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

Proceeding no.	91274910
Party	Plaintiff Brink Biologics, Inc.
Correspondence address	ALICIA M PASSERIN LEECH TISHMAN FUSCALDO & LAMPL 525 WILLIAM PENN PLACE, 28TH FLOOR PITTSBURGH, PA 15219 UNITED STATES Primary email: ipdocket@leechtishman.com Secondary email(s): apasserin@leechtishman.com, dpeake@leechtishman.com 412-261-1600
Submission	Motion to Amend Pleading/Amended Pleading
Filer's name	Alicia M. Passerin
Filer's email	ipdocket@leechtishman.com, apasserin@leechtishman.com, dpeake@leechtishman.com
Signature	/Alicia M. Passerin/
Date	02/13/2023
Attachments	BRINK-1-OPP Second Amended Combined Notice of Opposition.pdf(104512 bytes) BRINK-1-OPP - Exhibits 1-4 - Second Amended Combined Notice of Opposition.pdf(1276682 bytes) BRINK-1-OPP - Exhibits 5-8 - Second Amended Combined Notice of Opposition.pdf(6045334 bytes)

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

In re Application of:

Applicant : Agilent Technologies, Inc.
Serial No. : 90/642,875
Mark : NOVOPANEL
Application Date : April 13, 2021
Publication Date : November 9, 2021

Applicant : Agilent Technologies, Inc.
Serial No. : 90/842,567
Mark : NOVOPANEL
Application Date : July 22, 2021
Publication Date : November 9, 2021

BRINK BIOLOGICS, INC.)	
)	
Opposer,)	
)	
v.)	Opposition No. 91274910
)	
AGILENT TECHNOLOGIES, INC.)	
)	
Applicant.)	
_____)	

SECOND AMENDED COMBINED NOTICE OF OPPOSITION

Brink Biologics, Inc., an ImmunityBio (NASDAQ:IBRX) affiliate, is a corporation organized and existing under the laws of Delaware with a principal place of business at 3530 John Hopkins Court, San Diego, CA 92121 (“Opposer”). Opposer believes that it will be damaged by the issuance of a registration for the alleged mark for NOVOPANEL (“Proposed Mark”) shown in Application Serial Nos. 90/642,875 (“the ‘875 Application”) and 90/842,567 (“the ‘567 Application”). As grounds for opposition to the Proposed Mark, Opposer alleges as follows:

1. Opposer is a biotechnology solutions company focused on providing testing services and products, including cells and other reagents in the form of kits, for a range of laboratory testing applications.

2. Opposer offers natural killer cell lines that are established in stable cultures under Good Laboratory Practice (GLP) and current Good Manufacturing Practice (cGMP) conditions and further modified into multiple panels of cell lines suitable for a range of laboratory testing applications.

3. The natural killer cell lines include the NK-92® proprietary cell line which Opposer exclusively licenses from ImmunityBio, Inc. for use in *in vitro* and *in vivo* testing and diagnostic products and services.

4. The standardized panel of natural killer cell lines, together with media, media additives and other reagents, offered in conjunction with Opposer's NEUKOPANEL Mark, provide a means to establish a consistent, robust and reliable assay for use in scientific, laboratory or medical research, including the assessment of antibody-dependent cellular cytotoxicity ("ADCC") activity, a key tool in the development and manufacture of monoclonal antibody therapeutics.

5. In addition to these products, Opposer also offers research services for biologic testing, including technical support and biologic analysis.

6. Together, the products and services offered by Opposer provide Opposer's licensees with the tools to conduct discovery research, high-throughput screening, monoclonal antibody characterization, testing of clinical products, stability testing and assessment of product validation and efficacy. *See* Exhibit 1, which is a true and correct printout from Opposer's website.

7. Opposer is the owner of valid and subsisting United States Trademark Registration No. 4,187,822 on the Principal Register in the United States Patent and Trademark Office ("the '822 Registration") for the mark NEUKOPANEL ("Opposer's Registered Mark"). Opposer's '822 Registration identifies that the Registered Mark is used in conjunction with "cells for scientific, laboratory or medical research," (the "Registered Mark Goods"). Attached as Exhibit 2 is a true and correct printout from the United States Patent and Trademark Office electronic database showing the current status and title of the '822 Registration as of March 8, 2022.

8. The '822 Registration has become incontestable within the meaning of Section 15 of the Lanham Act, 15 U.S.C. § 1065

9. Opposer's Registered Mark has been used in interstate commerce in the United States continuously since at least as early as October 26, 2011, in connection with providing, offering for sale, selling, marketing, advertising and promoting the Registered Mark's Goods.

10. In addition, attached hereto as Exhibit 3 is a true and correct copy (redacted) of literature provided to Opposer's licensees, showing the NEUKOPANEL Mark used in connection with natural killer cells and other materials and as part of Opposer's technical support services.

11. Opposer also is the owner of valid and subsisting rights under the common law for its continuous use of the NEUKOPANEL Mark in commerce since at least as early as May 5, 2018 in conjunction with "cells for scientific, medical, and diagnostic laboratory use; cell-derived preparations for scientific, medical, and diagnostic laboratory use; cells for medical and diagnostic research; cell-derived preparations for medical and diagnostic research; kits comprised of cells, reagents and related instructions, for diagnostic, scientific, laboratory and medical research; kits comprised of cell-derived preparations; reagents and related instructions, for diagnostic, scientific, laboratory, and medical research; cells for calibration of laboratory assays, devices, and equipment; cell-derived preparations for calibration of laboratory assays, devices, and equipment; cells for use in development of laboratory assays, devices, and equipment; cell-derived preparations for use in development of laboratory assays, devices, and equipment," "cells for clinical, medical, therapeutic and diagnostic use; cell-derived preparations for medical and diagnostic use; products comprising cells; products comprising cell-derived preparations; kits comprised of cells, other reagents and related instructions for medical and diagnostic use; kits comprised of cell-derived preparations, other reagents and related instructions for medical and diagnostic use," and "scientific, medical, and diagnostic research services," (the "Common Law Goods and Services" and together with the Registered Marks Goods, "Opposer's Goods and Services").

12. Opposer's rights in and to the NEUKOPANEL mark in connection with these goods and services are embodied in Opposer's United States Trademark Application Serial No. 97/365,221 ("the '221 Application"), which was filed on April 15, 2022. Attached hereto as Exhibit 4 is a true and correct printout from the United States Patent and Trademark Office electronic database showing the current status and title of the '221 Application as of January 3, 2023.

13. Notwithstanding the inherent distinctiveness of Opposer's NEUKOPANEL Mark, the NEUKOPANEL Mark also has acquired secondary meaning to the public indicating Opposer as the source of goods and services bearing the NEUKOPANEL Mark.

14. Opposer has expended substantial time, money, and resources marketing, advertising, and promoting the Opposer's Goods and Services sold in various channels under the NEUKOPANEL Mark as identified in the '822 Registration and the '221 Application and as used under the common law.

15. Opposer's Registered Mark and its rights under the '221 Application and the common law are symbolic of extensive goodwill established by Opposer and serves as a unique identifier of the goods and services offered by Opposer.

16. Opposer has acquired a high degree of recognition through continued use and expenditures of time, effort and money in advertising and promotion.

17. The purchasing public has come to know, rely upon and recognize Opposer's NEUKOPANEL Mark as indicating a source of high quality and reliable goods and services in the life sciences and life science device industries.

18. As a result of its widespread, continuous, and exclusive use of the NEUKOPANEL Mark to identify its Goods and Services and Opposer as their source, Opposer has acquired and owns valid and subsisting federal statutory and common law rights to the NEUKOPANEL Mark.

19. To the best of Opposer's knowledge, the name and address of the Applicant of the NOVOPANEL Application is Agilent Technologies, Inc. ("Applicant"), a Delaware corporation having a principal place of business at 5301 Stevens Creek Boulevard, Santa Clara, CA 95051.

20. Upon information and belief, Applicant provides products and services in the life sciences and clinical and diagnostic testing industries, including cells and other reagents, computer software and equipment for monitoring cell populations, data analysis of cell populations, and related services.

21. Notably, Applicant offers goods and services that utilize not only NK-92® cells (i.e., Opposer's cells exclusively licensed from ImmunityBio, Inc., formerly known as Conkwest), but also reagents and other materials included in Opposer's Goods and offered for sale under the NEUKOPANEL Mark. *See Exhibits 5 to 8.*

22. In fact, Applicant even specifically refers to Opposer's NEUKOPANEL ADCC assay on page 3 of Exhibit 5.

23. On April 13, 2021, Applicant filed the '875 Application as an intent-to-use application for the Proposed Mark, NOVOPANEL. Applicant's Proposed NOVOPANEL Mark is applied for in International Class 005 for "reagents for medical use; medical diagnostic reagents; clinical medical reagents."

24. On July 22, 2021, Applicant filed the '567 Application as an intent-to-use application for the Proposed Mark, NOVOPANEL. Applicant's Proposed NOVOPANEL Mark is applied for in International Class 009 for "downloadable or recorded computer software for operating instrumentation that monitors cell or molecule populations, data acquisition from instrumentation that monitors cell or molecule populations, and data analysis of cell or molecule populations for research and development or diagnostic applications."

25. Opposer believes that it will be damaged by registration of the '875 Application and the '567 Application for the Proposed Mark NOVOPANEL and hereby opposes said Application.

COUNT I
LIKELIHOOD OF CONFUSION

26. Opposer hereby incorporates by reference and re-alleges each and every allegation set forth in Paragraphs 1 through 25.

27. Opposer has senior rights in and to the term NEUKOPANEL because Opposer's first use of its NEUKOPANEL Mark predates the filing of Applicant's Proposed Mark or any other date on which the Applicant has relied for the purposes of priority.

28. Opposer's continuous, extensive, and exclusive use in commerce establishes senior rights under the common law.

29. Opposer's '822 Registration is valid and subsisting and is *prima facie* evidence of Opposer's exclusive right to its NEUKOPANEL Mark in commerce in association with the goods and services specified in such registration and application.

30. Opposer's '221 Application serves as further evidence of Opposer's senior rights in and to the term NEUKOPANEL that predate any priority date of Applicant's Proposed Mark.

31. Applicant's Proposed Mark for NOVOPANEL contains the elements "N" and "OPANEL" which are identical to those in Opposer's Registered Mark for NEUKOPANEL.

32. Applicant's Proposed Mark for NOVOPANEL merely replaced the letters "EUK" in Opposer's Registered Mark for NEUKOPANEL with the letters "OV".

33. Applicant's Proposed Mark for NOVOPANEL and Opposer's Registered Mark for NEUKOPANEL each have four syllables.

34. Applicant's Proposed Mark for NOVOPANEL is phonetically similar to Opposer's Registered Mark for NEUKOPANEL, with each mark having phonetically identical final three syllables.

35. Applicant's Proposed Mark for NOVOPANEL is similar in appearance to Opposer's Registered Mark for NEUKOPANEL.

36. As illustrated in Exhibits 5 to 8, Applicant provides products and services that are similar to those offered by Opposer, including reagents, assays and kits that include NK cells or relate to cells and/or other reagents also included as part of Opposer's Goods and Services, as well as computer software and equipment for monitoring cell populations, data analysis of cell populations, and related services.

37. Applicant and Opposer market their respective products and services in the same or related industries and to the same or related consumers.

38. For at least these reasons, Applicant's Proposed NOVOPANEL Mark so resembles Opposer's NEUKOPANEL Registered mark as to be likely to cause confusion, or to cause mistake, or to deceive as to source by suggesting that Applicant's goods are associated with or approved, endorsed, affiliated, authorized or sponsored by Opposer.

COUNT II
DILUTION BY BLURRING

39. Opposer hereby incorporates by reference and re-alleges each and every allegation set forth in Paragraphs 1 through 38.

40. By reason of Opposer's continuous, extensive and exclusive use for many years, Opposer's NEUKOPANEL Registered Mark is distinctive and famous within the meaning of Section 43(c) of the Lanham Act, 15 U.S.C. § 1125(c).

41. Opposer's NEUKOPANEL Registered Mark became distinctive and famous prior to the filing of Applicant's application or any other date on which Applicant might attempt to rely for purposes of priority.

42. Registration by Applicant of the proposed NOVOPANEL Mark for Applicant's proposed goods and services would likely impair the distinctiveness and cause dilution by blurring of, and thereby damage, Opposer's famous NEUKOPANEL Mark in violation of 15 U.S.C. § 1125(c). Accordingly, pursuant to 15 U.S.C. § 1063, the Proposed NOVOPANEL Mark is not entitled to registration.

WHEREFORE, Applicant's Proposed NOVOPANEL Mark, Application Nos. 90/642,875 and 90/842,567, is damaging to Opponent and, accordingly, Opponent requests that the instant Notice of Opposition be granted and that registration of Applicant's Proposed NOVOPANEL Mark be refused.

Please charge any underpayment or credit any overpayment to Deposit Account 506516.

Date: February 13, 2023

Respectfully submitted,

By: /Alicia M. Passerin/
Alicia M. Passerin, Ph.D., Esq.
Ashley N. Crane, Esq.
E-mail: apasserin@leechtishman.com
LEECH TISHMAN FUSCALDO & LAMPL, LLC
525 William Penn Place, 28th Floor
Pittsburgh, Pennsylvania 15219
Telephone: 412/261-1600
Facsimile: 412/227-5551

Attorneys for Brink Biologics, Inc., Opposer

CERTIFICATE OF SERVICE

The undersigned affirms that OPPOSER’S SECOND AMENDED COMBINED NOTICE OF OPPOSITION was served on Applicant Agilent Technologies, Inc. by emailing a copy to Applicant’s attorney of record, Craig A. Beaker of Holland & Hart LLP, at docket@hollandhart.com, agarcia@hollandhart.com, cabeaker@hollandhart.com, and aepierce@hollandhart.com, as required pursuant to 37 CFR § 2.119 and TRADEMARK TRIAL AND APPEAL BOARD MANUAL OF PROCEDURE § 113.04, on the date set forth below.

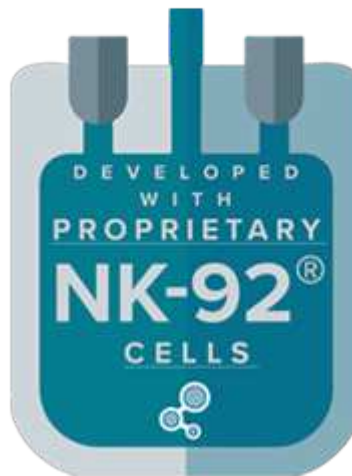
Dated: February 13, 2023

/Alicia M. Passerin/_____

Alicia M. Passerin

EXHIBIT 1

TO START A CELL LICENSING REQU



NEXT
GENERATION
BIOANALYTICAL
SOLUTIONS



TO START A CELL LICENSING REQU

OUR LICENSING PROCESS:

1

Fill out request form

2

Receive, review, and sign NDA

3

Review term sheet for license

4

Discuss details of license,
finalize, sign and pay fees

5

Obtain cells under license

NEUKOPANEL® CELL REQUEST FORM FOR PROPRIETARY NEUKOPLAST® NK-92 CELLS

Please complete form to initiate licensing process



CONTACT BRINK BIOLOGICS

TO START A CELL LICENSING REQU

Phone Number

Institution/Company Name *

I am a *

 ▼

Institution Type *

 ▼What is the jurisdiction of
incorporation of your organization? *

If you are a Contract Research Organization (CRO) please have your client contact us directly for a license and we can then arrange a direct sublicense with your CRO company for their specific project.

Are you a CRO? *

- Yes
 No

Corporate Address

Street Address *

City *

State / Province / Region



Postal Code *

Country *

Select your Neukopanel® cell lines of interest.

- aNK Control - NK-92.05 haNK CD16-V158.NK-92.05
- IaNK - CD16-F158.NK-92.05 GFP haNK- GFP+ CD16-
V158.NK-92.05
- GFP IaNK - GFP+ CD16-
F158.NK-92.05 NK-92 MI - NK-92 transfected
with human IL-2 cDNA in the
retroviral MFG-hIL-2 vector

TO START A CELL LICENSING REQU

appropriate licensing structure can be arranged. Incomplete explanations of use  

Is your description of intended use case detailed and complete? *

- Yes
- No

Does your use case involve making modifications to the cells? *

- Yes
- No

Do you plan to use the cells in house only, use a CRO, or both? *

- In-house
- Use a CRO
- Both

If you plan to use a CRO, which one?

Shipping Address

Shipping Carrier

Shipping Account Number



TAKING YOU BEYOND THE BRINK OF BINDING™ WITH OUR KILLER ASSAYS™.

THE NK-92® ASSAY COMPANY

Brink Biologics, Inc. (BBI) is a [ImmunityBio, Inc.](#) (NASDAQ: IBRX) affiliated biotechnology solutions company located in the prestigious Torrey Pines biotechnology science park in San Diego, CA.



KILLER CELLS – KILLER ASSAYS™

TO START A CELL LICENSING REQU

PROPRIETARY NEUKOPANEL® CELL LINES

A standardized panel of CD16 expressing NK-92® cell lines, provides for the first time the means to establish a consistent, robust and reliable assay for assessment of ADCC activity.

ADCC ASSAYS – ROUTINE HASSLE

Brink Biologics, Inc. holds the exclusive non-clinical rights to the NK-92® cell line and its CD16 expressing derivatives, all of which are uniquely and ideally suited for antibody-dependent cell-mediated cytotoxicity (ADCC) assays.

TO START A CELL LICENSING REQU

ROUTINE ASSAY

TIME CONSUMING AND A LACK OF OPTIONS

OUR SIMPLE SOLUTION

The exclusive global provider of NK-92® cell line testing services, products, and sublicenses within the life sciences and device industry.

Highly published cell lines – over 200 publications.

Relevant Examples:

Schnueriger, A., et al. (2011). "Development of a quantitative, cell-line based assay to measure ADCC activity mediated by therapeutic antibodies." *Mol Immunol* 48(12-13): 1512-1517.

Binyamin, L., et al. (2008). "Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy." *J Immunol* 180(9): 6392-6401.



TO START A CELL LICENSING REQU

adapted to mass production

Brink Biologics created the NK-92® master cell bank (MCB).

EXCLUSIVE

NK-92® MCB has been accepted as clinical grade material by regulatory agencies across the world, including the US FDA, EMEA and Canada Health.

RECOGNIZED QUALITY

The sole holder of homogeneously pure NK-92® cell cultures with superb retention of phenotypic characteristics in more than 6 months of continuous culture.



PURE CELL LINE

TO START A CELL LICENSING REQU

FIVE ADVANTAGES OF NEUKOPANEL® CELL LINES:

For the first time the means to establish a consistent, robust and reliable assay for assessment of true ADCC function.

COST & TIME SAVINGS

TRUE CYTOTOXICITY

EASE OF USE



ROBUST ASSAY

ELIMINATES PHENOTYPIC VARIABILITY

- Cost and time savings over common practice using donor-sourced (PBMC) NK cell isolation, expansion and maintenance in short term cultures
- Eliminates phenotypic variability of donor-sourced NK cells as Neukopanel cell lines offer clonal consistency, purity and reliability as an assay reagent
- Ease of use over donor-sourced NK cells and capable of long-term continuous cultures
- Ability to generate a readout spectrum with the use of our homozygous high and low affinity CD16 expressing lines alongside our negative control line for assay robustness
- True cytotoxicity rather than cell-binding readouts



RECENT NEWS

GLOBAL THERAPEUTIC CONTRACT AGREEMENT WITH SWISSBIOSIM GMBH

Sep 10, 2020

SAN DIEGO, Calif., September 10, 2020 – Brink Biologics, Inc. (“Brink”), a NantKwest, Inc. (NASDAQ: NK) affiliate and exclusively-licensed distributor of NantKwest’s proprietary off-the-shelf NK-92® natural killer cells in certain fields, announces the licensing of...

BRINK BIOLOGICS ANNOUNCES RESEARCH LICENSE AGREEMENT WITH WORCESTER POLYTECHNIC INSTITUTE FOR USE OF NANTKWEST’S NK-92® CELLS

May 1, 2019

NK-92® Cell Based Bioanalytical Solutions are the Key to Optimizing R&D and Commercial Product Development Efforts for Monoclonal Antibodies, Bi-Specifics and Related Biological Products SAN DIEGO--May 1, 2019-- Brink Biologics, Inc., a NantKwest,...

NANTKWEST ANNOUNCES NEW HANK NATURAL KILLER CELL PATENT ISSUANCE FURTHER EXPANDING INTELLECTUAL PROPERTY ESTATE

Apr 1, 2019

New haNK and T-haNK Based Natural Killer Cell Therapy Programs to Transition to Human Clinical Trials in 2019 CULVER CITY, CA - April 1, 2019 - NantKwest (Nasdaq:NK), a leading clinical-stage, natural killer cell based therapeutics company, today announced...



TO START A CELL LICENSING REQU



OFFICE

3530 John Hopkins Court
San Diego, CA 92121



HOURS

8am to 5pm M-F



CALL US

Main: (858) 633-0300

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EXHIBIT 2



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NEUKOPANEL

Word Mark	NEUKOPANEL
Goods and Services	IC 001. US 001 005 006 010 026 046. G & S: Cells for scientific, laboratory or medical research. FIRST USE: 20111026. FIRST USE IN COMMERCE: 20111026
Standard Characters Claimed	
Mark Drawing Code	(4) STANDARD CHARACTER MARK
Serial Number	85331370
Filing Date	May 26, 2011
Current Basis	1A
Original Filing Basis	1B
Published for Opposition	October 11, 2011
Registration Number	4187822
Registration Date	August 7, 2012
Owner	(REGISTRANT) Conkwest, Inc. CORPORATION ILLINOIS 3790 Via De La Valle, Suite 205 San Diego CALIFORNIA 92014 (LAST LISTED OWNER) BRINK BIOLOGICS, INC. CORPORATION DELAWARE 3530 JOHN HOPKINS COURT SAN DIEGO CALIFORNIA 92121

Assignment Recorded ASSIGNMENT RECORDED
Attorney of Record Alicia M. Passerin
Type of Mark TRADEMARK
Register PRINCIPAL
Affidavit Text SECT 15. SECT 8 (6-YR).
Live/Dead Indicator LIVE

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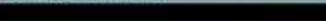
EXHIBIT 3

 Package Lot No. 201708
 Package Weight 13 KG
 Net Weight 13 KG
NAWB# 932 70156026
 Shipping Method: Standard Shipper, Liquid Nitrogen (-195 C)
 Temperature: -195 C (Standard, 200V)
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 3170 John Hopkins Court, San Diego, CA 92121
 (619) 594-1100

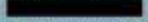

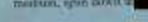











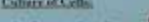















BRINK BIOLOGICS

THAWING AND CULTURE OF Brink Biologics® LIVING DRUGS IN A BAG®, NEUROPANELS®, or NEUROPLAST® CELLS (Standard Operation Procedure) Version 6.1 05/06/16



Materials:

- Human AB serum (male donor)
- Recombinant 
- 24-well plates, T-flasks (T25, T75, or higher)
- 

Thawing of Cells:

1. Take out frozen vial from a liquid N₂ tank. Rapidly thaw the tube in a 37°C water bath until only a  remains.
2. Transfer contents of vial dropwise to a  ml tube with  ml of  NK-92® cell growth medium, spin down at  rpm and  min.
3. Resuspend the cells pellet in  ml of  NK-92® growth medium and transfer to a  ml culture (or desired).
4. On day  replat this well into  wells with NK-92® cell growth medium  ml.
5. On day  pool the  wells ( ml) and transfer to a  ml remaining  ml of NK-92® cell growth medium (keep the  ml).
6. After  days, count the cells.
 - If cells number is below  cells/ml then collect cells by centrifugation  rpm and resuspend pellet in  ml of  NK-92® cell growth medium.
 - If cell number is less than  cells/ml then remove medium for another  days.
 - Otherwise use pass the  ml of  (containing  ml of NK-92® cell growth medium) to a  ml medium.

Culture of Cells:

1. Passage the cells every  days at a dilution of  with the cells by carefully pipetting up and down to break the clumps.

CONFIDENTIAL
 3170 John Hopkins Court, San Diego, CA 92121
 (619) 594-0500 Fax: (619) 594-1995 www.brinkbiologics.com

EXHIBIT 4



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NEUKOPANEL

Word Mark NEUKOPANEL

Goods and Services IC 001. US 001 005 006 010 026 046. G & S: Cells for scientific, medical, and diagnostic laboratory use; cell-derived preparations for scientific, medical, and diagnostic laboratory use; cells for medial and diagnostic research; cell-derived preparations for medical and diagnostic research; kits comprised of cells, reagents and related instructions, for diagnostic, scientific, laboratory, and medical research; kits comprised of cell-derived preparations, reagents and related instructions, for diagnostic, scientific, laboratory, and medical research; cells for calibration of laboratory assays, devices, and equipment; cell-derived preparations for calibration of laboratory assays, devices, and equipment; cells for use in development of laboratory assays, devices, and equipment; cell-derived preparations for use in development of laboratory assays, devices, and equipment. FIRST USE: 20180521. FIRST USE IN COMMERCE: 20180521

IC 005. US 005 006 018 044 046 051 052. G & S: Cells for clinical, medical, therapeutic and diagnostic use; cell-derived preparations for medical and diagnostic use; products comprising cells; products comprising cell-derived preparations; kits comprised of cells, other reagents and related instructions for medical and diagnostic use; kits comprised of cell-derived preparations, other reagents and related instructions for medical and diagnostic use. FIRST USE: 20180521. FIRST USE IN COMMERCE: 20180521

IC 042. US 100 101. G & S: Scientific, medical, and diagnostic research services. FIRST USE: 20180521. FIRST USE IN COMMERCE: 20180521

Standard Characters Claimed

Mark Drawing Code (4) STANDARD CHARACTER MARK

Serial Number 97365221

Filing Date April 15, 2022

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EXHIBIT 5

An Automated DELFIA ADCC Assay Method using a CD16.NK-92 Cell Line

Author

Brad Larson
Agilent Technologies, Inc.

Introduction

Pharmaceutical companies are conducting more monoclonal antibody (mAb) research, and the European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) have each drafted guidelines^{1,2} for biosimilar product development using mAbs. Both guidelines recommend performing extensive structural and functional characterization of the proposed product. Characterization studies often include cell-based antibody-dependent cell-mediated cytotoxicity (ADCC) assays.

ADCC is a prominent mechanism in the host immune defense where the Fab region of an antibody binds to a specific antigen on a target cell (Figure 1), commonly an infected cell or pathogen. The Fc region of the same antibody then binds to a Fc γ RIII or CD16 receptor on an effector cell, commonly a natural killer (NK) cell. The bound NK cell then secretes apoptosis-inducing agents, thus destroying the target cell.

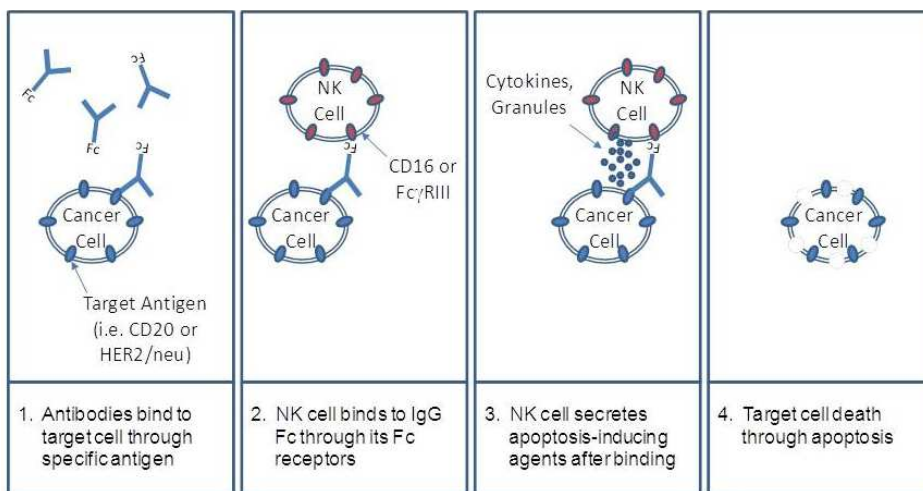


Figure 1. ADCC mechanism of action.

This application note discusses an automated, nonradioactive ADCC assay used in mAb immunotherapeutic development. The cell-based assay from PerkinElmer, Inc. (Waltham, MA), negates the need for dangerous radioactive materials and offers a simple, safe process. Additionally, when automated using Agilent BioTek instrumentation, the assay reduces active labor time and increases consistency and robustness over time and across users.

DELFI[®]A TRF assay theory

The dissociation-enhanced lanthanide fluorescent immunoassay (DELFI[®]A) calls for loading target cells with *bis*(acetoxymethyl) 2,2':6',2"-terpyridine-6,6"-dicarboxylate (BATDA), an acetoxymethyl ester of fluorescence enhancing ligand (Figure 2). Once inside the cell membrane, ester bonds hydrolyse to form 2,2':6',2"-terpyridine-6,6"-dicarboxylic acid (TDA), a non-cell permeable hydrophilic ligand. Upon cell lysis, the released TDA ligand now in solution binds to europium to form the highly fluorescent and stable chelate, EuTDA. The fluorescent signal is measured via time-resolved fluorescence (TRF), and directly correlates with the amount of target cell lysis.

In process, loaded target cells, titrated antibody, and effector cells are added to microplate wells (Figure 3). When the antibody binds to both target and effector cells, target cell lysis per the aforementioned mechanism of action is initiated. Following the appropriate incubation period, cells are pelleted. A supernatant aliquot containing TDA ligand is transferred to a separate microplate containing europium. The two compounds combine to form EuTDA, and the resulting signal is read using a microplate reader's TRF mode.

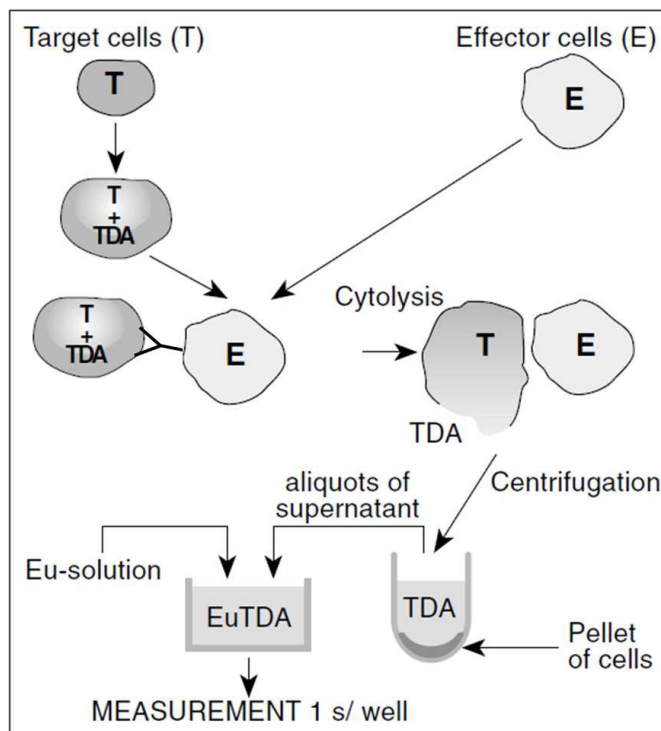
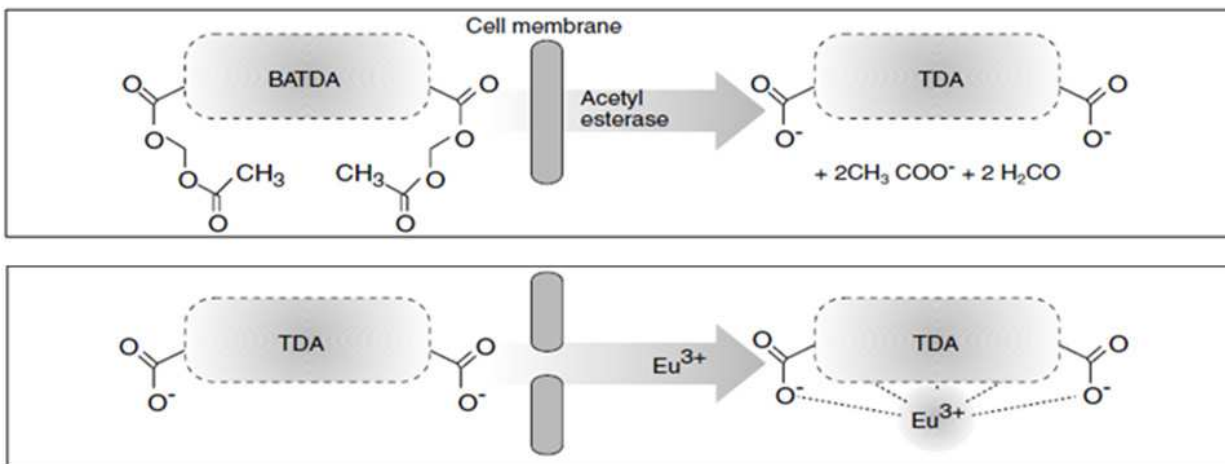


Figure 3. DELFIA assay, showing antibody bound to loaded target cells and effector cells; target cell lysis; pelleting; and TDA binding with europium to form the fluorescent chelate.



PerkinElmer Application Note: 1234-967-04 June 2001

Figure 2. DELFIA loading and detection principle.

Target and effector cell model

The assay model tested here incorporated ovarian carcinoma SKOV3 as target cells, as well as immortalized CD16.NK-92 cells from Conkwest (Del Mar, CA) as effector cells. These cells are derived from parental NK-92 cells, developed at the Fox Chase Cancer Center (FCCC), and are part of the Conkwest Neukopanel ADCC Assay System. They are transduced to express either the high affinity variant CD16-176V or the low affinity variant CD16-176F, which are allelic variants common in normal human populations. The advantage of CD16.NK-92 cells is that they have an easier cell preparation process, and therefore lower cost and required operator time, versus purifying fresh NK cells from blood. They also provide greater consistency in assay results versus repeated use of primary NK cells isolated from whole blood.

Materials

Cells and reagents

Ovarian carcinoma SKOV3 cells (part number AKR-253) were obtained from Cell Biolabs, Inc. (San Diego, CA). CD16-176F.NK-92 cells and CD16-176V.NK-92 cells were obtained from Conkwest. The Herceptin antibody was manufactured by Genentech, Inc. (South San Francisco, CA). Microplates were from Nunc (as included in the DELFIA complete assay kit) and Corning (part number 3361, Corning, NY). DELFIA EuTDA Cytotoxicity Reagent Kits, including BATDA Reagent, Lysis Buffer, and Europium Solution (part number AD0116) were purchased from PerkinElmer, Inc. (Waltham, MA).

Instrumentation

Precision microplate pipetting system

The compact Agilent BioTek Precision microplate pipetting system automates pipetting processes for walk-away operation. A user-configurable deck and four liquid handling transfer tools provide flexible experimental design for the DELFIA assay and many other microplate-based assays. Its small footprint allows for easy insertion into laminar flow hoods, making it especially suited for use with cell-based assay procedures. Per the DELFIA assay, Precision was used to perform all liquid handling steps, including test antibody titration and transfer, target and effector cell dispensing, supernatant transfer, and reagent dispensing.

Agilent BioTek Synergy H1 hybrid multimode microplate reader

A variety of Agilent BioTek Synergy multimode microplate readers are suitable for many budgets and applications. Each incorporates high precision and performance in

fluorescence, luminescence and absorbance modes, and are driven by integrated Agilent BioTek Gen5 data analysis software. The Agilent BioTek Synergy H1 hybrid multimode microplate reader is especially useful for the DELFIA assay. Synergy H1 provides high sensitivity and flexibility for cell-based and biochemical assays, with UV-visible absorbance, luminescence, and quadruple grating monochromator optics for top and bottom fluorescence. Synergy H1 may be upgraded at any time with an optional filter-based optics module for advanced read modes such as fluorescence polarization, TRF, and TRF energy transfer. Synergy H1 optics are devoid of fiber optic cables, reflective mirrors, and other components that limit light transmission and reduce sensitivity.

Methods

BATDA loading time determination

SKOV3 cells were washed with medium and adjusted to a concentration of 1×10^6 cells/mL. Three milliliters of cells were then placed into each of four different 15 mL conical tubes, followed by 5 μ L of BATDA reagent. The tubes were then placed into a 37 °C/5% CO₂ incubator for a total 5, 10, 20, or 30 minutes. Following incubation, the tubes were centrifuged to pellet the cells, medium was removed, and the cells were resuspended in wash buffer. The process was repeated three times to remove any ligand left in solution. After the final wash, the pellet was resuspended in culture medium and adjusted to concentrations of 1×10^5 or 2×10^5 cells/mL. Aliquots of 100 μ L of each cell concentration from the four different loading times, plus unloaded cells, were transferred to a microplate. Twenty-five microliters of lysis buffer were added to half of the replicates, while medium was added to the remaining wells. The plate was incubated for two hours using the aforementioned conditions to allow lysis to take place. The plate was then centrifuged for 5 minutes at 500 rpm. Twenty microliters of each supernatant was then combined with 200 μ L of europium solution in a separate plate, followed by a 15-minute room temperature incubation with shaking. The fluorescent signal from the wells containing lysed and unlysed cells was then quantified.

Target cell-mediated cytotoxicity test

One hundred microliters of preloaded SKOV3 cells, at a concentration of 1×10^5 cells/mL, were combined with 100 μ L of either CD16-176F.NK-92 or CD16-176V.NK-92 cells. NK-92 cells were seeded to create 10, 5, 3, 2, and 1:1 effector to target (E:T) cell ratios. Maximum release wells were also created by combining target cells and medium containing lysis buffer. The incubation and subsequent steps were followed as explained for the previous experiment.

DELFLIA ADCC assay

Multiple runs were performed using a 10:1 E:T cell ratio. 50 μL of preloaded SKOV3 cells at a concentration of 2×10^5 cells/mL and 100 μL CD16.NK-92 cells at a concentration of 1×10^6 cells/mL were added to each well of a 96-well assay plate. A 9-point 1:3 Herceptin antibody titration curve was created, and 50 μL of each 4x antibody concentration was added to each well. The plate was centrifuged for one minute at 750 rpm and incubated for 2 hours at $37^\circ\text{C}/5\% \text{CO}_2$ to allow opsonization and cell lysis to take place. After incubation, the plate was centrifuged again for five minutes at 750 rpm and 20 μL of supernatant was transferred to a detection plate. Two hundred microliters of europium solution were added to each well and the plate was shaken at 500 rpm for 15 minutes at room temperature. The fluorescent signal was then measured with Agilent BioTek Synergy H4 using a time-resolved format (data generated by Synergy H4 demonstrate similar data to that generated by Synergy H1). The assay was repeated using a 5:1 E:T ratio, as well as using a Corning 96-well plate in place of the Nunc assay plate provided in the DELFLIA kit to compare assay performance in the two microplate types.

Results and discussion

BATDA loading time determination

Per Figure 4, the optimal SKOV3 cell BATDA reagent loading time is seen at 20 minutes. This is the point where the highest ratio between the fluorescent signal from lysed and unlysed cells is seen without danger of cell overloading. Additionally, no advantage is seen when using cell concentrations higher than 1×10^5 cells/mL.

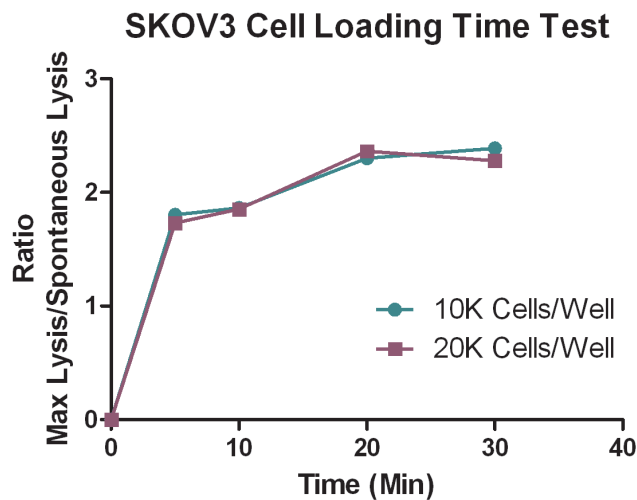


Figure 4. Optimal SKOV3 cell loading time showing peak BATDA loading at 20 minutes.

Target cell-mediated cytotoxicity test

When SKOV3 and engineered CD16.NK-92 cells were assayed in the absence of Herceptin, no appreciable cytotoxicity, or cell-mediated cytotoxicity, was noted (data not shown). This ensures that any increase in fluorescent signal seen when performing the ADCC assay is due to the cytotoxic properties of the test antibody.

Automated assay validation

Using a 10:1 E:T ratio (Figure 5), the high affinity CD16-176V.NK-92 strain was found to have increased cytotoxicity compared to the low affinity CD16-176F.

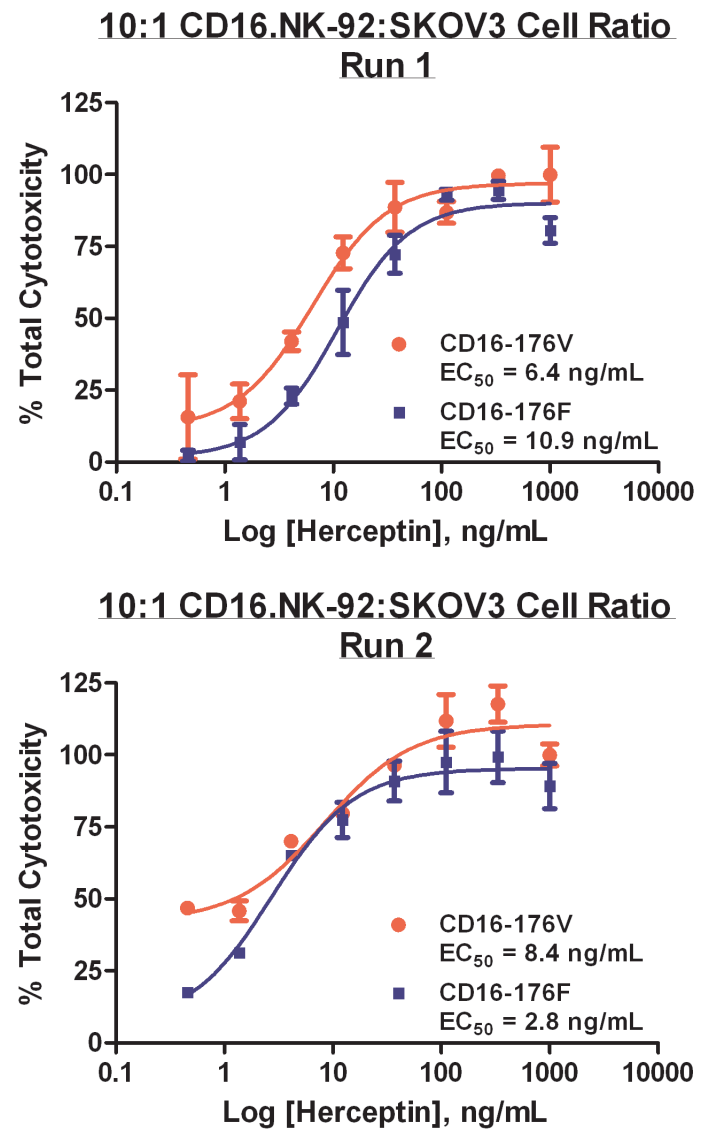


Figure 5. DELFLIA automated assay validation data using a 10:1 E:T ratio.

NK-92 strain at lower concentrations, consistent with previously generated data.³ Additionally, calculated EC₅₀ values in Figure 6 agree with previously generated values of 5.2 ng/mL and 15.4 ng/mL, respectively, using the chromium-based method.

A more realistic difference in cytotoxic effect between the CD16-176V.NK-92 and CD16-176F.NK-92 strains was seen using a 5:1 E:T ratio (Figure 6) rather than the 10:1 E:T ratio. Therefore, a 5:1 E:T ratio is recommended for CD16.NK-92 cell experiments.

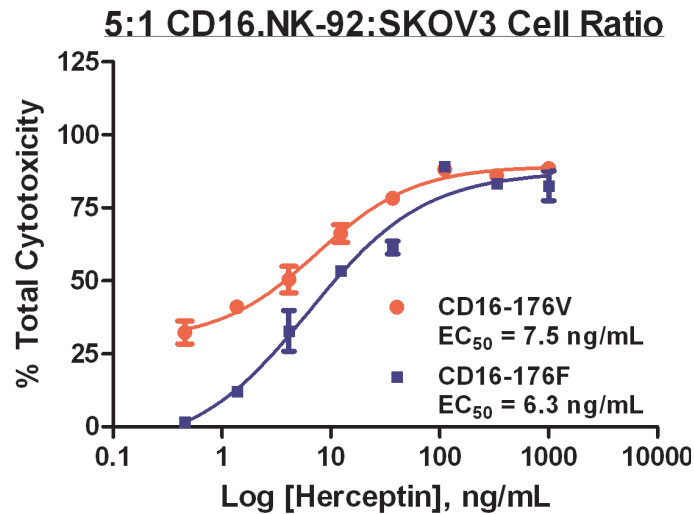


Figure 6. DELFIA automated assay validation data using a 5:1 E:T cell ratio.

Finally, microplate analysis using different manufacturers was evaluated. When comparing results from those obtained using the Nunc microplate included in the DELFIA complete assay kit, and a clear, flat-bottom, polystyrene Corning 3361 microplate (Figure 7), equivalent data were generated. This means that the Corning 3361 microplate may be a suitable substitute for the Nunc microplate when performing the DELFIA assay.

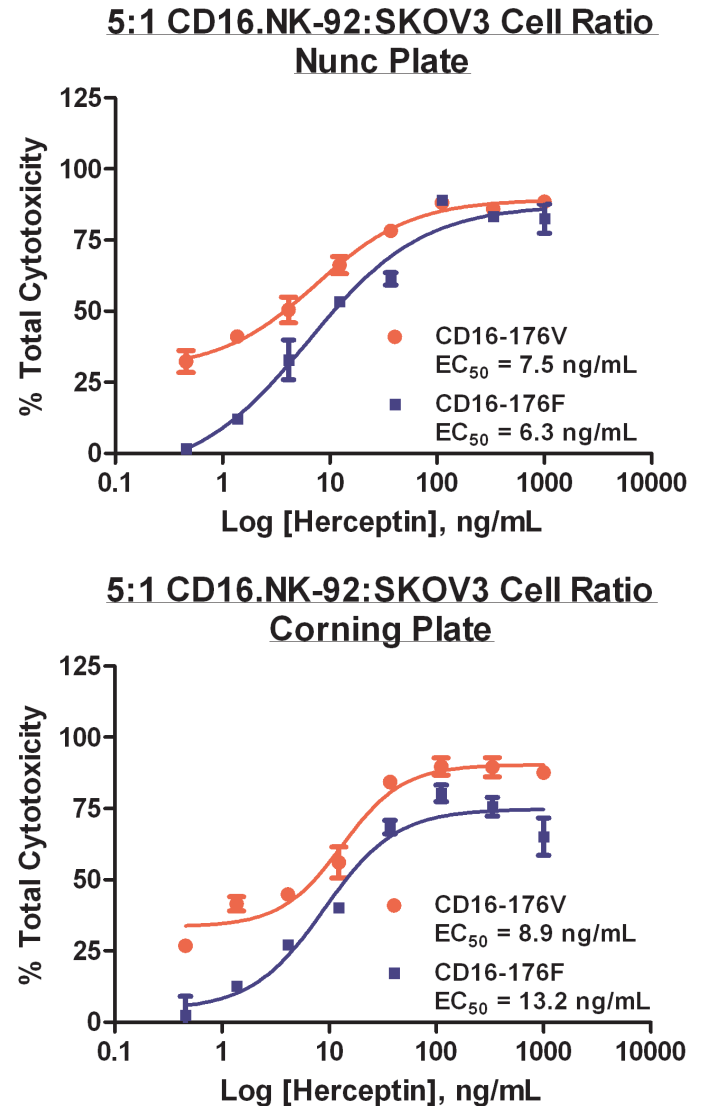


Figure 7. Comparison of DELFIA assay results using different microplate manufacturers.

Conclusion

This application note demonstrated the nonradioactive DELFIA assay for ADCC studies using TRF detection and CD16.NK-92 cell lines. The assay workflow may easily be automated with instruments such as the Agilent BioTek Precision microplate pipetting system to increase throughput and repeatability, and Agilent BioTek Synergy multimode microplate readers also increase overall laboratory efficiency. The propagatable effector cells lower cell preparation time and expense, and decrease variability. The combination of the nonradioactive assay chemistry, appropriate cell model, and automated instrumentation can increase productivity and throughput, simplify processes, and generate high-quality, reproducible results for the discovery of new antibody therapeutics.

References

1. Guideline on similar Biological Medicinal Products Containing Monoclonal Antibodies. *European Medicines Agency, Committee for Medicinal Products for Human Use*, EMA/CHMP/BMWP/403543/2010, Draft, **2010**.
2. Scientific Considerations in Demonstrating Biosimilarity to a Reference Product; U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), *Center for Biologics Evaluation and Research (CBER)*, Draft Guidance Feb **2012**.
3. Campbell, K. A Genetically-Modified NK Cell Line Expressing the Fc Receptor, CD16, for Use in ADCC Assays; *Fox Chase Cancer Center* **2008**, ff. http://www.fccc.edu/research/techtransfer/symposium/docs/900_920.pdf (accessed Dec 6, 2012).

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EXHIBIT 6

Semi-Automation of a Nonradioactive Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay

Part II: Comparisons with freshly isolated and cryopreserved human natural killer cells

Authors

Brad Larson and Peter Banks
Agilent Technologies, Inc.

Sumant Dhawan and
Shalini Wadwani
Cell Technology, Inc.

Abstract

This companion application note to Part I: Automation of a Bioluminescent ADCC Procedure with a Microplate Pipetting System, examines the effect of using different effector cell:target cell ratios and compares the results between freshly isolated human Natural Killer (NK) cells and commercially available cryopreserved human NK cells.

Introduction

In vitro antibody-dependent cell-mediated cytotoxicity (ADCC) assays are common tools for immunotherapeutic drug discovery and biosimilar development. Commonly, the search for a new immunotherapeutic or biosimilar drug uses a cancer cell line to model targeting proliferating cancer cells. In Part I of this companion application note, the use of Daudi cells (human Burkitt's lymphoma cell line) as a model for Rituxan-based biosimilar development was described. It demonstrated the utility of using a bioluminescent assay (aCella-TOX) to quantify the ability of rituximab to recruit freshly isolated human NK cells to induce the Daudi cells to undergo apoptosis.

The isolation of primary effector cells, such as human NK cells from peripheral blood, is a laborious, expensive process. Furthermore, donor-to-donor differences can lead to variations in ADCC assay performance. This application note examines the effect of using NK cells from different donors, reducing effector cell:target cell (E:T) ratios (20:1 to 10:1 NK cells to Daudi cells) and also the use of cryopreserved, commercially available NK cells, to relieve some of the issues associated with the isolation process. The use of cryopreserved NK cells tends to provide an off-the-shelf solution for users of ADCC assays by obviating the need for NK cell isolation.

Materials and methods

Materials

Daudi cells (human Burkitt's lymphoma cell line) were obtained from ATCC (part number CCL-213) and used as target cells in the ADCC assay. Rituximab, the monoclonal antibody in the drug Rituxan, and aCella-TOX (part number CLATOX 100-3) were provided by Cell Technology, Inc. Human primary NK cells were freshly isolated by Cell Technology for all experiments. NK cells from two individual donors were obtained and tested separately. Cryopreserved NK cells (part number PB012F) were provided by STEMCELL Technologies. These cells were thawed and used to manufacturer's instructions.

Daudi Cell Propagation Medium consisted of RPMI 1640 (Life Technologies, part number 11875), FBS, 10% (Life Technologies, part number 10437), NEAA, 1X (Life Technologies, part number 11140), and Pen-Strep-Glutamine, 1x (Life Technologies, part number 10378). ADCC Assay Medium consisted of the same components with the exception that Ultra-Low IgG FBS, 10% (Life Technologies, part number 16250), was substituted for the original FBS.

Instrumentation

The Agilent BioTek Precision microplate pipetting system combines an eight-channel pipetting head and an eight-channel bulk reagent dispenser in one instrument. The instrument was used to dispense all assay components, including target (Daudi) cells and complement, serially titrate antibody across a 96-well polypropylene plate, transfer samples from plate to plate, and dispense the aCella-TOX assay components.

The Agilent BioTek Synergy H4 hybrid multimode microplate reader combines filter- and monochromator-based detection systems in the same unit. A dedicated luminescence detection system was used to quantify the luminescent signal from each assay well. The plates were read in kinetic mode, using integrated Agilent BioTek Gen5 data analysis software, to capture the luminescent signal every 5 minutes.

Only semi-automated assays conducted with an Agilent BioTek Precision microplate pipetting system were performed.

Automated ADCC assay procedure

Daudi target cells, at a concentration of 2×10^5 cells/mL in 25 μ L were added to the 96-well assay plate. An 8-point titration curve was then created of the test antibody using serial 1:5 dilutions beginning at 1 μ g/mL. 25 μ L of each antibody dilution was added to the plate to start the reaction. The cells were allowed to opsonize for 15 minutes at 37 °C. NK effector cells, at a concentration of 4×10^6 cells/mL or 2×10^6 cells/mL, were then added (in 25 μ L) to give an E:T ratio of 20:1 or 10:1, respectively for both freshly isolated and cryopreserved NK cells. The plate was centrifuged for one minute, and incubated at 37 °C for 1.75 hours. The plate was removed from the 37 °C incubator and allowed to cool to room temperature for 5 to 10 minutes. The target cells in the maximum lysis control wells were lysed by adding 10 μ L of the lysis buffer, and the plate was incubated for an additional 5 minutes. 125 μ L of ADCC assay medium was then added to each well to bring the volume to 200 μ L. The plates were centrifuged for one minute. 50 μ L of enzyme assay diluent was then transferred to the appropriate wells of an opaque white luminescence plate. Then, 50 μ L of each reaction supernatant was transferred to wells containing the assay diluent. Then, 100 μ L of 2x enzyme assay reagent (containing G3P), followed by 50 μ L of 1x detection reagent was added to each diluted supernatant. The plates were immediately read using an Agilent BioTek Synergy H4 at 5-minute intervals. The RLU's were graphed and the data reduced by four-parameter fits for analysis.

Percent total cytotoxicity calculation

The luminescent signal from the wells containing media and other assay components was subtracted from all other wells to correct for background interference. Average nonlysed target cell-only control well signal was then subtracted from all sample wells. Percent total cytotoxicity was then calculated by dividing adjusted sample well signal by the average maximum lysis signal, and multiplying the result by 100.

Results and discussion

Figure 1 depicts the comparative performance of using different donors of freshly isolated NK cell and E:T ratios (20:1 and 10:1). It is apparent that for Donor 1, reducing the amount of NK cells by a factor of 2 has no discernable effect on the extent of cytotoxicity as the high doses of rituximab (i.e. >100 ng/mL) leads to complete (100%) Daudi cell toxicity. There is a small right shift in EC_{50} in using this lower amount of NK cells by nearly a factor of 2, however, but this may well not be pharmacologically relevant. A different situation is seen for the second donor of NK cells. High doses of rituximab do not provide complete Daudi cell toxicity using either 20:1 or 10:1 E:T ratios. Furthermore, reducing the NK cell amount lowers the extent of Daudi cell toxicity by approximately one third. The EC_{50} results for Donor 2 at both E:T ratios are significantly different from Donor 1 and remain the same for both ratios. It is apparent for both donors that higher E:T ratios tend to produce higher background toxicity (rituximab dosing <1 ng/mL).

As NK cells are primary cells, they can only be cultured for so long before they undergo senescence. Thus, pooling of donor samples has limited utility for reducing donor-to-donor variability in ADCC assays. The ability to cryopreserve NK cells, however, should assist in pooling a large number of donor samples and thus reduce variability in the ADCC assay. Figure 2 depicts the use of cryopreserved cells at both 20:1 and 10:1 E:T ratios. It is apparent that % total cytotoxicity responses are similar to Donor 2, where high doses of rituximab produce 60% and 40% total cytotoxicity for 20:1 and 10:1, respectively, but EC_{50} s are more consistent with Donor 1.

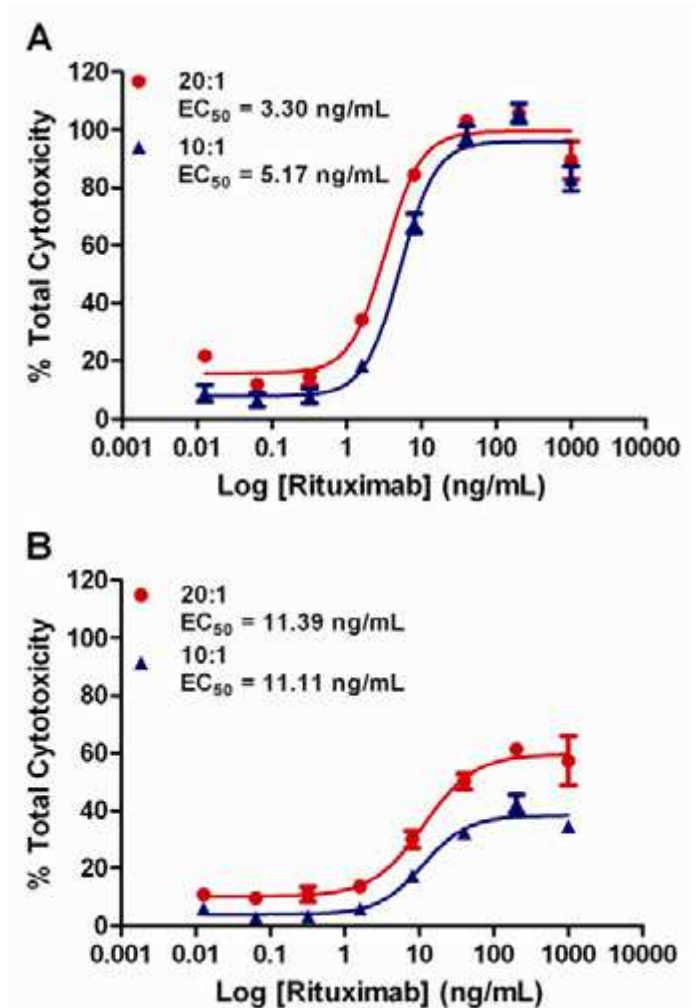


Figure 1. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay for Donor 1 (A) and Donor 2 (B).

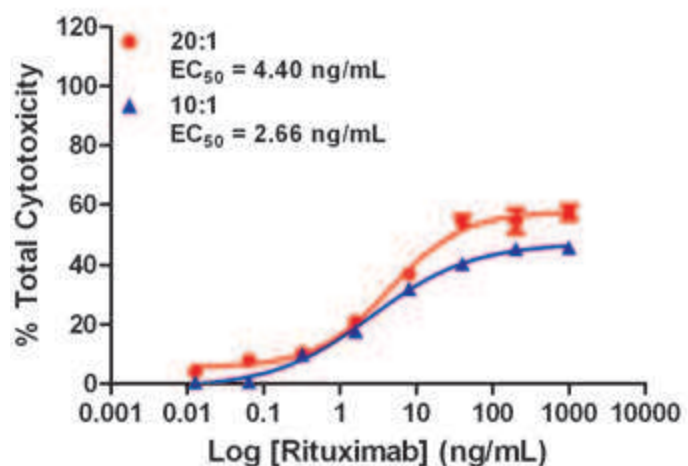


Figure 2. Comparative performance in 20:1 and 10:1 E:T ratios in rituximab dose-response ADCC assay using cryopreserved NK cells.

Conclusion

It has been shown that there can be differences in ADCC assay performance produced between donors of freshly isolated NK cells and from differing E:T ratios. This has some implication for immunotherapeutic drug discovery. It would be prudent for investigators to determine up front the range of donor-to-donor variability for the target cell they use in their ADCC model, to obtain consistent data between testing one antibody against another. The use of cryopreserved NK cells can relieve this problem to some extent, but one would also assume there would be lot-to-lot variability from the manufacturer, as well.

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EXHIBIT 7

Label-Free Assay for NK Cell-Mediated Cytolysis

xCELLigence real-time cell analysis

Author

Brandon Lamarche,
Joyce Velez, and Leyna Zhao
Agilent Technologies, Inc.

Introduction

Natural Killer (NK) cells are bone-marrow-derived lymphocytes originally identified by their large granular morphology. The NK cell lineage has been considered for cancer eradication due to its ability to kill a wide variety of tumor cells spontaneously, while sparing normal cells. While T cells must be educated by antigen-presenting cells before they recognize tumors, NK cells spontaneously lyse certain tumor targets *in vivo* and *in vitro* without requiring immunization or pre-activation. Several *in vivo* and *in vitro* studies have shown that, in addition to extravasation and the ability to infiltrate tumor tissues, NK cells have promising antitumor effects.

In addition, individuals lacking NK cells suffer from persistent viral infections and die prematurely as a consequence. The potency of uncontrolled or inappropriate NK cell responses is evident in disease conditions, such as allograft rejection, graft versus host disease, diabetes, various autoimmune and neurological diseases, and aplastic anemia/neutropenia. NK cells therefore play a prominent role in various physiological and disease states. The assessment of their cytolytic activity is important for monitoring immunocompetence in cancer, infectious diseases, and autoimmune diseases, and in determining the proteins that mediate the cytolytic effect.

The standard methods for measuring NK cell cytolytic activity are radioactive label release assays^{1,2} using chromium (⁵¹Cr), or indium (¹¹¹In).

In these assays, the target cells are radioactively labeled then mixed with effector cells. The release of the radioactive isotope, which correlates with NK cell-mediated cytotoxicity, is then measured at a given time point (less than four hours). Several nonradioactive labeling assays are also available, including flow cytometry, ELISA-based granzyme measurement, and morphometric analysis by microscopy.³

The Agilent xCELLigence system is an impedance-based real-time cell analysis (RTCA) technology. The attachment and interaction of adherent tumor cells with biosensors at the bottom of 96-well E-Plate leads to impedance changes, which correlate with cell number, size, and shape. In contrast, the addition of suspended NK cells to the wells results in negligible changes due to nonexistent or weak interaction with the biosensors. In this application note, we will summarize a series of experiments to demonstrate how the xCELLigence RTCA system can be applied to quantitatively, dynamically monitor NK cell-mediated cytotoxicity without labeling the target cells.

Materials and methods

Cells

The NK92, NIH 3T3, and all cancer cell lines used in these experiments were obtained from ATCC. The murine NK cell (mNK) was provided by Dr. Hui Shao of the University of Louisville. All the cell lines were maintained in a 37 °C incubator with 5% CO₂. The NK92 and mNK lines were maintained in Alpha MEM with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, 12.5% FBS, and 100 to 200 U/mL recombinant IL-2. Other cancer cell lines were maintained in RPMI media containing 5% FBS, 1% penicillin, and 1% streptomycin (Gibco). The NIH 3T3

cells were maintained in DMEM media containing 10% FBS, 1% penicillin, and 1% streptomycin.

Cytolytic analysis

Target cells were seeded (after establishing background with medium alone) into the wells of 96-well E-Plates in 100 µL of media. Cell growth was dynamically monitored with the xCELLigence RTCA system until they reached the log growth phase and formed a monolayer (24 to 34 hours, depending on the experiment). Effector NK cells at different concentrations were then directly added to the wells. Effector cells in wells without target cells are for the effector only background control. After the addition of the effector cells, the system continued to take measurements every 15 minutes for up to 20 hours.

Cell morphology analysis by microscopy

The effect of NK cell-mediated cytotoxicity on target cells was examined using a Nikon upright microscope. When the Cell Index (CI) dropped to 50% (relative to the control) after the addition of effector cells, cells were removed from the system, fixed in 80% methanol for five minutes, and stained with Giemsa blue. The morphology of the cells was examined under the microscope and imaged with a CCD camera.

Experiment data analysis

The integrated xCELLigence RTCA software displays the entire history of the experiment from seeding the cells to the end of cytotoxicity. The time- and effector-to-target ratio (E/T) dependent curves were displayed in real time, so NK cell activity was monitored continuously. The measured impedance is expressed in arbitrary CI units. The CI at each time point was defined as $(R_n - R_b)/15$, where R_n was the cell-electrode impedance of the well when it contains cells, and R_b was the background impedance of the well with the media alone.

To quantify the lysis at specific time points, the data were exported to Microsoft Excel, and the percentage cytotoxicity at specific E/T ratios was determined by comparing with the control.

Results and discussion

Dynamic monitoring of NK cell-mediated cytotoxicity

An effector murine NK cell line (mNK), and a target mouse cell line (NIH3T3) were used to assess NK cell-mediated cytotoxicity. 5,000 NIH 3T3 cells were seeded in wells of 96-well E-Plate. An xCELLigence RTCA system was used to monitor the cell growth every 60 minutes until the cells reached the growth phase at 34 hours. The effector murine NK cells were then directly added to the wells at different E/T ratios. The NK cell-mediated cytotoxicity was continuously monitored afterwards. As shown in Figure 1A, Cell Index declined significantly after adding mNK effector cells at the E/T ratio of 15:1, but no notable change was observed in wells without effector cells and negative control wells with YAC cells, which is a T lymphocyte line. This indicates that the decrease in the CI was due to the addition of the mNK cells and most likely mediated by cytotoxicity. This mNK cell-mediated cytotoxicity is time-dependent (Figure 1B). To further confirm the cytotoxicity effect, target cells were stained at eight hours after the addition of the mNK cells when the cytotoxicity reached approximately 50%, then examined under a microscope. As shown in Figure 1C, in the presence of mNK cells, a portion of target cells were effectively cleared away by the cytotoxic action of mNK cells, while adding the control YAC cells resulted in no change.

Furthermore, the kinetic analysis indicates that the mNK cell-mediated cytotoxicity can last longer than four hours, which is the standard incubation time for the radioisotope-based assay. The cytotoxicity was less than 30% after four hours, and can reach up to 70% by 12 hours. So, potentially, the standard incubation time of existing label-based assays could underestimate the NK cell's cytotoxic activity. Thus, the xCELLigence RTCA system not only offers label-free detection, but also provides more accurate assessment of cytotoxicity.

In summary, the xCELLigence RTCA system is one of the few available assay formats that can directly monitor NK cell-mediated cytotoxicity without labeling the target cells or using any chemical reporters. It enables scientists to monitor the entire cytotoxicity process dynamically, a feature that would be difficult to replicate with any label-based, endpoint assay format.

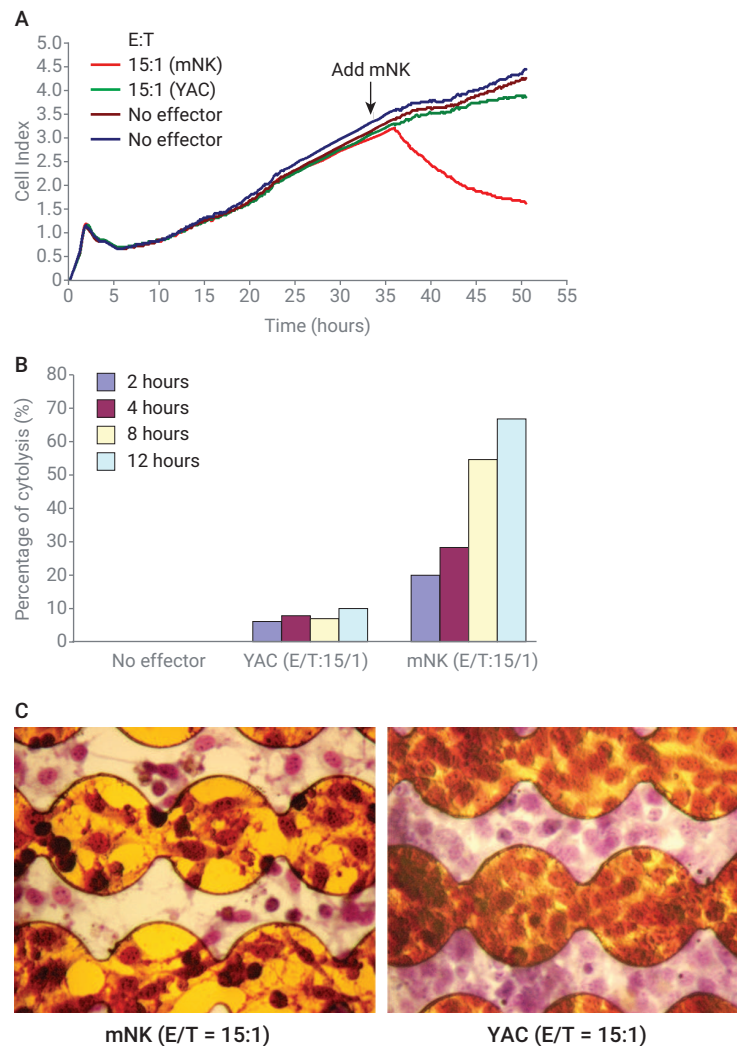


Figure 1. NK cell-mediated cytotoxicity. (A) Dynamic monitoring of NK cell-mediated cytotoxicity of NIH 3T3 cells. The NIH 3T3 cells were seeded in the 96-well E-Plate at 5,000 cells/well. Cell attachment, spreading, and proliferation were monitored in real time. 34 hours after seeding the cells, Cell Index values reached 3, which is equivalent to approximately 10,000 cells/well. 150,000 mNK cells or YAC (negative control) cells were added to wells in triplicate. (B) Time-dependent cytotoxic activity of mNK cells. The cytotoxic activity at a given time point was calculated and presented as the percentage of cytotoxicity $\{\% \text{ of cytotoxicity} = (CI_{\text{no effector}} - CI_{\text{effector}}) / CI_{\text{no effector}} \times 100\}$. (C) NIH3T3 target cell morphology at eight hours after addition of mNK and negative control YAC cells at 15:1 E/T ratio. Cells were stained with Giemsa blue and imaged with a 10x Nikon objective.

Quantitative measurement of NK cell-mediated cytotoxicity

The CI correlates with cell number,⁵ and has been used to quantitatively monitor cytotoxicity induced by chemical compounds, such as anticancer drugs, at different concentrations. To test whether NK cell activity can also be assayed quantitatively across a range of killing activity, cytotoxicity was monitored at different E/T ratios. We used both murine and human NK cell lines (mNK and NK92) as effectors. NIH 3T3 mouse cells and MCF7 cells (human breast cancer cells) were respective targets. As described previously, the target cells were first seeded to the 96-well E-Plate at 5,000 cells/well, and the cell growth was monitored with an xCELLigence

RTCA system. When the target cells reached the growth phase, the NK cells were directly added to wells at different concentrations. The NK cell-mediated cytotoxicity at different E/T ratios was then monitored in real time.

As shown in Figure 2, the normalized Cell Index (NCI) declined relative to the “no effector” control after the addition of mNK or NK92 cells to its target cells. The decline in the NCI values was E/T ratio-dependent. In both cases, the higher the E/T ratio, the lower the CI value. The decline was caused by a decrease in cell/electrode interaction that occurred during cytotoxicity. This demonstrates that the xCELLigence RTCA system permits specific and quantitative measurement of NK cell-mediated cytotoxic activity.

In addition, the dynamic monitoring of the cytotoxicity may also provide more insights into the underlying mechanisms of NK cell-mediated killing. In this study, the kinetic data show that NK92 cells are much more potent effectors than mNK cells. At E/T ratio of 4:1 or higher, NK92 cells killed more than 90% of MCF7 cells within four hours. Whereas for mNK cells, only 30% cytotoxicity occurs within that time. The difference in cytotoxic kinetics of NK cells indicates that the nature of the interaction between effectors and targets is cell-specific and may involve factors such as expression of NK receptors and ligands, or different mechanisms of NK cell-mediated cytotoxicity.

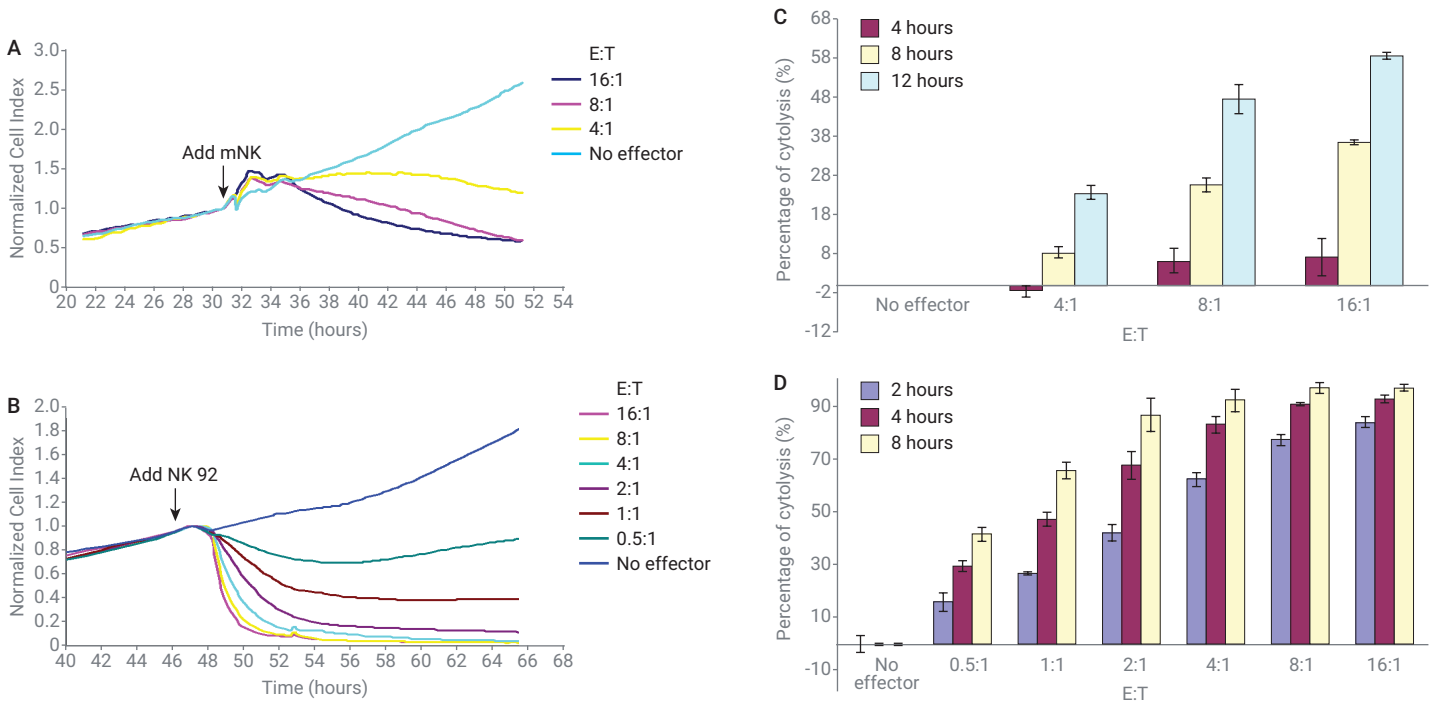


Figure 2. Label-free and quantitative measurement of cytotoxic activity of mNK cells and NK92 cells. (A). Quantitative measurement of cytotoxic activity of mNK cells. The NIH 3T3 cells were seeded to the 96-well E-Plate. Cell growth was monitored in real time on the xCELLigence RTCA system until the CI values reached 3, equivalent to 10,000 cells/well. The mNK cells were then added to target cells at different concentrations to generate a series of E/T ratios. The cytotoxicity of the target cells at different E/T ratios was dynamically monitored on the system. A normalized Cell Index was used, where the Cell Index values obtained after the addition of mNK cells were normalized against the Cell Index value from the same well before the addition of mNK cells. (C). Time-dependent cytotoxic activity of mNK cells at different E/T ratios. The percentage of cytotoxicity of the NIH 3T3 cells by mNK cells was calculated as described in Figure 1. The time-dependent cytotoxic activities are indicated by the curves. (B). Quantitative measurement of cytotoxic activity of NK92 cells. The MCF7 target cells were seeded, and the cell growth was monitored on the system as described above. The NK92 cells were then added to each well at different concentrations to generate the series of E/T ratios indicated. The cytotoxic activities of NK92 cells on MCF7 cells at different E/T ratios were dynamically monitored on the system. (D). Time-dependent cytotoxic activity of NK92 cells at different E/T ratios. The percentage of cytotoxicity of the MCF7 cells by NK92 cells was calculated as described in Figure 1.

Label-free assessment of NK cell cytolytic activity in various target cell lines

The cytolytic activities of mNK and NK92 were tested on nine cell lines, including eight different human cancer cell lines and the NIH 3T3 mouse cell line. The susceptibility of different target cell lines to NK92-mediated cytotoxicity is summarized in Table 1. NK92 showed a broad spectrum of cytolytic activity on cancer cell lines. The cytotoxicity mediated by NK92 occurred quickly, and reached the maximum killing activity in less than eight hours. Among seven cell lines tested, four cell lines (H460, HepG2, MCF7, and MDA-MB-231) reached 90% cytotoxicity (Figure 3A). In contrast, mNK cell-mediated cytotoxicity appeared to be more selective than NK92 (Figure 3B). Only four target cell lines (NIH 3T3, A549, HeLa, and MDA-MB-231) showed 30% to 65% cytotoxicity after 12 hours. The remaining five target cell lines, HT108, H460, HepG2, MCF7, and OVCAR4, displayed no or weak cytotoxicity (10%). In addition, the cytotoxicity mediated by mNK was much slower than that mediated by NK92, reaching the maximum approximately 12 hours after the addition of mNK cells (data not shown).

Table 1. NK92 cell-mediated cytotoxicity of seven cell lines.

Cell Name	Cell Type	Species	Maximum Cytotoxicity (%) at 12 Hours
HT1080	Fibrosarcoma	Human	42.2
H460	Nonsmall cell lung cancer	Human	95.4
HepG2	Hepatoma	Human	94.1
MCF7	Breast cancer	Human	96.5
A549	Nonsmall cell lung cancer	Human	52.2
HeLa	Cervix cancer	Human	51.0
MDA-MB-231	Breast cancer	Human	97.0

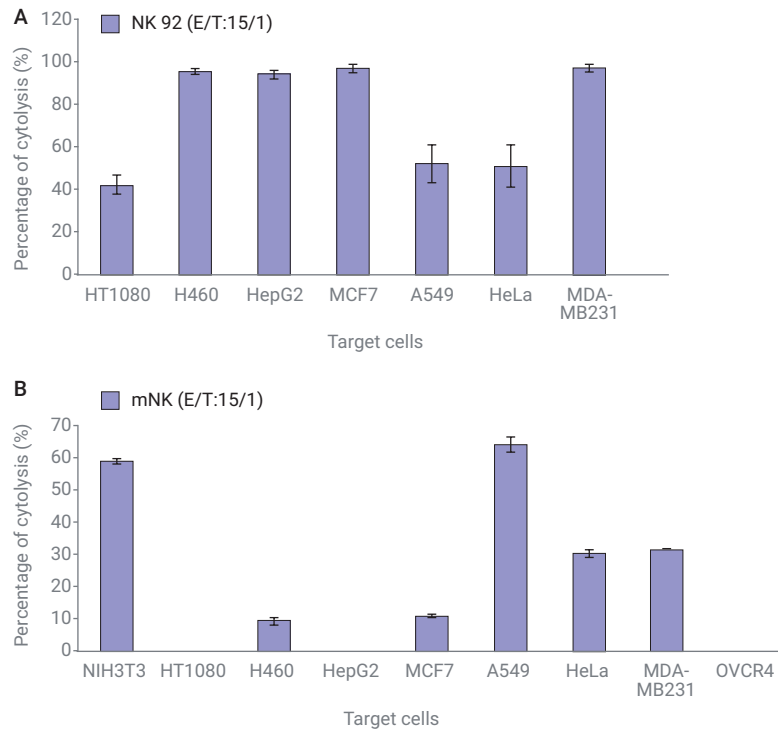


Figure 3. Label-free assessment of NK cell-mediated cytotoxicity using various cell lines. (A) The NK92-mediated cytotoxicity of seven different cancer cell lines. The percentage of cytotoxicity indicated for each cell line is calculated based on the Cell Index value of individual wells eight hours after NK92 cells were added. (B) The mNK-mediated cytotoxicity of nine different cancer cell lines. The percentage of cytotoxicity indicated for each cell line is calculated based on the Cell Index values of individual wells 12 hours after mNK cells were added. This was when the cytotoxicity reached maximum.

In summary, both human and murine NK cell lines were tested for their cytolytic activities on nine different target cell lines, including human cancer cell lines commonly used in the field. The experiments demonstrate that the xCELLigence RTCA system is capable of quantitatively assessing the NK cell-mediated cytolytic activity in real time, but without any labeling steps or additional reagents. This new technology also offers a full, automated solution, which could enable a large-scale screening of chemical compounds or genes responsible for the regulation of NK cell-mediated cytolytic activity.

References

1. Brunner *et al.* Quantitative Assay for the Lytic Action of Immune Lymphoid Cells on 51-Cr-Labelled Allogeneic Target Cells In Vitro; Inhibition by Sioantibody and by drugs. *Immunology* **1968**, *14*, 181–196.
2. Geldhof *et al.* Expression of B7-1 by Highly Metastatic Mouse T Lymphomas Induces Optimal Natural Killer Cell-Mediated Cytotoxicity. *Cancer Res.* **1995**, *55*, 2730–2733.
3. De Meyer *et al.* Morphometric Analysis of Cytolysis in Cultured Cell Monolayers: a Simple and Versatile Method for the Evaluation of the Lytic Activity and the Fate of LAK Cells. *J. Immunol. Methods* **2003**, *277*, 193–211.
4. Solly *et al.* Application of Real-Time Cell Electronic Sensing (RT-CES) Technology to Cell-Based Assays. *Assay Drug Dev. Technol.* **2004**, *2*, 363–72.

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EXHIBIT 8

Agilent xCELLigence Immunotherapy Kit

Monitor liquid tumor cell killing in real time



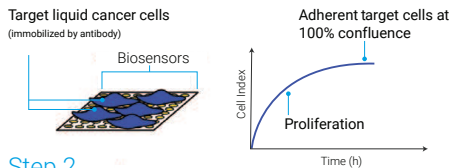


Monitor the efficacy of liquid cancer immunotherapies inside your incubator

Use Agilent xCELLigence immunotherapy kits with your real-time cell analysis (RTCA) system for a noninvasive solution to a broad range of applications. Improve reproducibility in your liquid cancer immunotherapy and suspension tumor cell killing applications, while maintaining cell health with continuous kinetic measurement.

- **Real time:** Quantitative monitoring of both fast (hours) and slow (days) killing
- **Simple workflow:** Reduce the number of sample handling steps
- **Improved sensitivity:** Physiologically relevant, low effector-to-target ratios
- **Automatic data plotting:** Eliminate subjective data

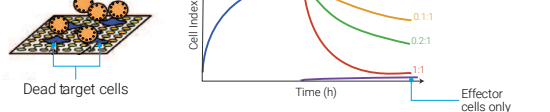
Step 1



Step 2

+ Nonadherent effector cells

Step 3



Step 1

The target liquid cancer cells are first seeded in the wells of an electronic microplate (E-Plate) that have been precoated with a tethering antibody. This biosensor signal, also known as Cell Index, increases as cells proliferate and then plateaus as cells approach 100% confluence.

Step 2

When added, nonadherent immune effector cells in suspension do not cause impedance changes (due to lack of adherence to the gold biosensors).

Step 3

If effector cells induce cell death of the target tumor cells, this cytolytic activity is precisely detected with high sensitivity. The continuous acquisition of impedance data for each well enables the generation of real-time killing curves for multiple conditions simultaneously.

Immune effector cells and liquid cancer target cells in use

Liquid Tumor Tethering Specificity	Effector Cells	Target Cells
anti-CD40	NK-92, CAR-T, primary CD8+ T cells	Daudi, Raji, Ramos, MEC2
anti-CD29	NK-92	K562, HEL 92.1.7
anti-CD19	NK-92, primary CD8+ T cells	Raji
anti-CD9	NK-92	NALM6, RS4;11, RPMI 8226
anti-CD71	NK-92	K562

Liquid tumor killing assay (anti-CD40) application data

The wells of an Agilent E-Plate are precoated with an anti-CD40 reagent, enabling liquid tumor targets to be immobilized on the plate bottom (Figure 1A). Immobilized target cells generate a robust impedance signal and proliferate to the point of confluence, resulting in a plateaued impedance signal. The growth of untethered target cells is essentially undetectable (Figure 1B). With or without tethering reagent coating the wells, the effector cells (NK-92 cells) produce minimal signal on their own (Figure 1B). The addition of effectors to the immobilized targets results in target cell death in a dose-dependent manner (Figure 1C).

Side-by-side, four-hour assays were performed for NK-92 cell mediated killing of Raji B cells that were immobilized (analyzed by Agilent xCELLigence) or in suspension (analyzed by flow cytometry). This assay was used to assess whether the physical immobilization of liquid tumor cells, via CD40 tethering, affects the efficiency with which they are killed. As shown in Figure 1D, the killing trends observed by these two methods correlate closely. The results were consistent with several previous publications, demonstrating that xCELLigence data reinforces data obtained by traditional assays.

Figure A.



Figure C.

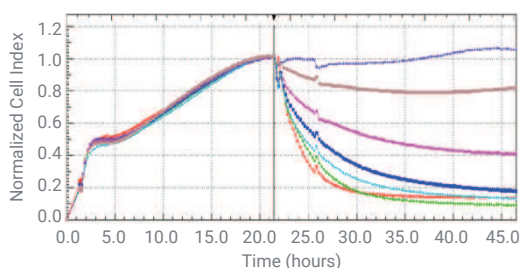


Figure B.

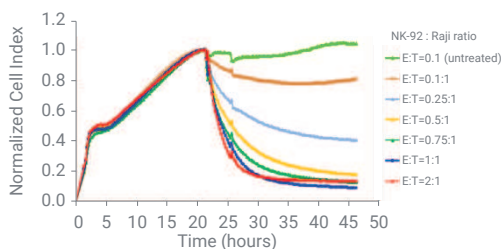
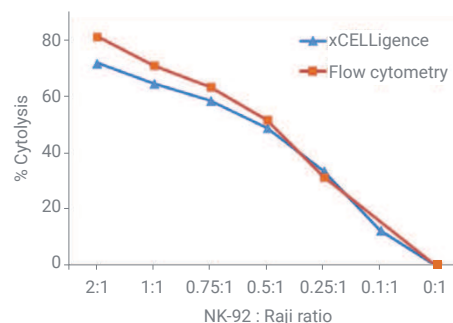


Figure D.



Agilent xCELLigence instruments for immunotherapy assays



Dual Purpose (DP)	Single Plate (SP)	Multiple Plates (MP)	High Throughput	Impedance and Imaging
3 × 16 wells	1 × 96 wells	6 × 96 wells	Up to 4 × 384 wells	Up to 5 × 96 wells





Liquid tumor killing assay (anti-CD40)

Tethering Kit (up to 6 × 96 well plates)
Part Number 8100005

125x Tethering reagent (anti-CD40) (250 µL)

10x tethering buffer (10 mL)

Cytolysis reagent (1.5 mL)

Sample Kit (up to 2 × 96 well plates)
Part Number 8100006

125x Tethering reagent (anti-CD40) (90 µL)

10x tethering buffer (10 mL)

Cytolysis reagent (1.5 mL)

Liquid tumor killing assay (anti-CD29)

Tethering Kit (up to 6 × 96 well plates)
Part Number 8100008

250x Tethering reagent (anti-CD29) (125 µL)

10x tethering buffer (10 mL)

Cytolysis reagent (1.5 mL)

Sample Kit (up to 2 × 96 well plates)
Part Number 8100009

250x Tethering reagent (anti-CD29) (45 µL)

10x tethering buffer (10 mL)

Cytolysis reagent (1.5 mL)

Liquid tumor killing assay (anti-CD19)

Tethering Kit (up to 6 × 96 well plates) Part Number 8100011	Sample Kit (up to 2 × 96 well plates) Part Number 8100012
125x Tethering reagent (anti-CD19) (250 µL)	125x Tethering reagent (anti-CD19) (90 µL)
10x tethering buffer (10 mL)	10x tethering buffer (10 mL)
Cytolysis reagent (1.5 mL)	Cytolysis reagent (1.5 mL)

Liquid tumor killing assay (anti-CD9)

Tethering Kit (up to 6 × 96 well plates) Part Number 8100014	Sample Kit (up to 2 × 96 well plates) Part Number 8100015
125x Tethering reagent (anti-CD9) (250 µL)	125x Tethering reagent (anti-CD9) (90 µL)
10x tethering buffer (10 mL)	10x tethering buffer (10 mL)
Cytolysis reagent (1.5 mL)	Cytolysis reagent (1.5 mL)

Liquid tumor killing assay (anti-CD71)

Tethering Kit (up to 6 × 96 well plates) Part Number 8100017	Sample Kit (up to 2 × 96 well plates) Part Number 8100018
125x Tethering reagent (anti-CD71) (250 µL)	125x Tethering reagent (anti-CD71) (90 µL)
10x tethering buffer (10 mL)	10x tethering buffer (10 mL)
Cytolysis reagent (1.5 mL)	Cytolysis reagent (1.5 mL)

Related products

Part Number	Part
300600890	E-Plate 16 PET (6 plates)
300600880	E-Plate 16 PET (36 plates)
5469830001	E-Plate 16 (6 plates)
5469813001	E-Plate 16 (36 plates)
300601140	E-Plate VIEW 16 (6 plates)
300601150	E-Plate VIEW 16 (36 plates)
300600910	E-Plate 96 PET (6 plates)
300600900	E-Plate 96 PET (36 plates)
5232368001	E-Plate 96 (6 plates)
5232376001	E-Plate 96 (36 plates)
300601020	E-Plate View 96 (6 plates)
300601030	E-Plate View 96 (36 plates)
5867681001	E-Plate 384 (10 plates)
5867673001	E-Plate 384 (40 plates)

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