Patients 2 years of age and above)

Cytotoxic bacterial culture	*illumigene C. difficile*			
	Positive	**Negative**	**Invalid*** **	**Total**
Positive	99	5**	4	108
Negative	27*	546	16	589
Total	126	551	20	697
			95% CI	
Sensitivity	99/104	**95.2%**	89.2 - 97.9%	
Specificity	546/573	**95.3%**	93.2 - 96.7%	
Correlation	645/677	**95.3%**	93.4 - 96.6%	
Invalid Rate	20/697	**2.9%**	N/A	

* 15/27 false positive results were positive by another FDA cleared molecular assay. Of the remaining 12 false positive results, 8 were positive by a FDA cleared assay for the detection of GDH.

** 2/5 false negative results were negative by another FDA cleared molecular assay.

*** Invalid results were obtained for 20/697 (2.9%) samples tested. Eleven (1.6%) of the invalids observed were categorized as Assay Invalids, indicative of improper sample preparation, reagent failure, instrument failure or internal control failure. One of the eleven specimens remained invalid after repeat testing from the original sample.

Table 2. Performance characteristics by site (Patients 2 years of age and above)

Site	**Positive Samples**			**Negative Samples**		
	***illumigene*/ Cytotoxic bacterial culture**	**Sensitivity %**	**95% CI**	***illumigene*/ Cytotoxic bacterial culture**	**Specificity %**	**95% CI**
Total	**99/104**	**95.2%**	**89.2 – 97.9%**	**546/573**	**95.3%**	**93.2 – 96.7%**
Site 1	4/5	80.0%	37.6 – 96.4%	58/60	97.6%	88.6 – 99.1%
Site 2	12/12	100%	75.7 – 100%	62/67	92.5%	83.7 – 96.8%
Site 3	20/20	100%	83.9 – 100%	87/92	94.6%	87.9 – 97.7%
Site 4	8/8	100%	67.6 – 100%	36/39	92.3%	79.7 – 97.3%
Site 5	55/59	93.2%	83.8 – 97.3%	303/315	96.2%	93.5 – 97.8%

(2) Patients less than 2 years of age: Independent clinical test sites located in the Midwestern and Southern regions of the United States and the manufacturer evaluated a total of 193 qualified patient samples. Samples were collected from 103 (53.4%) males and 90 (46.6%) females. The age groups of patients tested ranged from 0 months to 24 months. No differences in test performance were observed based on patient age, gender or geographical location. Overall Sensitivity was determined to be 93.3% (95% CI: 78.7 - 98.2%). Overall Specificity was determined to be 96.3% (95% CI: 92.2% - 98.3%). Subsequent tables show overall assay performance as well as performance by clinical site and patient age.

Table 3. Performance data (Patients less than 2 years of age)

Cytotoxic bacterial culture	*illumigene C. difficile*			
	Positive	**Negative**	**Invalid*** **	**Total**
Positive	28	2**	1	31
Negative	6*	156	0	162
Total	34	158	1	193
			95% CI	
Sensitivity	28/30	**93.3%**	78.7 - 98.2%	
Specificity	156/162	**96.3%**	92.2 - 98.3%	
Correlation	184/192	**95.8%**	92.0 - 97.9%	
Invalid Rate	1/193	**0.5%**	N/A	

* 3/6 false positive results were positive by another FDA cleared molecular assay. Of the remaining 3 false positive results, all were positive by a FDA cleared assay for the detection of GDH.

** 1/2 false negative results were negative by another FDA cleared molecular assay.

*** Invalid results were obtained for 1/193 (0.5%) samples tested. The invalid observed was categorized as an Assay Invalid, indicative of improper sample preparation, reagent failure, instrument failure or internal control failure. The specimen remained invalid after repeat testing from the original sample.

Table 4. Performance characteristics by site (Patients less than 2 years of age)

Site	Positive Samples			Negative Samples		
	illumigene / Cytotoxic bacterial culture	Sensitivity %	95% CI	*illumigene* / Cytotoxic bacterial culture	Specificity %	95% CI
Total	**28/30**	**93.3%**	**78.7 - 98.2%**	**156/162**	**96.3%**	**92.2 - 98.3%**
Site 1	8/8	100%	67.6 - 100%	48/49	98.0%	89.3 - 99.6%
Site 2	20/22	90.9%	72.2 - 97.5%	105/109	96.3%	90.9 - 98.6%
Site 4	0/0	N/A	N/A	2/3	66.7%	20.8 - 93.9%
Site 5	0/0	N/A	N/A	1/1	100%	20.7 - 100%

Table 5. Overall results by patient age

Patient age	Positive Samples			Negative Samples		
	illumigene / Toxigenic culture	Sensitivity %	95% CI	*illumigene* / Toxigenic culture	Specificity %	95% CI
< 2 years	28/30	93.3%	78.7 - 98.2%	156/162	96.3%	92.2 - 98.3%
≥ 2 to 12 years	10/11	90.9%	62.3 - 98.4%	75/79	94.9%	87.7 - 98.0%
> 12 to 21 years	5/5	100%	56.6 - 100%	53/56	94.6%	85.4 - 98.2%
> 21 years	83/87	95.4%	88.8 - 98.2%	417/437	95.4%	93.0 - 97.0%
Age Unknown	1/1	100%	20.7 - 100%	1/1	100%	20.7 - 100%

Analytical Sensitivity ***(Reference K100818)***

The analytical sensitivity of this assay for *C. difficile* was based on 20 replicates for each measurand and with a stated probability (e.g., 95% or 19/20 positive replicates) of obtaining positive responses at the following levels of the measurands:

Strain ID	Toxinotype	Phenotype	LoD/Test
VPI 10463	0	A+/B+	4 CFU/test
2007431	III (NAP1)	A+/B+	32 CFU/test
CF1	VIII	A-/B+	64 CFU/test
2006240	V (NAP7)	A+/B+	32 CFU/test
BI8	III	A+/B+	64 CFU/test
2007858	IX/XXIII	A+/B+	32 CFU/test
8864	X	A-/B+	64 CFU/test

Additional *C. difficile* stock cultures from different sources were tested and produced positive reactions at 64 CFU/test with ***illumigene C. difficile***. Strains and toxinotypes tested were as follows: **Type 0 Strains:** 10463, 2004111, 2004205, 2005070, 2005257, 2008029, 2008162, 2008341, 2008351, 2009066, 2009099, B1, G1, J7, K12, Y1; **Type III Strains:** 2004052, 2004118, 2007431, BI17, BI8; **Type V Strains:** 2005325, 2006240, 2008188, 2009018, 2009065, BK6; **Type VIII Strains:** 43598, 2008016, CF1; **Type X Strains:** 8864; **Type XII Strains:** 2007435; **Type IX/XXIII Strains:** 2007858; **Unknown Strains:** 2009132, 2009155, 2009277.

Reproducibility ***(Reference K100818)***

Blind coded panels of 10 samples were supplied to three independent laboratories for precision studies. Samples were randomly sorted within each panel to mask sample identities. The panels included contrived samples manufactured at the assay limit of detection (n = 3) and just below the limit of blank (i.e., high negative sample, n = 3). The panels also included uncharacterized positive (n = 2) and negative (n = 2) samples. Testing was performed by different operators at each site on the same day (intra-assay variability) for five days (inter-assay variability). Three lots of ***illumigene C difficile*** were used in this study. The results are given in the table below:

	Site 1		Site 2		Site 3		Total	
Sample Type	**Percent agreement**		**Percent agreement**		**Percent agreement**		**Percent agreement**	
Negative	20/20	100%	20/20	100%	19/19****	100%	59/59	100%
High Negative	25/30	83%	29/30	97%	28/30	93%	82/90	91%
Low Positive	30/30	100%	30/30	100%	30/30	100%	90/90	100%
Positive	20/20	100%	20/20	100%	20/20	100%	60/60	100%

***** 1 specimen generated an instrument invalid test result.*

Conclusions

The ***illumigene C. difficile*** assay used in conjunction with the ***illumipro-10*** can be used to detect toxigenic *C. difficile* in human stool samples from pediatric and adult patients. The test is diagnostic for toxigenic *C. difficile* infection.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993

Meridian Bioscience, Inc.
c/o Ms. Michelle L. Smith
Director Quality Systems
3471 River Hills Drive
Cincinnati, OH 54244

FEB 24 2011

Re: K110012
Trade/Device Name: illumigene™ *C. difficile* DNA Amplification Assay
Regulation Number: 21 CFR § 866.2660
Regulation Name: Microorganism differentiation and identification device
Regulatory Class: Class I
Product Codes: OMN
Dated: December 31, 2010
Received: January 3, 2011

Dear Ms. Smith:

We have reviewed your Section 510(k) premarket notification of intent to market the device referenced above and have determined the device is substantially equivalent (for the indications for use stated in the enclosure) to legally marketed predicate devices marketed in interstate commerce prior to May 28, 1976, the enactment date of the Medical Device Amendments, or to devices that have been reclassified in accordance with the provisions of the Federal Food, Drug, and Cosmetic Act (Act) that do not require approval of a premarket approval application (PMA). You may, therefore, market the device, subject to the general controls provisions of the Act. The general controls provisions of the Act include requirements for annual registration, listing of devices, good manufacturing practice, labeling, and prohibitions against misbranding and adulteration.

If your device is classified (see above) into class II (Special Controls), it may be subject to such additional controls. Existing major regulations affecting your device can be found in Title 21, Code of Federal Regulations (CFR), Parts 800 to 895. In addition, FDA may publish further announcements concerning your device in the Federal Register.

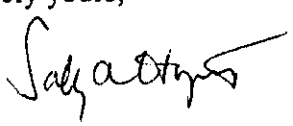
Please be advised that FDA's issuance of a substantial equivalence determination does not mean that FDA has made a determination that your device complies with other requirements of the Act or any Federal statutes and regulations administered by other Federal agencies. You must comply with all the Act's requirements, including, but not limited to: registration and listing (21 CFR Part 807); labeling (21 CFR Parts 801 and 809); medical device reporting (reporting of medical device-related adverse events) (21 CFR 803); and good manufacturing practice

requirements as set forth in the quality systems (QS) regulation (21 CFR Part 820). This letter will allow you to begin marketing your device as described in your Section 510(k) premarket notification. The FDA finding of substantial equivalence of your device to a legally marketed predicate device results in a classification for your device and thus, permits your device to proceed to the market.

If you desire specific advice for your device on our labeling regulation (21 CFR Parts 801 and 809), please contact the Office of *In Vitro* Diagnostic Device Evaluation and Safety at (301) 796-5450. Also, please note the regulation entitled, "Misbranding by reference to premarket notification" (21 CFR Part 807.97). For questions regarding the reporting of adverse events under the MDR regulation (21 CFR Part 803), please go to http://www.fda.gov/MedicalDevices/Safety/ReportaProblem/default.htm for the CDRH's Office of Surveillance and Biometrics/Division of Postmarket Surveillance.

You may obtain other general information on your responsibilities under the Act from the Division of Small Manufacturers, International and Consumer Assistance at its toll-free number (800) 638-2041 or (301) 796-7100 or at its Internet address http://www.fda.gov/cdrh/industry/support/index.html.

Sincerely yours,



Sally A. Hojvat, M.Sc., Ph.D.
Director
Division of Microbiology Devices
Office of *In Vitro* Diagnostic Device Evaluation and Safety
Center for Devices and Radiological Health

Indication(s) for Use Form

510(k) Number (if known): K110012

Device Name: *illumigene* Molecular Diagnostic Test System (***illumigene®*** *C. difficile* DNA Amplification Assay, ***illumipro-10™***)

Indications for Use:

The ***illumigene*** *C. difficile* DNA amplification assay, performed on the ***illumipro-10,*** is a qualitative *in vitro* diagnostic test for the direct detection of toxigenic *C. difficile* in human stool specimens from pediatric and adult patients suspected of having *Clostridium difficile*-associated disease (CDAD).

The ***illumigene*** *C. difficile* assay utilizes loop-mediated isothermal DNA amplification (LAMP) technology to detect the pathogenicity locus (PaLoc) of toxigenic *Clostridium difficile*. The *Clostridium difficile* PaLoc is a gene segment present in all known toxigenic *C. difficile* strains. The *C. difficile* PaLoc codes for both the Toxin A gene (*tcdA*) and the Toxin B gene (*tcdB*), has conserved border regions, and is found at the same site on the *C. difficile* genome for all toxigenic strains. The ***illumigene*** *C. difficile* assay detects the PaLoc by targeting a partial DNA fragment on the Toxin A gene. The *tcdA* target region was selected as an intact region remaining in all known A+B+ and A-B+ toxinotypes.

illumigene *C. difficile* is intended for use in hospital, reference or state laboratory settings. The device is not intended for point-of-care use.

Prescription Use ___X____ (Part 21 CFR 801 Subpart D) AND/OR Over-The-Counter Use _____ (21 CFR 801 Subpart C)

(PLEASE DO NOT WRITE BELOW THIS LINE-CONTINUE ON ANOTHER PAGE OF NEEDED)

Concurrence of CDRH, Office of In Vitro Diagnostic Devices (OIVD)

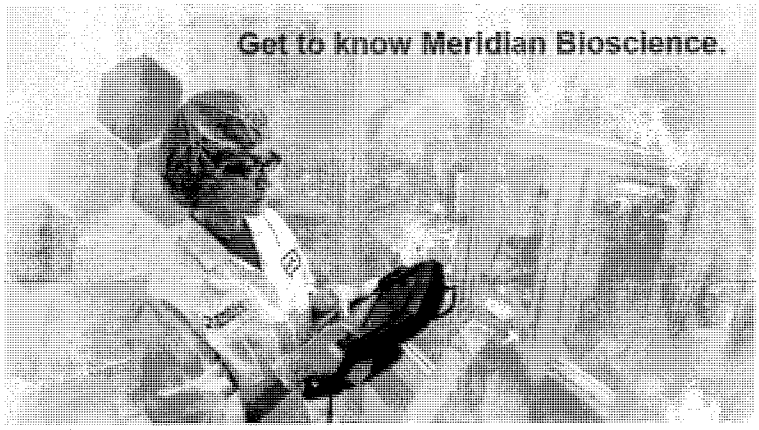
Freddie K. Poole

Division Sign-Off
Office of In Vitro Diagnostic Device
Evaluation and Safety

510(k) K110012

Exhibit 72

Get to Know Us



Meridian Bioscience is a fully integrated life science company that manufactures, markets and distributes a broad range of innovative diagnostic test kits, purified reagents and biopharmaceutical enabling technologies that help deliver answers. Our products provide accuracy, simplicity and speed for the early diagnosis and treatment of common medical conditions, such as *C. difficile*, *H. pylori*, foodborne diseases and respiratory infections.

In addition, we develop and manufacture a variety of biological and non-biological materials used in proficiency testing programs. Learn more about our Proficiency & Controls business unit.

Meridian diagnostic products are used outside of the human body and require little or no special equipment. We design our products to enhance the well being of the patient—while reducing the total outcome costs of healthcare.

Meridian has strong market positions in the areas of gastrointestinal infections, serology, parasitology and fungal disease diagnosis. We actively market our products to hospitals, reference laboratories, research centers, physician offices and diagnostics manufacturers in more than 60 countries around the world.

Our shares are traded on the NASDAQ National Market, symbol VIVO. Learn why Meridian Bioscience is a company to watch.

Learn more about our mission and core values.

Meridian Team

Meridian's Board of Directors and Management Team are committed to providing value to all of our stakeholders —our shareholders, our customers, and our employees.

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Interested in investment opportunities?

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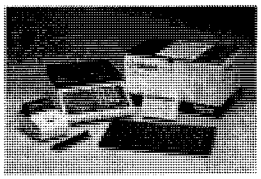
Products: *illumigene®*

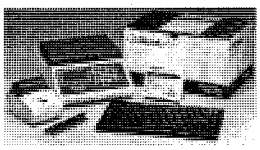
*illumi*gene® Molecular Diagnostic System



The *illumigene®* Molecular Diagnostic System is simple, delivering the power of molecular technology in a "rapid test" type format; flexible, integrating easily into any facility regardless of volume or expertise; and cost-effective, allowing addition of molecular assays without the cost of additional labor or capital expenditures.

illumigene® *C. difficile*

*illumi*gene® Molecular Diagnostic System



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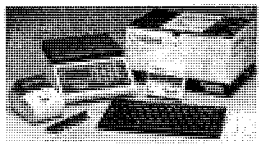
illumigene® Group B *Streptococcus*

*illumi*gene® Molecular Diagnostic System



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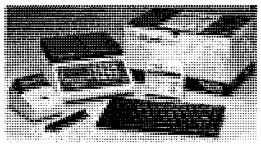
illumigene® Pertussis

*illumi*gene® Molecular Diagnostic System



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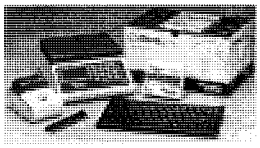
illumigene® Group A *Streptococcus*

*illumi*gene® Molecular Diagnostic System



The *illumigene®* Molecular Diagnostic System is simple, delivering the power of molecular technology in a "rapid test" type format; flexible, integrating easily into any facility regardless of volume or expertise; and cost-effective, allowing addition of molecular assays without the cost of additional labor or capital expenditures.

illumigene® Mycoplasma

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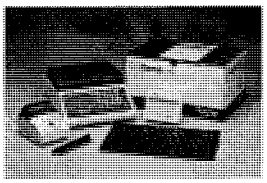
Products: *illumigene®* Molecular Diagnostic System

*illumi*gene® *C. difficile*



NEW molecular assay for the detection of *C. difficile* in stool.
Simple, flexible, cost-effective molecular assay for *C. difficile* detection. Allows addition of molecular assays without the cost of additional labor or capital expenditures.

Learn more about *illumigene®* and LAMP molecular technology

Product Details

Meridian Catalogue #:	280050
Kit Size	50 Tests
Shelf Life from date of manufacture	18 Months
Storage Requirements	21°-27° C/External Controls 2°-8° C
Specimen Type	Unformed stool
Assay Format	Isothermal DNA amplification
CE Marked	Yes
Sensitivity	95.2%
Specificity	95.3%
Hazardous	No
Country of Origin	USA
Available Within/Outside USA	Yes
Suggested CPT Code	87493 C. difficile amplified probe
Weight	
Results Interpretation	By *Illumipro*-10™ incubator/reader Negative = No turbidity Positive = Turbidity

More Product Information

Package Insert: *illumigene®* *C. difficile*

Package Insert: *illumigene®* *C. difficile* External Control

Procedure Card: *illumigene®* *C. difficile*

Procedure Video: *illumigene®* *C. difficile* Product Demonstration

Sales Sheet: *illumigene®* *C. difficile*

Product Sheet: *illumigene®* *C. difficile* work station

Product Sheet: *illumipro-10*™

CLSI: *illumigene®* *C. difficile*

MSDS: *illumigene®* S. Aureus Internal Control (ANSI)

MSDS: *illumigene®* Sample Preparation Apparatus (ANSI)

MSDS: *illumigene®* Stool Control Bead (ANSI)

MSDS: *illumigene®* Test Bead (ANSI)

MSDS: *illumigene®* Reaction Buffer (ANSI)

MSDS: *illumigene®* Positive Control (ANSI)

MSDS: *illumigene®* Negative Control (ANSI)

MSDS: *illumigene®* S. Aureus Internal Control (EU)

MSDS: *illumigene®* Sample Preparation Apparatus (EU)

MSDS: *illumigene®* Stool Control Bead (EU)

MSDS: *illumigene®* Test Bead (EU)

MSDS: *illumigene®* Reaction Buffer (EU)

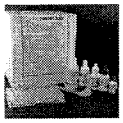
MSDS: *illumigene®* Positive Control (EU)

MSDS: *illumigene®* Negative Control (EU)

MSDS: *illumigene®* Negative Control Plasmid Stock (EU)

Related Products

NEW molecular assay for the detection of *Group B Streptococcus*.
View Product



ELISA for the detection of *C. difficile* toxins A&B in stool specimens.
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Rapid EIA for the detection of *C. difficile* toxins A&B in stool specimens.
View Product

next ❯

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THE PROMISE OF *illumigene*®
Molecular Simplified

The Promise of *illumigene*® empowers your lab with the speed and accuracy of molecular testing.

illumigene® delivers the power of molecular technology in a simplified and affordable format. Meridian Bioscience has employed Loop-Mediated Isothermal Amplification (LAMP) technology to design and manufacture the ***illumipro-10***™, a compact, automated detection system with a very simple assay process that is the heart of ***illumigene***®. Based on feedback from hundreds of laboratory professionals, ***illumigene***® is the molecular diagnostic system clinical labs have been waiting for.

Learn more about innovative LAMP technology

illumigene® is:

- Simple procedure with a smaller footprint
- Faster and More Flexible
- More cost-effective

illumigene® makes it possible for any laboratory—regardless of space, personnel, or budget limitations—to implement molecular diagnostic capabilities.

Simpler and Smaller
The simple 7-step sample preparation process takes less than 2 minutes of hands-on time per sample. The ***illumipro-10***™ offers walk-away automation. When you place the sample into the ***illumipro-10***™ and press the "run" button, your work is done.

With ***illumigene***® there is:

- No centrifugation, precision pipetting
- No thermal cycling
- No gel electrophoresis

The ***illumipro-10***™:

- Has a very small footprint—approximately the size of an 8.5" x 11" sheet of paper
- Internal and external keyboard as well as an internal bar code reader for flexible sample ID entry
- Provides continuous access while running up to 10 samples at a time in 2 independent 5-sample chambers
- Does not require an auxiliary computer

Learn more about the *illumipro-10*™ instrument

Learn more about the *illumigene*® *C. difficile* 7-step process

Faster and More Flexible
Because there is no thermal cycling, ***illumigene***® can provide **results for up to 10 samples in less than an hour.** Results are displayed directly on the ***illumipro-10***™ LED readout screen and can be printed out on the system printer. ***illumigene***® integrates easily into any facility, regardless of work volume or staff experience:

- Flexibility to stop and restart the assay procedure
- Adaptable to single sample or batch processing
- Optional work station adapts to any user or work flow

More Cost Effective
illumigene® allows your lab to add molecular diagnostics without additional costs for labor, training, and capital equipment. In your lab, ***illumigene***® means:

- Less than 2 minutes hands-on time per sample
- Minimal labor requirement
- Low maintenance with no additional service costs and 24-hour equipment replacement
- Reduced waste disposal costs from environmentally friendly, mercury-free process

Add *illumigene*® Capability to Your Lab
illumigene® is revolutionary technology that is simple, fast, and affordable. It puts molecular diagnostics within the reach of every lab.

Product Information

illumigene® System Brochure

Product Sheet: ***illumipro-10***™

Sell Sheet: *C. difficile* Assay

Product Sheet: ***illumigene***® work station

Sell Sheet: Lamp Process

Sell Sheet: LAMP vs PCR

Sell Sheet: Pediatric

Video: ***illumigene***® Assay Procedure

Video: LAMP Technology

Clinical Literature

ASM Abstract 1: A Novel New Molecular Approach for the Detection of *Clostridium difficile*

ASM Abstract 2: Reactivity of *Clostridium difficile* Toxinotypes with the ***illumigene***® *C. difficile* Molecular Assay

ASM Abstract 3: A New Molecular Approach for the Detection of *Clostridium difficile*

CVS Abstract: A New Molecular Approach for the Detection of *Clostridium difficile*

illumigene®: Summary of Features and Benefits	
Features	**Benefits**
Simple 7-Step Procedure	Assay able to be performed by any technologist regardless of experience No off-site training required Fast implementation into laboratory workflow Minimizes hands-on time (~2 minutes/sample)
Highly Accurate Results	No algorithms needed to achieve high performance Peace of mind/confidence in results
Rapid turnaround time—results in under an hour	Adapts to any laboratory's workflow Provides quicker results to physicians—better patient care
Small system footprint	Fits into any lab space No remodeling of lab required to add systems/assays
No capital equipment burden	Very cost-effective solution for any facility No additional charge for service agreement 24 hour unit replacement
Room temperature storage of kits	No refrigeration space required No equilibration steps required—ready to run at all times
Optional System Accessories	Flexible printing options in minimal bench space QWERTY keyboard and built-in barcode reader allows for flexible sample ID entry Workstation allows for organization of procedural workflow

For more information or to order your *illumigene*® System, call: **800-543-1980**.

Return to "Diagnosing *C. diff* Infection"

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illumipro-10™

Automated isothermal amplification and detection system that provides fast, accurate results in a flexible package that fits into any lab.

- Optimal flexibility for stat or batch runs
- True walk away capability for optimized workflow
- Very low maintenance requirements enhance efficiency
- Instrument verifications can be performed with in-house personnel

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illumipro-10™

Digital display guides users through process

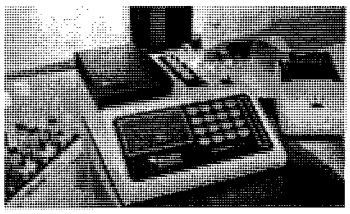
Reaction blocks operate independently

Automatic system self-check

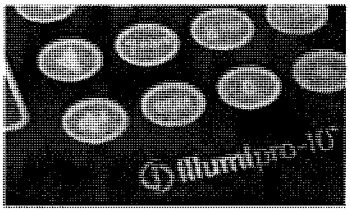
Small footprint of instrument is only 8.5" x 11"

Barcode reader for sample ID entry

Procedure



1. Load samples



2. Press "RUN"

3. Enter patient ID

Ordering information

illumipro-10™ Incubator Reader	610172
illumipro-10™ Printer	610173
illumipro-10™ Keyboard	610174
illumipro-10™ Operations Manual	11007

For more information, contact an *illumigene*® specialist at 1-888-763-6769 or visit us on the web at www.meridianbioscience.com.

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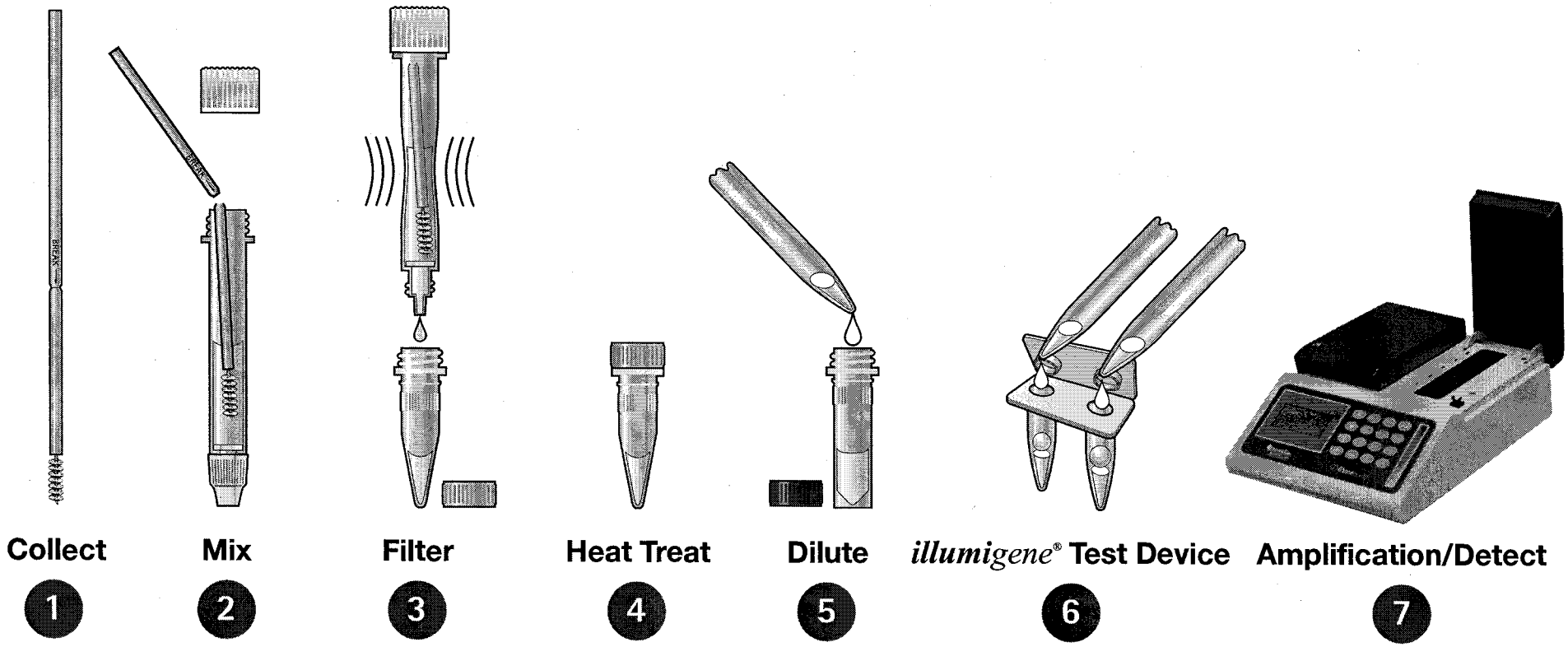
illumigene® C. difficile Assay Procedure



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- Comply with testing guidelines
- Molecular performance provides increased sensitivity
- A new standard for patient care

DECREASE WORKLOAD

- Simplified procedure
- Reduced turnaround time

DEFINITIVE ANSWERS, CONFIDENT RESULTS

- Accurate results for improved patient care
- Optimal information for the clinician

OPTIMAL FOR YOUR LABORATORY

- Rapid results means better patient care
- Procedural flexibility to adapt to workflow
- Molecular accuracy

OPTIMAL FOR YOUR BOTTOM LINE

- Adaptable to centralized or decentralized testing
- Cost-effective per test price (includes capital equipment)
- Reduces costs associated with slow and inaccurate results

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SPEED AND ACCURACY OF MOLECULAR TESTING

— THE NEW STANDARD FOR PATIENT CARE —

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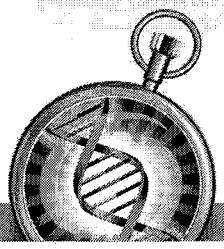

TEST TOMORROW

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ORDERING INFORMATION

PRODUCT	MERIDIAN CATALOG NUMBER
illumipro-10™ Incubator Reader	610172
illumipro-10™ Printer	610173
illumipro-10™ Keyboard	610174
illumigene® Workstation	610169
AVAILABLE ASSAYS	
illumigene® C. difficile Assay	260050
illumigene® External Controls C. difficile	270030
illumigene® Group B Streptococcus Assay	260050
illumigene® External Controls Group B Streptococcus	270600

For more information, contact an *illumigene®* specialist at 1-888-763-6769 or visit us on the web at www.meridianbioscience.com.

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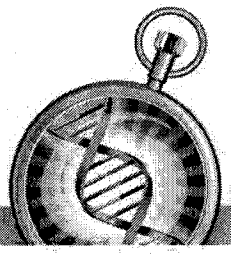
Australian Technology Park
Eveleigh NSW 2015
Telephone . . . +61 (0)2 9209 4180
Fax . . . +61 (0)2 9209 4763
Email: info.aust@bioline.com



ORDER TODAY TEST TOMORROW

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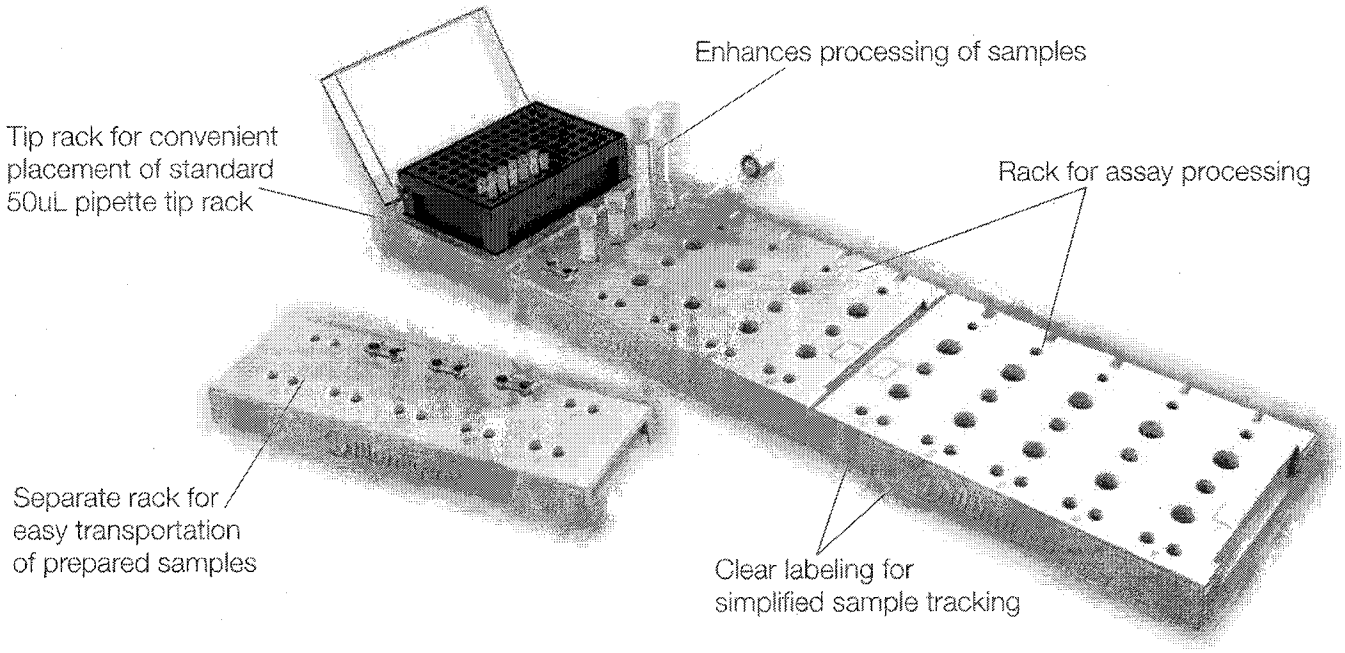
illumigene® Assay Workstation

Optimizing flexibility and efficiency for your molecular assay processing.

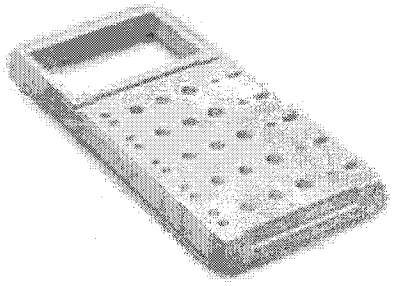


- **Optimized flexibility**
  - — Configurable to small or large batch sizes
  - — Adjustable to left- or right-handed operators
- **Enhanced efficiency**
  - — Simple tracking of samples throughout workflow
  - — ***illumigene®*** device transport rack matches ***illumipro-10™*** configuration
- **Incredible ease of use**
  - — Clean/decontaminate through simple bleach submersion
  - — Quick assembly/disassembly/reconfiguration of all parts

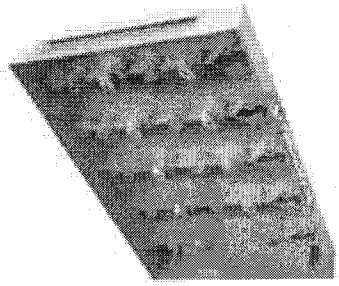
THE PROMISE OF illumigene® Molecular Simplified

Meridian Bioscience, Inc.® Inspired Science. Trusted Solutions.®

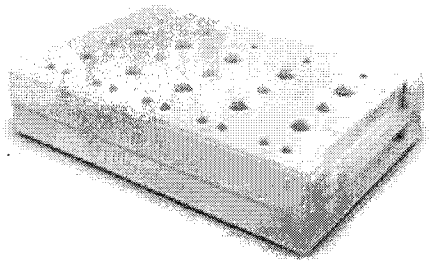
illumigene® Molecular Diagnostic System



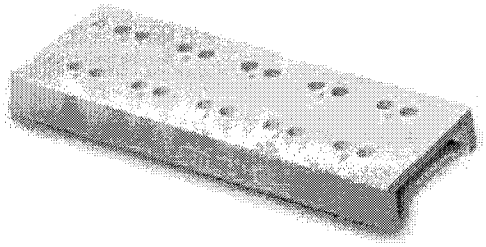
Flexible interlocking design allows for adaptation to run sizes as well as left- or right-handed operation.



Flow through wells for ease of cleaning and draining for optimized contamination control.



Interlocking stackable design to facilitate storage and maintain low footprint of ***illumigene***® system.



Separate sample transport block matched with ***illumipro-10***™ design to enhance ease of use and tracking of patient samples.

The ***illumigene***® workstation is designed to enhance the simplicity, efficiency, and workflow of the ***illumigene***® assay system. Another innovation from the unparalleled global leader in medical diagnostics.

Ordering information

illumigene® Workstation	610169

For more information, contact an ***illumigene***® specialist at 1-888-763-6769 or visit us on the web at www.meridianbioscience.com.

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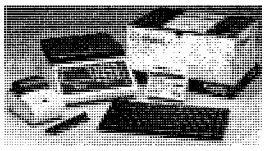
Products: *illumigene®* Molecular Diagnostic System

*illumi*gene® Group A *Streptococcus*



NEW molecular assay for the detection of Group A *Streptococcus*
Group A *Streptococcus* is a bacterium commonly found in the human throat which causes acute pharyngitis, or strep throat. Accurate diagnosis of streptococcal pharyngitis followed by appropriate antibiotic therapy is important for the prevention and control of Group A Strep infections. ***illumigene®*** Group A Strep detects *Streptococcus pyogenes* in throat swab specimens. The test procedure is remarkably simple and highly sensitive. ***illumigene®*** Group A Strep is ideal for helping in early diagnosis and proper patient management of this common, sometimes serious disease.

Learn more about *illumigene®* and LAMP molecular technology

Product Details

Meridian Catalogue #:	280150
Kit Size	50 Tests
Shelf Life from date of manufacture	18 Months
Storage Requirements	2°-27° C/External Controls 2°-8° C
Specimen Type	Throat swab sample (Liquid Amies, without charcoal ; Liquid Stuart)
Assay Format	Isothermal DNA amplification
CE Marked	Yes
Sensitivity	98.0%
Specificity	97.7%
Hazardous	No
Country of Origin	USA
Available Within/Outside USA	Yes
Suggested CPT Code	87651
Results Interpretation	By *illumipro*-10™ incubator/reader Negative Positive

More Product Information

Sales Sheet: ***illumigene®*** Group A *Streptococcus*

Package Insert: ***illumigene®*** Group A *Streptococcus*

Package Insert: ***illumigene®*** Group A *Streptococcus* External Control

Procedure Card: ***illumigene®*** Group A *Streptococcus*

Procedure Video: ***illumigene®*** Group A *Streptococcus* Product Demonstration

CLSI: ***illumigene®*** Group A *Streptococcus*

MSDS: ***illumigene®*** Group A *Streptococcus* Positive Control (ANSI)

MSDS: ***illumigene®*** Group A *Streptococcus* Control Bead (ANSI)

MSDS: ***illumigene®*** Group A *Streptococcus* Sample Preparation Apparatus (ANSI)

MSDS: ***illumigene®*** Group A *Streptococcus* Test Bead (ANSI)

MSDS: ***illumigene®*** Group A *Streptococcus* External Positive Control (EU)

MSDS: ***illumigene®*** Group A *Streptococcus* Control Bead (EU)

MSDS: ***illumigene®*** Group A *Streptococcus* Sample Preparation Apparatus (EU)

MSDS: ***illumigene®*** Group A *Streptococcus* Test Bead (EU)

MSDS: ***illumigene®*** Group A *Streptococcus* Negative Control (EU)

MSDS: ***illumigene®*** Assay Control Bulk (EU)

Related Products

NEW molecular assay for the detection of *C. difficile* in stool.
View Product

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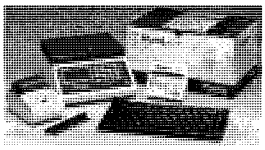
Products: *illumigene®* Molecular Diagnostic System

*illumi*gene® Group B *Streptococcus*



NEW molecular assay for the detection of *Group B Streptococcus*
Fully comply with the 2010 CDC guidelines by using the most sensitive assay on the market for *Group B Streptococcus* detection. The simplified procedure allows labs to report accurate, definitive results in less time than culture. This simple, cost-effective menu addition make this innovative test ideal for helping in early diagnosis and proper management of this preventable disease.

Learn more about *illumigene®* and LAMP molecular technology

Product Details

Meridian Catalogue #:	280350
Kit Size	50 Tests
Shelf Life from date of manufacture	18 Months
Storage Requirements	2°-27° C/External Controls 2°-8° C
Specimen Type	Vaginal/rectal swab specimens, antepartum
Assay Format	Isothermal DNA amplification
CE Marked	Yes
Sensitivity	98.6%
Specificity	93.2%
Hazardous	No
Country of Origin	USA
Available Within/Outside USA	Yes
Suggested CPT Code	87081,87653
Weight	
Results Interpretation	By *illumipro*-10™ incubator/reader Negative Positive

More Product Information

CLSI: *illumigene®* Group B *Streptococcus*

Sales Sheet: *illumigene®* Group B *Streptococcus*

Sales Sheet: LAMP Process

Product Sheet: illumipro-10™

Product Sheet: *illumigene®* workstation

Package Insert: *illumigene®* Group B *Streptococcus*

Package Insert: *illumigene* External Control® Group B *Streptococcus*

Procedure Card: *illumigene®* Group B *Streptococcus*

Procedure Video: *illumigene®* Group B *Streptococcus* Product Demonstration

MSDS:*illumigene®* Control (ANSI)

MSDS: *illumigene®* Group B *Streptococcus* Control Bead (ANSI)

MSDS: *illumigene®* Group B *Streptococcus* External Control (ANSI)

MSDS: *illumigene®* Group B *Streptococcus* Test Bead (ANSI)

MSDS: *illumigene®* Negative Control (ANSI)

MSDS: *illumigene®* Reaction Buffer (ANSI)

MSDS: *illumigene®* Control (EU)

MSDS: *illumigene®* Group B *Streptococcus* Control Bead (EU)

MSDS: *illumigene®* Group B *Streptococcus* External Control (EU)

MSDS: *illumigene®* Group B *Streptococcus* Test Bead (EU)

MSDS: *illumigene®* Negative Control (EU)

MSDS: *illumigene®* Negative Control Plasmid Stock (EU)

MSDS: *illumigene®* Reaction Buffer (EU)

Related Products

NEW molecular assay for the detection of *C. difficile* in stool.
View Product

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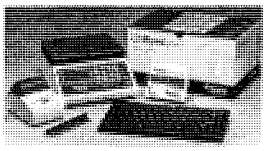
Products: *illumigene*® Molecular Diagnostic System

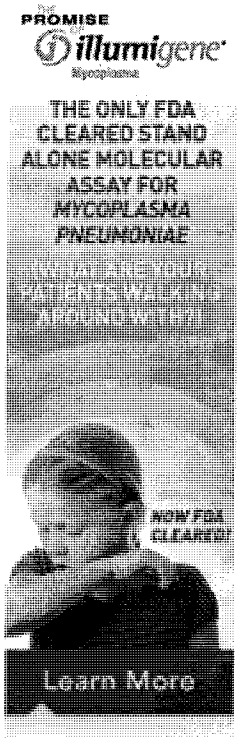
*illumi*gene® Mycoplasma



NEW molecular assay for the detection of *Mycoplasma pneumoniae*

M. pneumoniae has been associated with up to 40% of community-acquired pneumonias. Accurate diagnosis of *Mycoplasma pneumonia* is critical to provide appropriate antibiotic therapy. ***illumigene***® Mycoplasma detects *Mycoplasma pneumonia* in throat swab and nasopharyngeal swab specimens. ***illumigene***® Mycoplasma is ideal for enabling a more rapid diagnosis, providing earlier identification of outbreaks and prevention of secondary cases through implementation of control measures.

Learn more about *illumigene*® and LAMP molecular technology



Meridian Bioscience, Inc.

Product Details

Meridian Catalogue #:	280550
Kit Size	50 Tests
Shelf Life from date of manufacture	18 Months
Storage Requirements	2°- 8° C/External Controls 2°- 8° C
Specimen Type	Throat and nasopharyngeal swab specimens (0.85% Saline, M4, M4-RT, M5, UTM-RT)
Assay Format	Isothermal DNA amplification
CE Marked	Yes
Sensitivity	NP Swab: 100% ; Throat Swab: 100%
Specificity	NP Swab: 100% ; Throat Swab: 100%
Hazardous	No
Country of Origin	USA
Available Within/Outside USA	Yes
Suggested CPT Code	87581
Weight	
Results Interpretation	By *illumipro*-10™ Incubator/Reader Negative Positive

More Product Information

Sales Sheet: ***illumigene***® *Mycoplasma*

Package Insert: ***illumigene***® *Mycoplasma*

Package Insert: ***illumigene***® *Mycoplasma* External Control

Procedure Card: ***illumigene***® *Mycoplasma*

Procedure Video: ***illumigene***® *Mycoplasma* Product Demonstration

Product Sheet: LAMP Process

Product Sheet: illumipro-10™

Product Sheet: ***illumigene***® workstation

MSDS: ***illumigene***® Control II (ANSI)

MSDS: ***illumigene***® Control II (EU)

MSDS: ***illumigene***® Reaction Buffer II (ANSI)

MSDS: ***illumigene***® Reaction Buffer II (EU)

MSDS: ***illumigene***® *Mycoplasma* Control Bead (ANSI)

MSDS: ***illumigene***® *Mycoplasma* Control Bead (EU)

MSDS: ***illumigene***® *Mycoplasma* Test Bead (ANSI)

MSDS: ***illumigene***® *Mycoplasma* Test Bead (EU)

MSDS: ***illumigene***® *Mycoplasma* External Positive Control (ANSI)

MSDS: ***illumigene***® *Mycoplasma* External Positive Control (EU)

MSDS: ***illumigene***® External Negative Control II (ANSI)

MSDS: *illumigene*® External Negative Control II (EU)

MSDS: *illumigene*® Assay Control Bulk (EU)

Related Products

NEW molecular assay for the detection of *Group A Streptococcus.*
View Product

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Products: *illumigene®* Molecular Diagnostic System

*illumi*gene® Pertussis

NEW molecular assay for the detection of *Bordetella pertussis*.
This innovative test is the only FDA cleared stand-alone molecular assay for *Bordetella pertussis* providing a solution to the unmet need of today's healthcare market. ***illumigene®*** provides results in less than 1 hour. Collect, test and treat same day for optimal patient management.

Learn more about ***illumigene®*** and LAMP molecular technology

Product Details

Meridian Catalogue #:	280750
Kit Size	50 Tests
Shelf Life from date of manufacture	18 Months
Storage Requirements	2°- 30° C/External Controls 2°- 8° C
Specimen Type	Nasopharyngeal Swab Specimens
Assay Format	Isothermal DNA amplification
CE Marked	Yes
Sensitivity	87.8%
Specificity	97.8%
Hazardous	No
Country of Origin	USA
Available Within/Outside USA	Yes
Suggested CPT Code	87798
Weight	
Results Interpretation	By *illumipro*-10™ Incubator/Reader Negative = No turbidity Positive = Turbidity

More Product Information

CLSI: ***illumigene®*** *Pertussis*

Package Insert: ***illumigene®*** *Pertussis*

Package Insert: ***illumigene®*** *Pertussis* External Control

Sales Sheet: ***illumigene®*** *Pertussis*

Procedure Video: ***illumigene®*** *Pertussis*

Product Sheet: LAMP Process

Product Sheet: illumipro-10™

Product Sheet: ***illumigene®*** workstation

MSDS: ***illumigene®*** *Pertussis* Control Lyosphere (ANSI)

MSDS: ***illumigene®*** *Pertussis* Test Lyosphere (ANSI)

MSDS: ***illumigene®*** *Pertussis* Negative Control (ANSI)

MSDS: ***illumigene®*** *Pertussis* Mineral Oil (ANSI)

MSDS: ***illumigene®*** *Pertussis* Positive Control (ANSI)

MSDS: ***illumigene®*** *Pertussis* Sample Buffer (ANSI)

MSDS: ***illumigene®*** *Pertussis* Control Bulk (ANSI)

MSDS: ***illumigene®*** Control II (EU)

MSDS: ***illumigene®*** Reaction Buffer II (EU)

MSDS: ***illumigene®*** External Negative Control II (EU)

MSDS: ***illumigene®*** *Pertussis* Control Bead (EU)

MSDS: ***illumigene®*** *Pertussis* Test Bead (EU)

MSDS: ***illumigene®*** *Pertussis* Negative Control (EU)

MSDS: ***illumigene®*** *Pertussis* Mineral Oil (EU)

MSDS: ***illumigene®*** *Pertussis* Positive Control (EU)

MSDS: ***illumigene®*** *Pertussis* Sample Buffer (EU)

MSDS: ***illumigene®*** Assay Control Bulk (EU)

Related Products

NEW molecular assay for the detection of Mycoplasma.
View Product

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Overview

Overview of *C. difficile* infection (CDI)*

Clostridium difficile is a gram positive, spore-forming bacillus that produces two exotoxins: toxin A and toxin B. These toxins are responsible for the diarrhea and inflammation seen in infected patients. Toxin A is an enterotoxin that can cause extensive mucosal damage and is an activator of mast cells and neutrophils, which trigger inflammatory mediators. Toxin B is a cytotoxin that is known to cause morphological changes in tissue culture cells.[4]

C. diff is the most commonly recognized cause of diarrhea in hospitalized patients and in patients in long term care facilities. Approximately 15% of antibiotic associated diarrhea can be attributed to CDI.[5] Most CDI cases are healthcare facility acquired.[6] However, a recent study has noted a trend toward community associated disease.[7] The estimated annual cost of CDI in 2007 ranged from 1.14 to 1.62 billion dollars.[8]

In the last decade, a *C. diff* strain, BI/NAP1/027, thought to be more virulent has become widely prevalent, possibly due to its facility for sporulation.[4] It produces more of toxins A and B than other strains as well as a 3rd binary toxin.[4] However, there is evidence from clinical studies that BI/NAP1/027 may not produce disease that is more virulent than that produced by other *C. diff* strains when factors such as age are controlled.[9,10]

*Some sources refer to the illness as *C. difficile*-associated disease (CDAD).

Association of Professionals in Infection Control (APIC) statistical portrait of CDI patients[11]

- 55.9% were female, 44.1% male
- 84.7% were on the medical service
- 69.2% were >60 years of age
- 57.9% had an initial episode of mild or moderate disease
- 10.94% had severe to complicated disease
- 72.5% were considered healthcare-associated infections
- 45.5% were detected >48 hours after admission
- 26.6% required ICU admission, 18.2% had shock, and 16.5% required vasopressors
- 79.4% had antimicrobial before onset
- 47.4% had hospitalization within 90 days of onset
- 46.5% had resolution of diarrhea within six days

Find information on the differential diagnosis and diagnostic protocol for CDI

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Overview

Overview of Group A *Streptococcus*

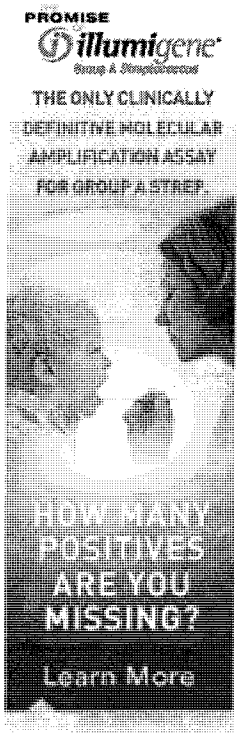
Streptococcus pyogenes (also known as Group A *Streptococcus*, or GAS) is a gram-positive, beta hemolytic bacteria. It is comprised of at least 80 serotypes, which makes developing a vaccine difficult.[1] Group A Streptococcus is a bacterium commonly found in the human throat or on skin and causes a wide variety of diseases in humans, the most common being acute pharyngitis or strep throat. Group A *Streptococcus* infections may result in mild illness (e.g. pharyngitis, impetigo) or may lead to invasive, life-threatening illness such as acute rheumatic fever, cellulitis, bacteremia, necrotizing fasciitis, or *Streptococcal* toxic shock syndrome.

Accurate diagnosis of streptococcal pharyngitis followed by appropriate antibiotic therapy is important for the prevention and control of Group A Strep infections.

Person-to-person transmission of Group A Strep can occur from contact with mucus of the nose or throat from infected persons and wounds or sores on the skin. Asymptomatic carriers less likely to pass infection but is possible.

Acute pharyngitis is one of the most frequent illnesses for which pediatricians and other primary care physicians are consulted, with an estimated 15 million visits per year in the United States. Approximately 20-30% of children seeking physician care are diagnosed with Group A Strep pharyngitis. Traditional laboratory diagnosis is performed by rapid antigen testing or throat swab culture. Studies show that culture may miss up to 10% of true positives.[3]

The societal cost of Group A Strep among children in the United States ranges from $224 to $539 million per year.[2] Worldwide, approximately 18.1 million people suffer from a serious Group A Strep disease.[4]



Meridian Bioscience, Inc.

Related Products

NEW molecular assay for the detection of *C. difficile* in stool.
View Product

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Overview

Overview of Group B *Streptococcus*

Invasive Group B *Streptococcal* disease emerged in the 1970's as the leading cause of infectious disease in infants. Infants with early onset infection (<7 days of age) may show symptoms of respiratory distress, apnea or sepsis. Early-onset GBS disease is most commonly associated with sepsis and pneumonia, however it may lead to meningitis.

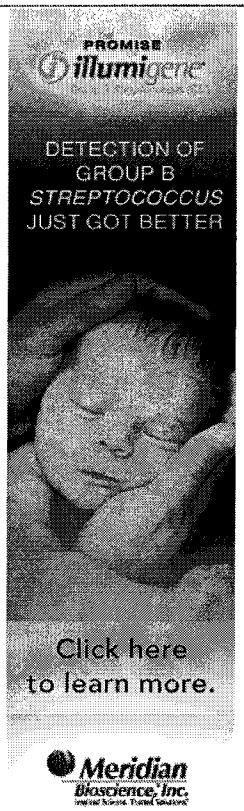
The fatality rate for infants with early-onset GBS disease is currently estimated at 4%-6%. Surviving infants may experience long-term disabilities including hearing loss, vision loss, or mental retardation. The primary risk factor for early-onset GBS disease is maternal colonization.

If detected, intrapartum transmission of Group B *Streptococcus* or early-onset GBS disease can be prevented by administration of antibiotic prophylaxis prior to delivery. Traditional culture has been the standard of care for most laboratories in North America for many years, but the significant potential for false negatives is creating a shift in testing.

Molecular Testing for Group B *Streptococcus*

Molecular testing is growing due to its ability to provide more accurate results than traditional culture methods. ***illumigene*®** Group B *Streptococcus* (GBS) exceeds the performance of current Group B Streptococcus test methods. ***illumigene*®** GBS fully complies with the CDC guidelines for antepartum testing at 35 - 37 weeks gestation while allowing the flexibility of testing with multiple broth-enriched specimen types (Lim, TransVag, Carrot).

***illumigene*®** GBS provides definitive answers and confident results, putting the right information at the right time in the hands of physicians. Reliable results make it possible for mothers to be treated at the time of delivery, ensuring the best outcome for baby.



Related Products

NEW molecular assay for the detection of *C. difficile* in stool.
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Exhibit 73

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Mark: ILLUMINOTES

ILLUMINOTES

US Serial Number: 86373206

Application Filing Date: Aug. 21, 2014

Register: Principal

Mark Type: Service Mark

Status: New application will be assigned to an examining attorney approximately 3 months after filing date.

Status Date: Sep. 04, 2014

Mark Information

Mark Literal Elements: ILLUMINOTES

Standard Character Claim: Yes. The mark consists of standard characters without claim to any particular font style, size, or color.

Mark Drawing Type: 4 - STANDARD CHARACTER MARK

Goods and Services

Note: The following symbols indicate that the registrant/owner has amended the goods/services:

- Brackets [..] indicate deleted goods/services;
- Double parenthesis ((..)) identify any goods/services not claimed in a Section 15 affidavit of incontestability; and
- Asterisks *..* identify additional (new) wording in the goods/services.

For: Non-downloadable electronic newsletters in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics; online electronic newsletters delivered via email in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics

International Class(es): 041 - Primary Class

U.S Class(es): 100, 101, 107

Class Status: ACTIVE

Basis: 1(a)

First Use: Apr. 20, 2006

Use in Commerce: Apr. 20, 2006

Basis Information (Case Level)

Filed Use:	Yes	**Currently Use:**	Yes	**Amended Use:**	No
Filed ITU:	No	**Currently ITU:**	No	**Amended ITU:**	No
Filed 44D:	No	**Currently 44D:**	No	**Amended 44D:**	No
Filed 44E:	No	**Currently 44E:**	No	**Amended 44E:**	No
Filed 66A:	No	**Currently 66A:**	No		
Filed No Basis:	No	**Currently No Basis:**	No		

Current Owner(s) Information

Owner Name: Illumina, Inc.

Owner Address: 5200 Illumina Way
San Diego, CALIFORNIA 92122
UNITED STATES

Legal Entity Type: CORPORATION

State or Country Where Organized: DELAWARE

Attorney/Correspondence Information

Attorney of Record

Attorney Name: Lisa M. Martens

Docket Number: 39319-008700

Attorney Primary Email Address: tmdoctc@fr.com

Attorney Email Authorized: Yes

Correspondent

Correspondent Name/Address: LISA M. MARTENS
FISH & RICHARDSON P.C.
PO BOX 1022
MINNEAPOLIS, MINNESOTA 55440-1022

UNITED STATES

Phone: (858) 678-5070 **Fax:** (858) 678-5099

Correspondent e-mail: tmdoctc@fr.com **Correspondent e-mail Authorized:** Yes

Domestic Representative - Not Found

Prosecution History

Date	Description	Proceeding Number
Sep. 04, 2014	NEW APPLICATION OFFICE SUPPLIED DATA ENTERED IN TRAM	
Aug. 25, 2014	NEW APPLICATION ENTERED IN TRAM	

TM Staff and Location Information

TM Staff Information - None

File Location

Current Location: NEW APPLICATION PROCESSING **Date in Location:** Sep. 04, 2014

PTO Form 1478 (Rev 9/2006)
OMB No. 0651-0009 (Exp 12/31/2014)

Trademark/Service Mark Application, Principal Register

Serial Number: 86373206
Filing Date: 08/21/2014

The table below presents the data as entered.

Input Field	Entered
SERIAL NUMBER	86373206
MARK INFORMATION	
***MARK**	ILLUMINOTES
STANDARD CHARACTERS	YES
USPTO-GENERATED IMAGE	YES
LITERAL ELEMENT	ILLUMINOTES
MARK STATEMENT	The mark consists of standard characters, without claim to any particular font, style, size, or color.
REGISTER	Principal
APPLICANT INFORMATION	
***OWNER OF MARK**	Illumina, Inc.
***STREET**	5200 Illumina Way
***CITY**	San Diego
***STATE (Required for U.S. applicants)**	California
***COUNTRY**	United States
***ZIP/POSTAL CODE (Required for U.S. applicants only)**	92122
LEGAL ENTITY INFORMATION	
TYPE	corporation

STATE/COUNTRY OF INCORPORATION	Delaware
GOODS AND/OR SERVICES AND BASIS INFORMATION	
INTERNATIONAL CLASS	041
*** IDENTIFICATION**	Non-downloadable electronic newsletters in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics; online electronic newsletters delivered via email in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics
FILING BASIS	SECTION 1(a)
FIRST USE ANYWHERE DATE	At least as early as 04/20/2006
FIRST USE IN COMMERCE DATE	At least as early as 04/20/2006
SPECIMEN FILE NAME(S)	
ORIGINAL PDF FILE	SPE0-6715112910-184934995_._TDMK_Specimen.pdf
CONVERTED PDF FILE(S) (1 page)	\\TICRS\EXPORT16\IMAGEOUT16\863\732\86373206\xml1\APP0003.JPG
SPECIMEN DESCRIPTION	Copy of electronic newsletter
ATTORNEY INFORMATION	
NAME	Lisa M. Martens
ATTORNEY DOCKET NUMBER	39319-0087001
FIRM NAME	Fish & Richardson P.C.
STREET	P.O. Box 1022
CITY	Minneapolis
STATE	Minnesota
COUNTRY	United States
ZIP/POSTAL CODE	55440-1022

PHONE	(858) 678-5070
FAX	(858) 678-5099
EMAIL ADDRESS	tmdoctc@fr.com
AUTHORIZED TO COMMUNICATE VIA EMAIL	Yes
OTHER APPOINTED ATTORNEY	Erin M. Hickey, Nancy L. Ly, Timothy A. French, Cynthia Johnson Walden
CORRESPONDENCE INFORMATION	
NAME	Lisa M. Martens
FIRM NAME	Fish & Richardson P.C.
STREET	P.O. Box 1022
CITY	Minneapolis
STATE	Minnesota
COUNTRY	United States
ZIP/POSTAL CODE	55440-1022
PHONE	(858) 678-5070
FAX	(858) 678-5099
EMAIL ADDRESS	tmdoctc@fr.com
AUTHORIZED TO COMMUNICATE VIA EMAIL	Yes
FEE INFORMATION	
NUMBER OF CLASSES	1
FEE PER CLASS	325
***TOTAL FEE DUE**	325
***TOTAL FEE PAID**	325
SIGNATURE INFORMATION	
SIGNATURE	/Will Morrison/
SIGNATORY'S NAME	Will Morrison
SIGNATORY'S POSITION	Attorney

DATE SIGNED	08/20/2014

PTO Form 1478 (Rev 9/2006)
OMB No. 0651-0009 (Exp 12/31/2014)

Trademark/Service Mark Application, Principal Register

Serial Number: 86373206
Filing Date: 08/21/2014

To the Commissioner for Trademarks:

MARK: ILLUMINOTES (Standard Characters, see mark)
The literal element of the mark consists of ILLUMINOTES.
The mark consists of standard characters, without claim to any particular font, style, size, or color.

The applicant, Illumina, Inc., a corporation of Delaware, having an address of
5200 Illumina Way
San Diego, California 92122
United States

requests registration of the trademark/service mark identified above in the United States Patent and Trademark Office on the Principal Register established by the Act of July 5, 1946 (15 U.S.C. Section 1051 et seq.), as amended, for the following:

International Class 041: Non-downloadable electronic newsletters in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics; online electronic newsletters delivered via email in the fields of nucleic acid sequencing and genotyping, medical diagnostics, medical research, life sciences, biology, molecular pathology, molecular diagnostics, laboratory medicine, biotechnology, and genetics

In International Class 041, the mark was first used by the applicant or the applicant's related company or licensee or predecessor in interest at least as early as 04/20/2006, and first used in commerce at least as early as 04/20/2006, and is now in use in such commerce. The applicant is submitting one(or more) specimen(s) showing the mark as used in commerce on or in connection with any item in the class of listed goods and/or services, consisting of a(n) Copy of electronic newsletter.

Original PDF file:
SPE0-6715112910-184934995_._TDMK_Specimen.pdf
Converted PDF file(s) (1 page)
Specimen File1

The applicant's current Attorney Information:
Lisa M. Martens and Erin M. Hickey, Nancy L. Ly, Timothy A. French, Cynthia Johnson Walden of Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022

United States

The attorney docket/reference number is 39319-0087001.

The applicant's current Correspondence Information:

Lisa M. Martens
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022
(858) 678-5070(phone)
(858) 678-5099(fax)
tmdoctc@fr.com (authorized)

A fee payment in the amount of $325 has been submitted with the application, representing payment for 1 class(es).

Declaration

The signatory believes that: if the applicant is filing the application under 15 U.S.C. Section 1051(a), the applicant is the owner of the trademark/service mark sought to be registered; the applicant or the applicant's related company or licensee is using the mark in commerce on or in connection with the goods/services in the application, and such use by the applicant's related company or licensee inures to the benefit of the applicant; the specimen(s) shows the mark as used on or in connection with the goods/services in the application; and/or if the applicant filed an application under 15 U.S.C. Section 1051(b), Section 1126(d), and/or Section 1126(e), the applicant is entitled to use the mark in commerce; the applicant has a bona fide intention to use or use through the applicant's related company or licensee the mark in commerce on or in connection with the goods/services in the application. The signatory believes that to the best of the signatory's knowledge and belief, no other person has the right to use the mark in commerce, either in the identical form or in such near resemblance as to be likely, when used on or in connection with the goods/services of such other person, to cause confusion or mistake, or to deceive. The signatory being warned that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. Section 1001, and that such willful false statements and the like may jeopardize the validity of the application or any registration resulting therefrom, declares that all statements made of his/her own knowledge are true and all statements made on information and belief are believed to be true.

Declaration Signature

Signature: /Will Morrison/ Date: 08/20/2014
Signatory's Name: Will Morrison
Signatory's Position: Attorney
RAM Sale Number: 86373206
RAM Accounting Date: 08/21/2014

Serial Number: 86373206
Internet Transmission Date: Thu Aug 21 12:31:43 EDT 2014
TEAS Stamp: USPTO/BAS-67.151.129.10-20140821123143169
9772-86373206-500cb1d862942c9294da5b3c3a
f12bfaba8beae823b203eecc92dbd557ceea379d
7-DA-11403-20140820184934995170

ILLUMINOTES

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From: Community [redacted]
To: [redacted]
Cc:
Subject: April Illuminotes: Cancer Research Products, SDS Search Tool, HiSeq v4 Reagents, and PsychArray BeadChip

Sent: Tue 4/15/2014 10:11 AM

www.illumina.com

Illuminotes

April 2014

Illumina Product Portfolio Expands to Support Cancer Research

Recent additions to the Illumina portfolio are designed to empower oncological research:

- The TruSeq® RNA Access Library Prep Kit reduces the sequencing depth required for FFPE and other degraded samples by isolating coding regions from human RNA.
- Using expert-defined content, the TruSight™ Myeloid Sequencing Panel enables clinical research labs to interrogate 54 genes and exonic regions associated with

Exhibit 74

TiE South Coast

Fostering Entrepreneurship Globally

SEARCH

This Site All TiE

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About TiE & TiE South Coast

From its modest beginnings in Silicon Valley, The Indus Entrepreneurs (TiE) has evolved into one of the most vibrant and well-known non-profit organizations in the world. Created in 1992, TiE's mission is to enrich foster and facilitate entrepreneurship throughout the community. TiE has evolved into a vibrant global organization of more than 13,000 members and 56 chapters in 12 countries. While TiE itself is a non-profit organization, the original chapter, TiE Silicon Valley, has long been recognized as the launch pad for hundreds of successful companies that have created an estimated $75 billion in value. Today TiE has become a powerful network of worldwide influence and is growing at a breathtaking pace.

"TiE" stands for "The Indus Entrepreneurs," and while its roots originated with US entrepreneurs from India, TiE also stands for "Talent, Ideas, Enterprise." TiE's membership and benefits are open to all with members consisting of entrepreneurs, executives, investors, academics, consultants, service providers and mainstream professionals from a multitude of industries.

TiE San Diego was inaugurated in June 2000 largely in response to tremendous economic growth and entrepreneurial spirit in "America's Finest City." The Chapter achieves its goals by hosting monthly events in which prominent speakers share their experiences, wisdom and insights. There is also ample opportunity for business networking which is an integral part of the chapter.

In May 2011, TiE Orange County merged with TiE San Diego and the new chapter is called - TiE South Coast. TiE South Coast is the 7th largest TiE Chapter from the 56 chapters worldwide.

Objectives:

* Foster entrepreneurship within the community
* Nurture entrepreneurs through mentoring
* Provide a networking platform for TiE-South Coast members
* Enhance business acumen via TiE Institute educational programs
* Encourage global networking with other TiE chapters around the world

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Exhibit 75

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- The Queens award for enterprise international trade 2013
- LIFE SCIENCE EMPLOYER OF THE YEAR AWARD WINNER 2014

Join the community

Working for Scientists

SelectScience, with its expert scientific reviews, allows for fast, impartial opinion about laboratory equipment and techniques. Through SelectScience, scientists can easily select and contact manufacturers

Working for Manufacturers What Our Customers Say

SelectScience is a game changer, as it facilitates more meaningful conversations with scientific buyers. This allows brand promotion, marketing effectiveness, and actually creates demand for products and increased sales.

Working for SelectScience What Our Staff Say

SelectScience has attracted the brightest and best professionals in the industry and we enable our staff to achieve their own personal career aspirations and be the best they can be.

We foster an environment where talented and ambitious individuals can flourish and employees are empowered to directly contribute to business results.

Our mission is to inspire and motivate, celebrate and reward success, and to help people to fulfill their potential and the goals of the company.

Meet the team >>

Career development

We have a culture of success, achievement and development for all staff, nurtured from the top level down and enabled by our significant growth.

Competitive salary

You can expect exciting earning potential and salary reviews that reflect the results you deliver.

Working environment

We look after the wellbeing of staff - you choose between a standard office chair or gym ball.

Full benefits

Along with a comprehensive benefits package, you'll experience a culture where personal development is nurtured and encouraged, a fabulous location, free parking and pension scheme.

Social events

We enjoy brunch, casual Fridays, gifts to celebrate birthdays and regular staff social events.

Location

With employees spread globally, our UK head office is nestled in beautiful countryside between Bath and Bristol.

Award winning

Join a leading, award-winning team that recently received the Queen's Award and was named Life Sciences Employer of the Year.

Training

We'll send you on any training you identify, as long as it benefits you and your job.

Current Vacancies

> US Sales Manager, Mid-Atlantic

Following business growth, we have a great opportunity in sales covering the Mid-Atlantic territory. You will be responsible for selling online marketing solutions and advertising to businesses in the science industry.

More info

> European Sales Manager, home based

We have an exciting opportunity for an exceptional individual to lead our sales team in Europe. With your own territory in Europe and a growing sales team to manage, this is a real opportunity to contribute to a growing, dynamic company

More info

> Editorial: Life Sciences, Bath, UK

Are you a scientist in Life Sciences looking for an exciting career in publishing? We are seeking to recruit Editorial staff to join our expanding team, with responsibility for defining and delivering a dedicated editorial/marketing plan.

More info

> Marketing Manager, Bath, UK

A new and exciting opportunity has arisen for a Marketing Manager within SelectScience. You will define and execute tactical marketing initiatives to help the global sales team achieve its plan and support a team to deliver innovative online campaigns and exceptional service to international clients.

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About

Our Mission

Xconomy is dedicated to providing business and technology leaders with timely, insightful, close-to-the-scene information about the local personalities, companies, and technological trends that best exemplify today's high-tech economy.

We are the authoritative voice on the exponential economy, the realm of business and innovation characterized by exponential technological growth and responsible for an increasing share of productivity and overall economic growth.

We deliver this valuable content through a unique global network of localized news sites, events, conferences, and other initiatives designed to better connect people and ideas.

Robert Buderi
Founder, CEO, Editor in Chief

Before launching Xconomy, Bob was a research fellow in MIT's Center for International Studies. He previously served as Editor in Chief of MIT's *Technology Review*, leading the magazine to numerous editorial and design awards and overseeing its expansion into three foreign markets, its introduction of electronic newsletters, and its organization of highly successful conferences. Earlier, as *BusinessWeek*'s technology editor, he shared in the 1992 National Magazine Award for "The Quality Imperative," a special issue of the magazine. Bob is also the author of three books about technology and innovation. *Guanxi* (2006) looks at Microsoft's Beijing research lab as a metaphor for global competitiveness. *Engines of Tomorrow* (2000) describes the evolution of corporate research. *The Invention That Changed the World* (1996) examines the work of a secret lab at MIT during WWII. Bob served on the Council on Competitiveness-sponsored National Innovation Initiative and as an advisor to the Draper Prize Nominating Committee. He has been a regular guest of CNBC's Strategy Session and has spoken about innovation to many organizations, including the Business Council, Amazon, eBay, Google, IBM, and Microsoft.

Rebecca Zacks
Cofounder, COO, Executive Editor

Rebecca was previously the managing editor of *Physician's First Watch*, a daily e-newsletter from the publishers of the *New England Journal of Medicine*. Before helping launch *First Watch*, she spent a decade covering innovation for *Technology Review*, *Scientific American*, and *Discover Magazine*'s TV show. In 2005-2006 she was a Knight Science Journalism Fellow at MIT. Rebecca holds a bachelor's degree in biology from Brown University and a master's in science journalism from Boston University.

Gregory T. Huang
Deputy Editor, and Editor, Xconomy Boston

Greg has covered science, technology, and business as a journalist. He was a features editor at *New Scientist* magazine, where he edited and wrote articles on physics, technology, and neuroscience. Previously he was senior writer at *Technology Review*, where he covered advances in computing, robotics, and devices. His writing has appeared in *Wired*, *Nature*, and *The Atlantic Monthly*'s website. Greg is the co-author of *Guanxi* (Simon & Schuster, 2006), about Microsoft in China. He was named a New York Times professional fellow in 2003. Before becoming a journalist, he did research at MIT's Artificial Intelligence Lab, and published 20 papers in scientific journals and conferences. He has a Master's and Ph.D. in electrical engineering and computer science from MIT, and a B.S. in electrical engineering from the University of Illinois, Urbana-Champaign.

Alex Lash
National Biotechnology Editor

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Our Team

Alex covers the life sciences with an emphasis on the San Francisco Bay Area. Before joining Xconomy, he ran biopharma coverage at the Elsevier publication Start-Up and wrote for its sister publications In Vivo and The Pink Sheet. Alex covered the first dot-com boom with CNET News and the Industry Standard and co-founded the high-tech satire magazine In Formation. In between (and sometimes during) staff jobs, he has also written for publications including Wired, ReadyMade, Popular Science, Architecture, SF Weekly, the San Francisco Bay Guardian, Chow, and Business 2.0. He lives in his hometown of San Francisco with his wife and daughters, plays baseball in a 30-and-over league, and grows lettuce in the summer fog.

Martin LaMonica
National Correspondent

Martin is a veteran journalist covering science, technology, and business from Cambridge, MA. He writes about energy and technology for MIT Technology Review as a contributing editor, the Boston Globe, the Guardian, Scientific American, IEEE Spectrum, and others. For ten years, he was senior editor at CNET where he covered clean tech, the Web, and tech companies. During the dotcom boom and bust, he was executive editor at enterprise IT publication InfoWorld and previously was the Paris correspondent for the IDG News Service. He graduated from Cornell University.

Curt Woodward
Senior Editor

Curt is an experienced journalist who has covered government, politics and business as a beat reporter—with some sports, agriculture, natural disasters and crime thrown in for good measure. He spent more than eight years with The Associated Press, mostly in Olympia, WA, covering the statehouse, political campaigns, and public policy. Highlights include being footnoted in a state Supreme Court ruling, having a story on government budget gimmicks cited in a college accounting text, and covering visits by the president, vice president, and several presidential candidates. His previous postings with AP included Seattle, Bismarck, ND, and Helena, MT. Curt has a bachelor's degree in journalism from Western Washington University in Bellingham, WA.

Ben Fidler
Deputy Biotechnology Editor

Ben is a seasoned business journalist that comes to Xconomy after a nine-year stint at The Deal, where he covered corporate transactions in industries ranging from biotech to auto parts and gaming. Most recently, Ben was The Deal's senior healthcare writer, focusing on acquisitions, venture financings, IPOs, partnerships and industry trends in the pharmaceutical, biotech, diagnostics and med tech spaces. Ben wrote features on creative biotech financing models, analyses of middle market and large cap buyouts, spin-offs and restructurings, and enterprise pieces on legal issues such as pay-for-delay agreements and the Affordable Care Act. Before switching to the healthcare beat, Ben was The Deal's senior bankruptcy reporter, covering the restructurings of the Texas Rangers, Phoenix Coyotes, GM, Delphi, Trump Entertainment Resorts and Blockbuster, among others. Ben has a bachelor's degree in English from Binghamton University.

Bruce V. Bigelow
Editor, Xconomy San Diego

Bruce joins Xconomy from the *The San Diego Union-Tribune*, where he was a longtime business reporter covering technology, aerospace, and other subjects. He was a member of the newsroom team awarded the 2006 Pulitzer Prize in National Reporting, and received other awards for disclosing the extraordinary casualty rate among San Diego-based Titan Corp.'s employees in Iraq, and for "The Toymaker," a 14-part chronicle of a San Diego startup company. He graduated from U.C. Berkeley with a B.A. in English Literature and has a M.S.J. from the Columbia University Graduate School of Journalism. Before joining the *Union-Tribune* in 1990, he worked for the Associated Press in Los Angeles and *The Kansas City Times*.

Michael Davidson
Editor, Xconomy Boulder/Denver

Michael Davidson is an awarding-winning journalist whose career as a business reporter has taken him from the garages of aspiring inventors to assembly centers for billion-dollar satellites. Most recently, Michael covered startups, venture capital, IT, cleantech, aerospace and telecoms as a reporter for the Boulder County Business Report, which gave him a close up view of the Boulder and Denver area's most innovative companies and entrepreneurs. Before switching to business journalism, Michael covered politics and the Colorado Legislature for the Colorado Springs Gazette and the government, police and crime beats for the Broomfield Enterprise, a paper in suburban Denver. He also worked for the Boulder Daily Camera, and his stories have appeared in the Denver Post and Rocky Mountain News. Career highlights include an award from the Colorado Press Association, doing barrel rolls in a vintage fighter jet and learning far more about public records than is healthy. Michael started his career as a copy editor for the Colorado Springs Gazette. Michael has a bachelor's degree in English from the University of Michigan.

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Jeff Engel
Editor, Xconomy Wisconsin

Jeff joins Xconomy from The Milwaukee Business Journal, where he covered manufacturing and technology and wrote about companies including Johnson Controls, Harley-Davidson and MillerCoors. He previously worked as the business and healthcare reporter for the Marshfield News-Herald in central Wisconsin. He graduated from Marquette University with a bachelor degree in journalism and Spanish. At Marquette he was an award-winning reporter and editor with The Marquette Tribune, the student newspaper. During college he also was a reporter intern for the Muskegon Chronicle and Grand Rapids Press in west Michigan.

Benjamin Romano
Editor, Xconomy Seattle

Benjamin has covered the intersections of business, technology and the environment in the Pacific Northwest and beyond for more than a decade. At The Seattle Times he was the lead beat reporter covering Microsoft during Bill Gates' transition from business to philanthropy. He also covered Seattle venture capital and biotech. Most recently, Benjamin followed the technology, finance and policies driving renewable energy development in the Western US for Recharge, a global trade publication. He has a bachelor's degree from the University of Oregon School of Journalism and Communication.

João-Pierre S. Ruth
Editor, Xconomy New York

After more than thirteen years as a business reporter in New Jersey, João-Pierre S. Ruth joins the ranks of Xconomy serving as a correspondent for its New York City branch. He has covered telecom players such as Verizon Wireless, device makers such as Samsung, and developers of organic LED technology such as Universal Display Corp. João-Pierre earned his bachelor's in English from Rutgers University.

Sarah Schmid
Editor, Xconomy Detroit

Sarah joins Xconomy Detroit after working in Communications for the Michigan Economic Development Corporation, the state's business development office, and the Michigan House of Representatives. She's an award-winning journalist who cut her teeth at the small but fiercely muckraking Missoula Independent, where she carved out a beat covering issues critical to Native American people living in the state of Montana. Her reporting resulted in institutional changes that forced local school districts to better serve Native students. She holds a bachelor's degree in journalism from the University of Montana and proudly calls Detroit "the most fascinating city I've ever lived in."

Angela Shah
Editor, Xconomy Texas

Angela has written about startups along a wide entrepreneurial spectrum, from Silicon Valley transplants to Austin partying in the '90s tech boom to 20-something women defying cultural norms as they seek to build vital IT infrastructure in a war-torn Afghanistan. As a foreign correspondent based in Dubai, her work appeared in The New York TImes, TIME, Newsweek/Daily Beast and Forbes Asia. With the launch of Xconomy Texas, she's returned to her hometown of Houston and now writes about innovation in the Lone Star State.

Bernadette Tansey
Editor, Xconomy San Francisco

Bernadette has covered information technology, biotechnology, business, law, environment, and government as a Bay area journalist. She has written about edtech, mobile apps, social media startups, and life sciences companies for Xconomy, and tracked the adoption of Web tools by small businesses for CNBC. She was a biotechnology reporter for the business section of the San Francisco Chronicle, where she also wrote about software developers and early commercial companies in nanotechnology and synthetic biology.

Wade Roush
Contributing Editor

Wade is a veteran journalist with a focus on information technology. As a staffer with MIT's Technology Review from 2001 to 2006, he served as senior editor, San Francisco bureau chief, and executive editor of TechnologyReview.com. Before joining TR, Wade was the Boston bureau reporter for Science, managing editor of supercomputing publications at NASA Ames Research Center, and Web editor at e-book pioneer NuvoMedia. He has a B.A. in the history of science from Harvard University and a PhD in the history and social study of science and technology from MIT. His work has appeared in Science, Technology Review, IEEE Spectrum, and Encyclopaedia Brittanica, and he has been a guest commentator on CNN, CNBC, NECN, WGBH, NPR, and PBS.

John Carey
Contributing Editor

John Carey is a freelance writer covering the environment, energy, science, technology and medicine. Until 2010, he was senior correspondent in Business Week's Washington Bureau. In his 21 years at the magazine, he wrote stories on everything from sequencing the human genome and global warming to tobacco regulation, election technology, cholesterol-lowering drugs and renewable energy. Prior to Business Week, Mr. Carey spent a year as an editor of The Scientist, three years as a writer and editor for National & International Wildlife magazines, and six years at Newsweek, where he covered science, technology, and health. He now writes for publications like Scientific American, Conservation, and National Wildlife. His stories have won awards from the American Association for the Advancement of Science, the Wistar Institute, the Association of Health Care Journalists, the Deadline Club, the American Institute of Biological Sciences, the Oversees Press Club, and others. He was also a National Magazine Award finalist. Mr. Carey has degrees in biochemistry (B.S., Yale University), marine biology (M.Sc., University College of North Wales), and forest ecology (M.F.S., Yale School of Forestry & Environmental Studies).

Frank Vinluan
Contributing Editor

Frank is a business journalist with more than a decade of experience covering various aspects of technology and life sciences. Based in Raleigh, he was a staff writer at the Triangle Business Journal covering technology, biotechnology and energy before joining MedCityNews.com as North Carolina bureau chief. Prior to moving to North Carolina's Research Triangle in 2007 he held business reporting positions at The Des Moines Register and The Seattle Times. Frank has a bachelor's degree in history from Carleton College and a master's degree in public affairs reporting from the University of Illinois Springfield.

Jim Edwards
Publisher

Jim has spent the last 10 years leading the business development efforts for firms in the consulting and performance improvement industry. He was Vice President, Client Relationships for The Madison Consulting Group, enhancing their national presence working with Fortune 100 companies. Most recently, he held the position of Senior Account Executive for AchieveGlobal, the world's largest performance improvement organization. He graduated from Colgate University with a concentration in Philosophy.

William A. Ghormley
Senior Vice President, Business Development

Bill has worked in marketing and new business creation in a number of industries, including media. His past employers include Polaroid's OEM organization, Harvard Pilgrim Health Care, and Kendall Healthcare. Bill also served as president of the Marketing Science Institute, a Cambridge-based research center created by the Harvard Business School and the Wharton School to measure media impact on customer attitudes and behaviors. Bill studied economics at Stanford, history at Boston College, and business at Dartmouth.

Gregory Calkins
Associate Publisher and Vice President, Business Development

Greg is a seasoned sales and marketing executive who brings over 17 years of experience in the area of business development and start-up management. Prior to Xconomy, Greg founded Calkins Advisors, a business content company focused on connecting thought leaders with business leaders. Greg also spent eight years as a business developer, strategic marketer, and alliance builder for Interactive Sports—a NYC-based media company. His efforts resulted in relationships with nationally recognized media, financial, sports, technology, and entertainment companies. Greg attended Hartwick College studying political science and history.

Richard Freierman
Business Manager

Richard has 30 years experience keeping the wheels on the bus, starting at Computer Design & Applications, where he created and managed an engineering support group charged with software technical publications, beta testing, and software release. His subsequent management and operations experience has included the Image and Meaning Conference (MIT 2001), retail management for Video Signals, a 7 store regional chain, and campaign management for four successful municipal elections in Cambridge, MA. Around the dawn of the World Wide Web, Richard was Web guru and creative partner for the Family Surfboard, a website for kids' online activities. He also co-authored *Save the Earth at Work* (Bob Adams Inc., 1991) and *Corporate Realities and Environmental Truths* (Wiley, 1993). While Richard doesn't get to apply his SUNY Forestry Degree very often in Cambridge, it does help him see the forest for the trees.

Geoa Geer
Events and Marketing Coordinator

On a whim, Geoa attended an 2014 Xconomy Forum and immediately loved the friendly staff and well-organized event. After signing on as event support, then part-time administrative assistant, then full-time logistics coordinator for Xconomy – it seems the happiness was mutual. Geoa ran Ultimate Frisbee tournaments, leagues, teams, and community programs in America and Australia from 2007-2013, handling everything from hyper 3rd graders learning to throw to recruiting and training coaches, running world-class tournaments, and managing some of the largest youth Ultimate programs in the world. Keep an eye out for wordy explanations and Victorian English shout-outs as her treasured marks of passage from a BA in English Literature from University of Pittsburgh.

Samantha Lam
Events and Marketing Coordinator

Prior to joining Xconomy, Samantha managed events for non-profits in Maine and South Africa. She was a PR intern for Fuse, a leading youth marketing agency. She holds a bachelor's degree in Mass Communication from Champlain College in Burlington, VT.

Andrew Koyfman
Chief Technology Officer Emeritus

Andrew has eight-plus years of experience in the software industry. He recently completed his MBA at MIT Sloan, where he was involved with the MIT Venture Capital and Private Equity Club and the MIT Sales Club. Prior to Sloan, he was a cofounder and the Chief Architect of Swapthing.com, an online bartering site. Andrew earned a B.Sc. in Math and Computer Science and an M.Sc. in Computer Science at Brown University.

Alun Anderson
Alun was variously the Editor, Editor-in-Chief and Publishing Director of *New Scientist* magazine from 1992 up until 2005, and was a member of the Board of IPC Media, Europe's largest magazine house and now a part of Time Warner. During his time as Publishing Director at *New Scientist*, he successfully launched the magazine in the United States, first as an internet-based business and then as a print publication. *New Scientist*'s internet business has been particularly successful and its strategic development is used as a business case study for the London Business School MBA course. Earlier he held senior roles at the journals *Nature* and *Science*. He has published two books on science and technology in Japan and several scientific papers in the field of behavior and neurobiology.

Alun Anderson has been a Member of the Royal Society Committee on the Public Understanding of Science and a Member of the Council of the University of Sussex and is currently a Member of the Council of the Royal Institution, Britain's most prestigious science communication organization. He has three times been voted 'Editor of the Year' by the British Society of Magazine Editors (1993, 1995 and 1997). In 1997 he was also voted the 'Editors' Editor of the Year'. In 2000, the *London Evening Standard* listed him as one of London's most influential people and *The Australian* newspaper named him as one of the world's top 100 thinkers.

Robert Buderi
(See Bob's bio above.)

Jason Chudnofsky
For over three decades Jason Chudnofsky has been at the core of the ever-expanding technology events business launching flagship trade shows and expositions in venues across North and South America, Europe, Asia, Africa, and the Pacific Rim. Jason served as President of the Interface Group, Comdex Events World Wide, and the Sands Expo and Convention Center in Las Vegas. He has served on the Boards of Directors for: Folio Exhibits, Inc., Tech Corps, SOFTBANK Japan, Inc., Ziff Davis, Inc., Key3Media Group, Quantum Clicks, Logo Wire, Hook Media, Pulver Media, Next Step Technology, Visiting Nurses Association, the Anti-Defamation League, and the Museum of Science.

Chudnofsky is a nationally known sales and management training consultant. Born in Boston, Chudnofsky holds a B.S. degree in Business Management from Northeastern University. He and his wife Judy have two daughters and two grandchildren and make their home in Needham, MA.

Chris Sheehan
Chris is a former managing director of CommonAngels, where he invested in technology startups. He led CommonAngels investments in Carbonite (IPO: CARB), Linkable Networks, Practically Green, OwnerIQ, Xconomy, Insightix, Blaze Software, Offerpop, Yieldbot, Kibits, and Powerhouse Dynamics. His investment focus is in Internet, digital media, and software sectors. Previously, he was a venture partner at Industry Ventures where he was actively involved in the acquisition and management of secondary venture investment portfolios.

Our Board

Prior to Industry Ventures, Chris was a Director of Corporate Development for BEA Systems, a leading enterprise software company with over $1B in sales. Prior to BEA, Chris led the private equity practice for the startup research firm, Stax Inc. Chris also spent six years with the top ranked investment bank in Australia and co-founded an investment firm, Northstate Partners. He started his career as an equity analyst. He also serves on the board for the Center for Women & Enterprise, is a mentor at TechStars, and holds a Bachelor of Commerce degree from the University of Queensland, Australia.

Susan Hunt Stevens
Susan Hunt Stevens is the Founder/CEO of Practically Green, an early stage start-up that helps people live greener and healthier by using technology and social networking to educate, motivate and recognize people for making green changes in their lives. Previously, Stevens spent nine years at The New York Times Company, most recently as senior vice president, digital for Boston Globe Media, where she ran Boston.com, one of the largest news and information sites on the web. She began her career as a management consultant, working for the same leadership team at three different firms, including Alexander & Alexander, Mercer Management Consulting (now Oliver Wyman) and APM. Stevens joined The New York Times in 1998 as director of marketing for NYTimes.com, left the company briefly in 2000 to co-found and serve as President for Abridge, Inc, a venture-funded e-mail data mining start-up, and rejoined The Times in 2002 as Vice President of Circulation Marketing for The Boston Globe. She was promoted in 2005 to Vice President, Consumer Marketing taking leadership for consumer public relations and brand marketing for The Boston Globe and Boston.com. In 2006, she was promoted the Senior Vice President, circulation and marketing. Ms. Stevens received her MBA from The Amos Tuck School of Business at Dartmouth College; she also holds a BA in Government with high honors from Wesleyan University and is a candidate for a graduate certificate in sustainable design at the Boston Architectural College. She serves on the board of the Center for Women & Enterprise, a non-profit that helps women start and grow businesses, and Inspiring Kids, a non-profit in New England that helps schools raise funds in a way that promotes environmental stewardship and social philanthropy.

William C. Taylor (special advisor)
William C. Taylor is an agenda-setting thinker, writer, and entrepreneur. His new book, *Mavericks at Work*, has been a *New York Times* and *Wall Street Journal* bestseller, and was named a "Best Business Book of 2006" by *The Economist*, the *Financial Times*, and Amazon.com. As cofounder and founding editor of *Fast Company*, he launched a magazine that earned a passionate following among executives and entrepreneurs around the world and won two coveted National Magazine Awards. His management blog, "Game Changer," appears weekly on HarvardBusiness Online, and his column, "Bill Taylor on Big Ideas," runs in London's *Guardian* newspaper. He is a member of the Xconomy board of directors.

Steve Woit (Founding Publisher and Special Advisor)

Steve is the founder of Riga Ventures, a venture firm that invests in media, software, and Internet businesses. He helped fund *02138*, a magazine and website focused on the Harvard University alumni community, which was purchased by the Atlantic Media Group (publishers of the *Atlantic Monthly*) in 2006. He was a leading investor in Bitpipe, where he served as EVP of Sales and Marketing; the company was acquired by TechTarget for $40 million in 2004.

Steve also worked for 17 years for Patrick J. McGovern, Chairman and founder of International Data Group (IDG), the largest global publisher of technology publications and websites and a leading global technology venture investor. While at IDG, he was responsible for overseeing the launch of new publication and Web-based businesses. He was a General Partner at IDG Ventures and served as Publisher and President of *Federal Computer Week*, President of *Web Shopper*, EVP of *Computerworld*, and Director of Business Development and Vice President/New Products for IDG worldwide. Since 1999, Steve has been an active member of the angel investment group CommonAngels and formerly sat on the group's Board of Directors.

Our editorial principles are old-fashioned and simple. We believe that our content is only of value to our audience if we ensure that it is trustworthy. To that end, all editorial decisions will be made by our writers and editors and will remain independent of our business operations. We promise that our underwriters, advertisers, investors, and partners will have no special influence on the content we present, either on the site or at the events we hold—although at certain events, we may invite some sponsors to introduce speakers or moderate panels. What's more, to avoid real or perceived conflicts of interest, staff and freelance writers will not write about companies or fields in which they invest or for which they perform any non-journalistic work.

We recognize that there's an important distinction between content generated by career journalists who are committed to the ethical standards of the profession and that generated by experts and insiders like the Xconomists (see below). The Xconomists offer a unique and important perspective on

Ethics

business, technology, and society, but they are also enmeshed in many of the potential conflicts of interest from which journalists strive to free themselves. We think the best strategy here is transparency, and so we have designed the site to clearly indicate which content comes from which type of author, the professional journalist or the expert. We also encourage readers to find out exactly where the Xconomists are coming from by clicking through the links below to their bios.

The Xconomists are an unrivaled group of leading technologists, scientists, and business innovators. They will share their views and insights — and from time to time their pet peeves and perhaps a bit of gossip — in the Xconomist Forum.

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Joe Chung
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Laura Strong
Matt Younkle

Xconomists at Large

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Rishi Jaitly
Todd Park
John Seely Brown
Vinod Khosla
Dane Stangler
Desh Deshpande
Joel S. Marcus

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Website development support from Andrew Koyfman with design support from Rob Hunter.

Exhibit 77

FierceMarkets

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Life Sciences

From discovery, clinical development, and manufacturing to post-approval stages, our publications touch on every aspect of the life sciences industry. Anchored by FierceBiotech, FiercePharma, and FierceMedicalDevices, this group of 10 publications provides advertisers with unparalleled daily reach across 300,000 effective e-mail subscribers and 250,000 unique monthly visitors representing every major biopharmaceutical company and thousands of startup drug and device developers.

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Audience: Senior business executives and clinical operations leaders at biotech, pharmaceutical, and related drug development companies.

Key Topics: Clinical stage drug development, FDA/EMEA regulations, drug licensing pacts, mergers & acquisitions involving developers, biotech & pharma deals

Newsletter Frequency: Daily

Email Subscribers: 148,000+

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Audience: Senior business executives, brand managers, operations directors, and sales and marketing leaders at pharmaceuticals.

Key Topics: Pharma company news, generic drug companies, pharmaceutical marketing and sales, FDA regulations and warnings, Phase IV clinical trials, contract manufacturing, label expansions, patent expirations, post-approval market development

Newsletter Frequency: Daily

Email Subscribers: 150,000+

Monthly page views: 580,000

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Audience: Pre-clinical scientists, lab directors, and corporate leadership early stage drug developers.

Key Topics: Commercial and academic R&D programs, scientific breakthroughs in drug discovery, R&D trends and financing, stem cell news, preclinical research, the growing bridge between research and clinical proof-of-concept studies

Newsletter Frequency: Weekly

Email Subscribers: 45,000+

Monthly page views: 46,000

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Audience: Corporate executives and R&D leaders at vaccine developers and pharmaceuticals of all sizes.

Key Topics: Vaccine R&D, sales and marketing, research breakthroughs, vaccine production, disruptive technologies, next-generation vaccine therapeutics

Newsletter Frequency: Weekly

Email Subscribers: 25,000+

Monthly page views: 60,000

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Audience: CIOs, CTOs, clinical operations leaders, and IT-managers at drug developers and clinical research organizations of all sizes.

Key Topics: Clinical trials IT, patient recruitment, data management, clinical trial design & management, pharmacovigilance, cloud computing, study startup

Newsletter Frequency: Weekly

Email Subscribers: 20,000+

Monthly page views: 39,000

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Audience: Site managers, process engineers, purchasing and outsourcing directors, and corporate executives at pharmaceuticals and contract manufacturers.

Key Topics: Contract manufacturing, quality and yield assurance, FDA regulations, supply chain disruptions, product recalls, biopharma packing & security, supply chain software & systems, manufacturing technology advancements

Newsletter Frequency: Twice Weekly

Email Subscribers: 30,000+

Monthly page views: 85,000

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Audience: Corporate executives and design leaders at device and diagnostic manufacturers.

Key Topics: Diagnostics, new product development, regulatory issues, breakthrough innovations, deal-making

Newsletter Frequency: Daily

Email Subscribers: 80,000+

Monthly page views: 140,000

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Audience: Corporate executives at biotechnology, pharmaceutical, and drug-delivery technology companies of all sizes.

Key Topics: Emerging delivery technologies & methodologies, market impact, breakthroughs in delivery methods for new and existing therapeutics

Newsletter Frequency: Weekly

Email Subscribers: 18,000+

Monthly page views: 27,000

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View Rates (http://www.fiercemarkets.com/rates/new?industry=life-sciences-2#cro)

Audience: Biotech executives who purchase outsourcing Pharma executives who purchase outsourcing Biopharma executives providing outsourcing services

Key Topics: CRO Industry News, Strategic partnerships between CROs and drug developers, Clinical trials outsourcing, Lab services outsourcing, Efficacy of outsources R&D models

Newsletter Frequency: Weekly

Email Subscribers: 18,000+

Monthly page views: 33,000

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View Rates (http://www.fiercemarkets.com/rates/new?industry=life-sciences#diagnostics)
Sign up (http://www.fiercediagnostics.com/signup)

Audience: Senior level diagnostic developers and manufacturers

Key Topics: Market launches, M&A and partnership deals, financing achievements, clinical milestones, diagnostics at the early stage, diagnostic talkers, and regulatory issues.

Newsletter Frequency: Weekly

Email Subscribers: 20,000

Monthly page views: 22,000

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Audience: CMOs, Brand Managers, Marketing Directors, Agency Executives, Pharmaceutical Sales Reps

Key Topics: Consumer advertising, DTC trends, off label marketing settlements, ad budgets, new drug launches, agency moves, and payer formularies.

Newsletter Frequency: Weekly

Email Subscribers: 18,000

Monthly page views: 30,000

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Exhibit 78

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