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APPLICATION NOTE

Implementing MOA-Reflective ADCC Assays using Ready-to-Use KILR Target and Effector Cells from Screening to Lot Release

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INTRODUCTION

The development of antibody-based therapeutics to target the killing of tumor cells has revolutionized the space of immuno-oncology and the field of cancer therapeutics. Mechanisms by which a therapeutic antibody is able to target and kill tumor cells is critical to capture and demonstrate for Investigational New Drug (IND) applications. One of the mechanisms by which a therapeutic antibody can leverage the host immune defense and evoke target cell death is the antibody-dependent cell-mediated cytotoxicity (ADCC). Quantifying ADCC potential of an antibody therapeutic being evaluated relies on employing assays that include either radiolabeling (posing safety challenges), dye-based assays (risking the spontaneous release of dyes from the cells), or reporter gene assays (indirect MOA (mechanism-of-action)-reflective downstream detection only), all of which compromise the quality of the final data or provide non-functional output. This creates the need for a secondary bridging ADCC assay that measures direct target cell death by immune effector cells. The availability of a direct MOA-reflective ADCC assay that can be implemented from screening to characterization to potency-testing in a QC lot-release program is of paramount importance to address the market need.

This application note focuses on demonstrating the ability to measure direct target cell death mediated by ADCC via implementing an MOA-reflective cell-based assay format that offers several advantages over existing methods. This cell-based assay format (also known as the KILR® cytotoxicity assay platform) provides robustness, precision, accuracy, and demonstrates its fit-for-purpose nature from screening therapeutic antibody candidates in early drug development to characterization and potency testing in QC lot-release programs. The KILR assay also demonstrates versatility by being compatible with different cell models and a variety of effector cells. A KILR target cell model (Raji) was adapted to a ready-to-use (RTU) format to significantly reduce assay development and assay performance time. The KILR Raji ADCC bioassay demonstrates excellent robustness and assay reproducibility. In this application note, we present phase-appropriate qualification data to conclude that the KILR Raji ADCC bioassay is fit-for-purpose for screening and relative potency testing in lot-release testing.

BACKGROUND

Antibody-Dependent Cell-Mediated Cytotoxicity

ADCC is an important mechanism by which an antibody drug can kill an antigen presenting target cell by utilizing the host immune defense mechanism. In ADCC, a therapeutic antibody binds to antigen-expressing target tumor cells with its Fab region, and engages with an immune effector cell via the Fcγ receptor (FcγR) on the effector cell surface. This engagement causes the immune effector cells (natural killer (NK) cells, macrophages, monocytes, and eosinophils) to secrete apoptotic substances (e.g., perforin, granzymes, etc.) that mediate target cell lysis. Figure 1 shows an illustration of the mechanism of ADCC.

Assays for Measuring ADCC

Cytotoxicity assays, such as ADCC, are commonly used in immuno-oncology. The ability of a therapeutic antibody to utilize ADCC as a primary MOA and promote target cell death can be demonstrated using *in vitro* assays to predict and mimic a physiological response.

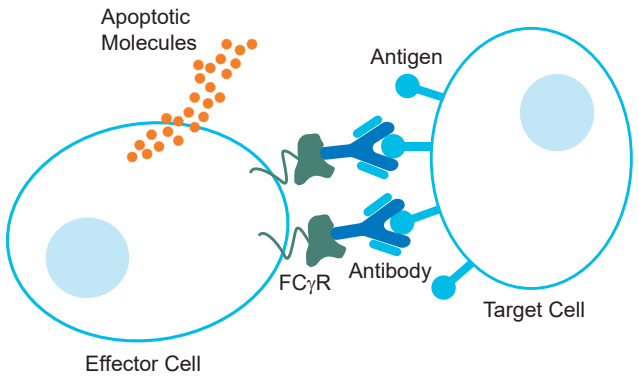


Figure 1. Antibody mediated cell cytotoxicity occurs when FcγR of effector cell binds to the Fc portion of the antibody, which also binds to antigen expressing target cell via Fab region. This association causes the immune effector cell to release perforins, granzymes which causes target cell lysis.

Commonly used assays include reporter gene and fluorescent dye-based assays. However, the reporter gene assays suffer from drawbacks as they serve to predict the ADCC potential and typically require a bridging ADCC assay to quantify target cell death. Other assays, such as radiolabeling pose safety challenges, while fluorescent and dye-based assays run the risk of spontaneous release of dyes from the cells causing leaks, thus impacting the accuracy and reproducibility of the data. MOA-reflective assays are being increasingly required by regulatory agencies (for MOA profiling in IND filings) as a crucial format for evaluating a therapeutic antibody's impact on target cancer cells. When implemented as robust, easy-to-run, and reproducible formats, these assays can fast-track the development timeline of antibody therapeutics.

Due to the existing gaps between current assay offerings that quantify and evaluate ADCC of the therapeutic antibody and industry requirements, the availability of an assay that is MOA-reflective and can be implemented from screening to potency-testing in lot-release program is of paramount importance.

TECHNOLOGY

KILR Cytotoxicity Assay Platform

The Eurofins DiscoverX KILR® (Killing Immune-Lysis Reaction) cytotoxicity assay platform enables a true measurement of ADCC activity while delivering highly sensitive detection in a high-throughput capacity reproducibility with precision and accuracy using a simple assay protocol. As regulatory authorities are increasingly requiring data on the impact of Fc-mediated effector cell mechanisms for the submitted antibody therapeutic via true MOA-reflective assays that offer robustness, precision, and accuracy, the KILR cytotoxicity assay format fits the purpose of being able to demonstrate the efficacy of a biotherapeutic's Fc-mediated target cell death induced by ADCC.

The KILR cytotoxicity assay format specifically measures the killing of antigen-expressing target cells in a co-culture with immune effector cells based on a proprietary Enzyme Fragment Complementation (EFC; discoverx.com/EFC) technology. This technology provides a simple, dye-free, non-radioactive assay format to measure ADCC and other immune effector cell-mediated cytotoxicity applications such as ADCP, CDC, and T-cell redirection. This assay format offers accuracy, precision, and specificity beyond traditional ADCC methods that involve radioisotope or dye-based formats. The KILR ADCC assay platform is biologically relevant, robust for measuring cytotoxicity, and serves as a format for developing regulatory-compliant cytotoxicity assays for the evaluation of ADCC activity from the screening phase to potency testing in lot-release programs. These assays are qualified with the marketed drug and available in a RTU bioassay format that minimizes variability due to target cells and ensure long-term assay reproducibility.

Principle of KILR ADCC Assay

Target cells expressing the relevant antigen are engineered to stably express a housekeeping protein tagged with an EFC reporter fragment called enhanced ProLabel® (ePL). This reporter fragment is a small enzyme donor fragment of β-galactosidase (β-gal) that is inactive when not paired with its larger enzyme acceptor (EA) fragment. The ePL-tagged reporter protein (also called the KILR Reporter Protein) is detected in the medium by the addition of detection reagents containing the EA fragment of β-gal and a substrate. When the target cell is incubated with appropriate antibody and effector cells, the effector-mediated killing of target cells releases the tagged reporter protein into the medium. This release leads to the formation of the active β-gal enzyme that hydrolyzes the substrate to give a chemiluminescent output that can be detected on any benchtop luminescence reader. A stronger chemiluminescent quantitative signal corresponds to a higher percentage of ADCC-mediated killing of target tumor cell. Overall, the KILR ADCC assay is versatile, and can be used with many different effector cell types (PBMCs, purified NK cells, CD16 engineered effectors, etc).

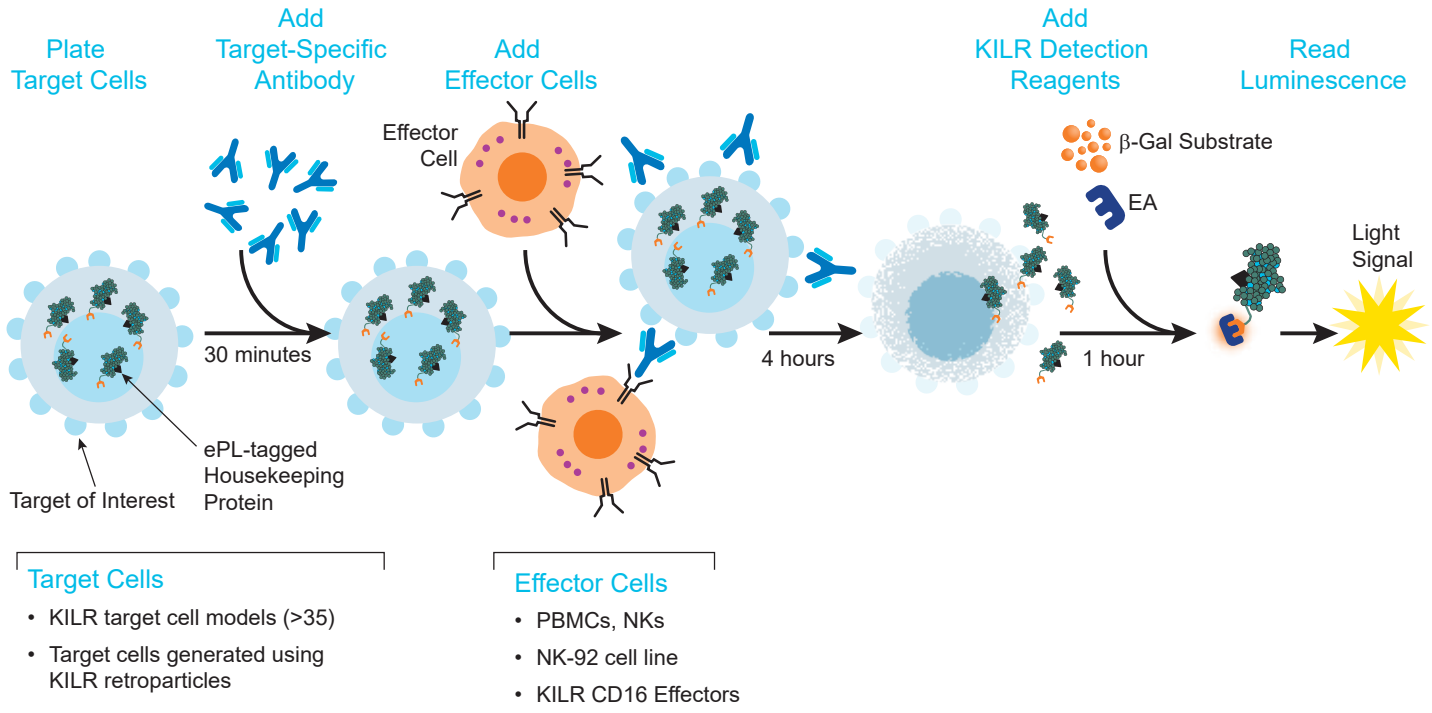


Figure 2. KILR ADCC assay principle. Effector cells such as PBMCs, NK cells, or KILR CD16 Effector Cells engineered to stably express a portion of the tagged reporter protein with β-gal enzyme fragment are added to the antibody coated target cells expressing the antigen. Lysis of the target cells by immune effectors mediates the release of tagged reporter protein by the target cells. Following the addition of detection reagents containing the substrate and the complementary portion of the β-gal enzyme, the active β-gal enzyme is formed and the substrate is hydrolyzed to generate a chemiluminescent output detectable on any bench top luminescence reader.

The assay principle schematic in Figure 2 demonstrates the simplicity of the KILR® ADCC assay and represents a typical scheme of steps followed to measure cytotoxicity using the KILR platform.

METHODS

The following flow-chart (Figure 3) depicts an overview of the main steps (KILR target cells preparation, antibody and effector cell incubation, and quantifying ADCC) involved in performing the KILR ADCC assay. For the detailed protocol, please refer to the "Assay Protocol" section in the Appendix.

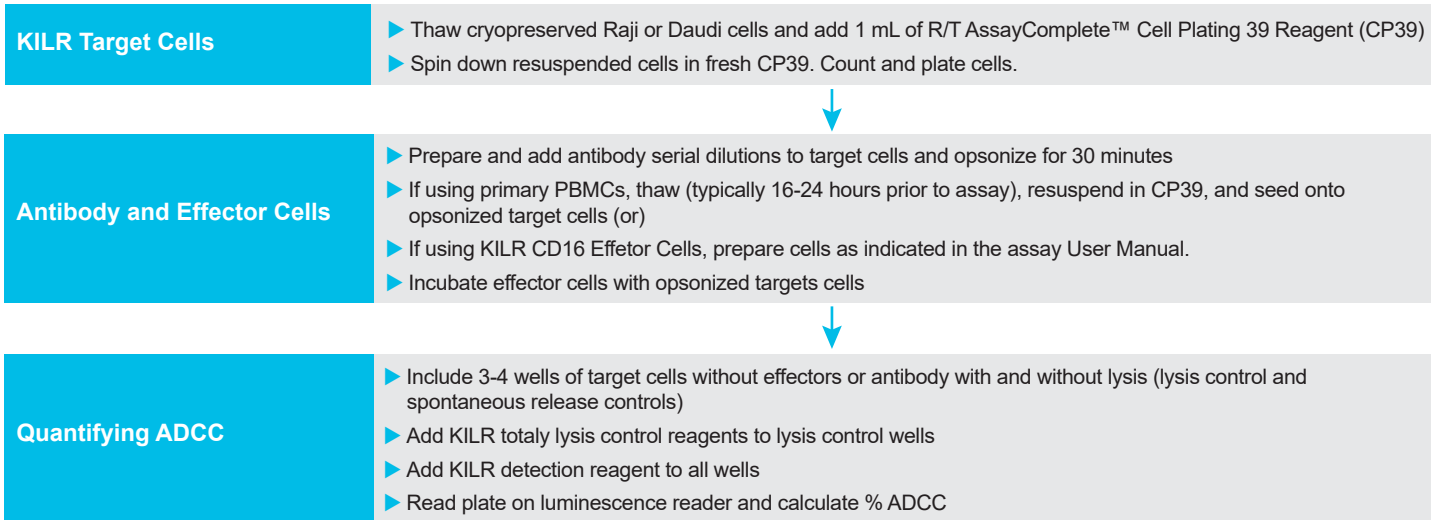


Figure 3. Flow-chart of ADCP Assay. This flow-chart depicts the main steps involved in performing the ADCC assay. For the detailed protocol and materials used, please refer to the Appendix section.

KILR ADCC Bioassay Format

Raji is the first KILR® target cell model launched in the RTU bioassay kit format. This assay format is rendered as fit-for-purpose for screening and measurement of relative potency in lot-release testing. The advantages of KILR ADCC bioassay include the following:

a. Suitable for the development of ADCC assays for multiple antigens (e.g. HER2, CD20, CD38, EGFR etc.) and can be used with a variety of effector cell types.

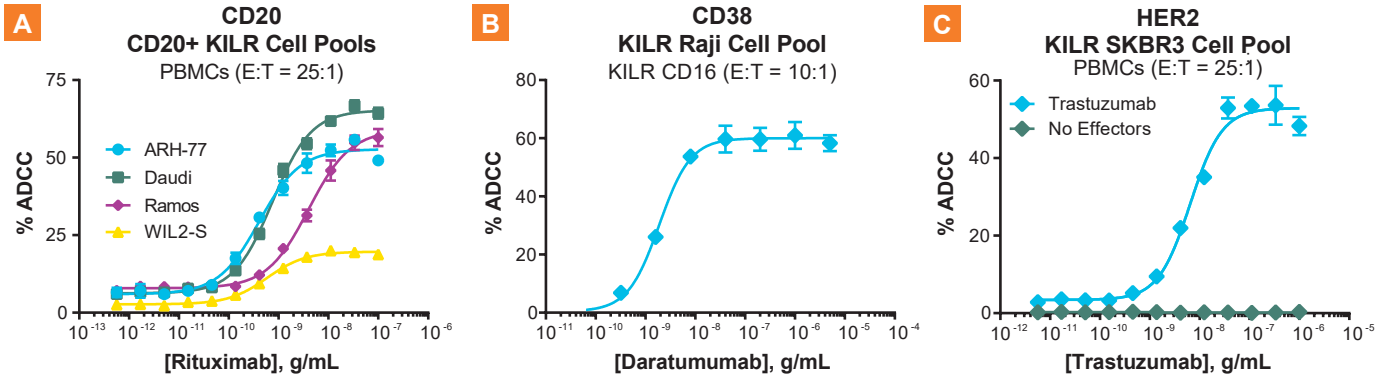
b. Able to demonstrate excellent inter-assay repeatability when used in combination with engineered KILR CD16 Effector Cells.

c. Amenable for phase-appropriate qualification as demonstrated with the KILR Raji bioassay model.

RESULTS AND DISCUSSION

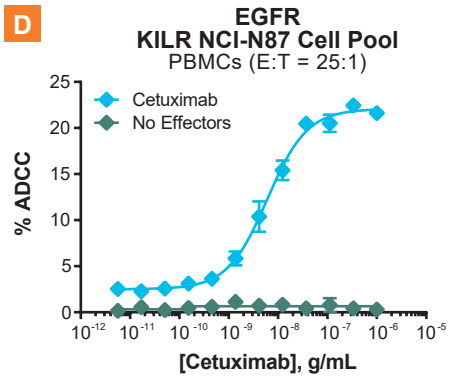
Evaluation of ADCC Mediated by Antibodies in Diverse Cancer Models

The evaluation of ADCC targeting multiple antigens in diverse cancer models (stable pools representing homogeneous population of cells expressing the KILR reporter to maintain heterogeneity of the native cell line) has been demonstrated. The figures below show anti-CD20, anti-CD38, anti-HER2, and anti-EGFR mediated ADCC in different KILR cell models, and the compatibility of KILR ADCC assay with multiple effector cells.



Sample	E_{MAX}	EC_{50}, ng/mL
Daratumumab	60%	1.89

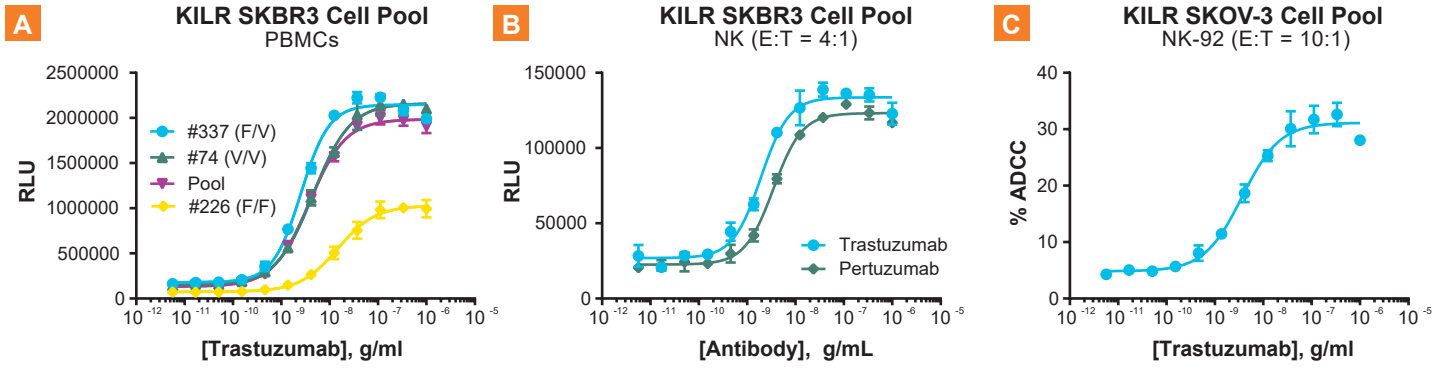
Sample	E_{MAX}	EC_{50}, ng/mL
Trastuzumab	53%	6.44



Sample	E_{MAX}	EC_{50}, ng/mL
Cetuximab	22%	6.04

Figure 4. Detection of ADCC-mediated by antibodies. A. Rituximab-mediated ADCC in four different CD20⁺ B-lymphoblast KILR models (ARH-77, Daudi, Ramos, and WIL2-S) using primary PBMCs at an effector-to-target ratio (E:T) of 25:1. B. ADCC mediated by the anti-CD38 therapeutic antibody, Daratumumab, in the KILR Raji cell model using engineered effector cells (KILR CD16 Effector Cells) at an E:T of 10:1. C. ADCC mediated by the anti-HER2 therapeutic antibody, Trastuzumab (approved for treatment of metastatic breast cancer), in the KILR SKBR3 cell model using primary human PBMCs at an E:T of 25:1. D. ADCC mediated by the anti-EGFR therapeutic antibody, Cetuximab (approved for treatment of metastatic colorectal cancer), in the KILR NCI-N87 cell model using primary human PBMCs at an E:T of 25:1.

As shown in Figure 4, Rituximab, Daratumumab, Trastuzumab, and Cetuximab mediated dose-dependent cell death in selected cell pools is evident by the increase in % ADCC with increasing antibody concentration. Cytotoxicity was not observed when effector cells were not included in the assay as shown in Figure 4. C. and D. for anti-HER2 and anti-EGFR antibody treatments, respectively. These results demonstrate and confirm the applicability of the KILR® platform in diverse cancer models for ADCC read-outs and shows the compatibility of the assay platform with different cell types, antibodies, and multiple effector cells as shown in Figure 5.



Donor	S/B	HillSlope	EC_{50}, ng/mL
#226 (F/F)	13.7	1.113	14.6
#337 (F/V)	13.9	1.486	2.53
#74 (V/V)	15.2	1.128	4.54
Pool	15.5	1.161	3.76

Sample	S/B	HillSlope	EC_{50}, ng/mL
Trastuzumab	5	1.501	1.92
Pertuzumab	5.5	1.463	3.48

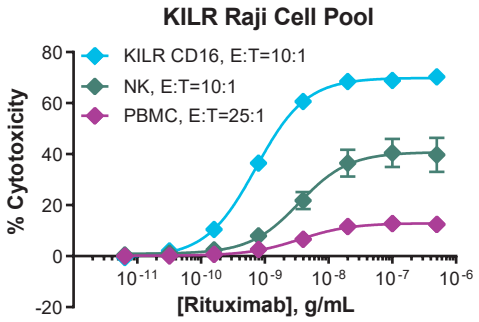
Sample	S/B	HillSlope	EC_{50}, ng/mL
Trastuzumab	7.6	1.107	3.63

Figure 5. Compatibility of KILR ADCC assays with multiple effector types. A. ADCC using primary PBMCs from 3 different donors characterized as a F158 homozygote (F/F; #226), a F158/V158 heterozygote (F/V; #337) or a V158 homozygote (V/V; #74). B. ADCC with two HER2-targeting antibodies using primary NK cells in the KILR SKBR3 model. C. ADCC with Trastuzumab using an engineered NK-92 cell line as effector cells with the NK-92-resistant cell line SKOV3.

Fc receptor-mediated ADCC is an important MOA as it mediates target cell killing using target specific antibodies. The human FcγRIIIa gene displays a polymorphism in the position coding for amino acid residue 158. This translates to a higher affinity FcγRIIIa variant having a valine at amino acid 158 (V158) and a lower affinity FcγRIIIa variant carrying phenylalanine F158. As shown in Figure 5, PBMCs derived from donors with different genotypes were evaluated for ADCC using Trastuzumab (Figure 5. A.). PBMCs with V/V (homozygous) or F/V (heterozygous) genotypes are expected to have higher affinity for the Fc portion of the IgG1-based antibody, and therefore should produce a more potent ADCC response. In contrast, the F/F donor is expected to have the least potent response, as observed in Figure 5. A. This evidence of strong ADCC was observed when using primary NK cells or an engineered NK-92 cell line as effector cells in the SKBR3 and SKOV-3 cell pools, respectively, confirming the compatibility of KILR ADCC assays with multiple effector cell types.

Robust ADCC Assay Performance

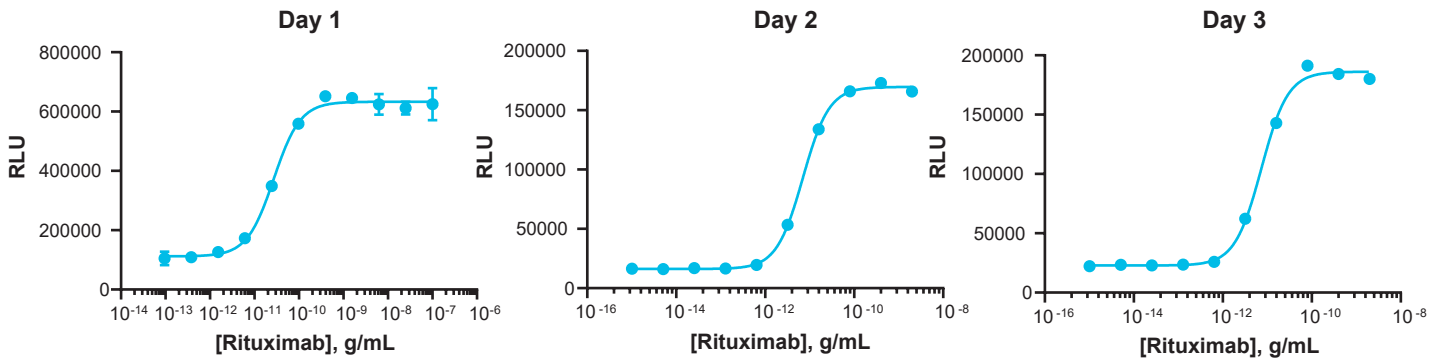
For bioassay qualification and lot release testing, it is important that the assay demonstrates acceptable robustness, precision (repeatability and intermediate precision), linearity, and range. To ensure a robust ADCC assay, the identification of appropriate effector cells is paramount. In Figure 6, we compared the performance of the single-donor derived KILR CD16 Effector Cells to primary effector cells (e.g PBMCs or NK cells) using KILR Raji target cells. When treated with Rituximab, the KILR CD16 Effector Cells produced a 2.5-fold higher assay window and E_{MAX} compared to the primary NK cells (seen in Figure 6) when used at the same E:T ratio (E:T = 10:1). The difference in assay window and E_{MAX} is also more pronounced when comparing primary PBMCs (used at an E:T = 25:1).



Effector Cells	EC_{50}, ng/mL	S/B	E_{MAX}
KILR CD16 (10:1)	0.73	28.2	70%
Primary NK's (10:1)	3.46	10.9	41%
PBMCs (25:1)	3.51	4.9	13%

Figure 6. Robust ADCC assay performance with KILR CD16 Effector Cells. Rituximab mediated ADCC shown when using KILR CD16 Effector Cells, NK cells, and PBMCs demonstrating larger assay windows with KILR CD16 Effector Cells compared with primary NK cells and even more compared to PBMCs.

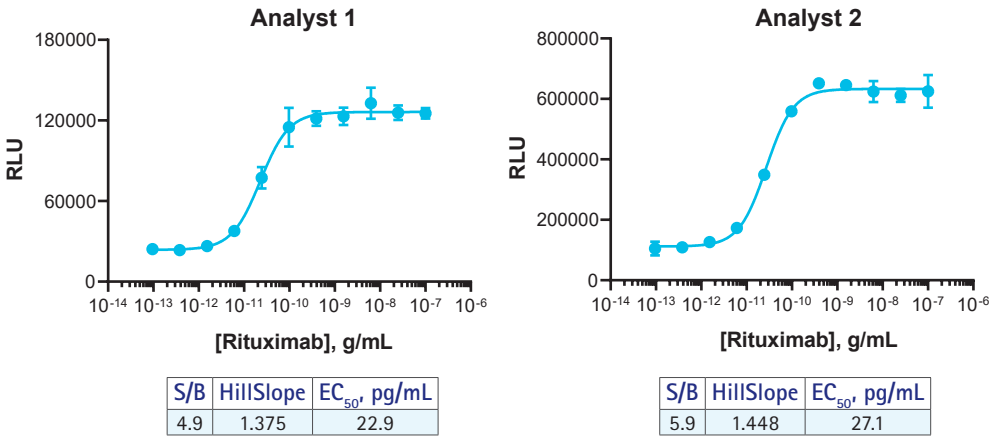
When KILR® bioassay target cells are used in combination with KILR CD16 Effector Cells, the ADCC assays demonstrate high repeatability as evident from assay performance by a single analyst on three different days (Figure 7. A.), where EC_{50} varied by less than 10%, and assay window varied by less than 15%, as shown in the table below the graphs. Comparable data was obtained with the KILR Daudi model (data not shown). The reproducibility of the assay is demonstrated by comparable ADCC performance by two independent analysts on two different days (Figure 7. B.) with the same lot of KILR Raji bioassay cells and with KILR CD16 Effector Cells, but using independent vials of both cell types. Excellent concordance in curve shape, S/B (signal-to-background), and EC_{50} for the two analysts was observed with the assay ready cells and KILR CD16 Effector Cells (E:T = 10:1). These results demonstrate the highly reproducible nature of the assay platform as evident from the low variability of EC_{50} observed among different plates, combined with low inter-day variation.



Parameter	Day 1	Day 2	Day 3	% RSD
EC_{50}, pg/mL	6.2	7.03	7.4	8.90%
S/B	9.8	10.6	8.3	12.20%
E_{MAX}	135%	114%	75.80%	27.70%

Figure 7. Repeatability of KILR ADCC Bioassays on different days. Repeatability of KILR ADCC assays conducted over three days using KILR Raji bioassay target cells and KILR CD16 Effector Cells (E:T = 10:1). Dose-response of the anti-CD20 antibody Rituximab by a single analyst on 3 different days. Each day represents an independent vial of the KILR Bioassay target cells and effector cells from a single vial of KILR CD16 Effector Cells. The table summarizes the low variability in EC_{50}, S/B, and E_{MAX} values over the three days indicating the high repeatability of the ADCC assay.

The table in Figure 7 summarizes the results and demonstrates the low variability in EC_{50}, S/B, and E_{MAX} values over the three days indicating the high repeatability of the ADCC assay. Execution of the KILR® Raji ADCC Bioassay with the anti-CD20 antibody Rituximab over two days and by two different analysts (Figure 8) revealed highly similar dose-response curves and EC_{50} values, reflecting on the reproducibility of the assay.



S/B	HillSlope	EC_{50}, pg/mL
4.9	1.375	22.9

S/B	HillSlope	EC_{50}, pg/mL
5.9	1.448	27.1

Figure 8. Reproducibility of KILR ADCC Bioassays by different analysts. Functional response analysis of the KILR ADCC assay using KILR Raji ready-to-assay cells and KILR CD16 Effector Cells (E:T = 10:1) by two different analysts, run on independent days, showing excellent concordance in curve shape, S/B, and EC_{50}.

Qualification of KILR ADCC Bioassays

The purpose of qualifying a bioassay (method qualification) is to demonstrate its suitability for determining the relative potency of the relevant drug product. Phase-appropriate qualification per The International Council for Harmonisation (ICH) guidelines should include evaluation of the indicated drug, with a minimum of nine determinations over a minimum of three nominal concentrations (NC) that cover the specified range (e.g., three concentrations / three replicates each) to establish accuracy of the method. These ICH guidelines require assessment of intermediate method precision that should incorporate multiple sources of experimental variation, including multiple days and analysts and additional sources of variation such as multiple release lots of reagents (e.g., different lots of target and effector cells).

The KILR Raji Bioassay was qualified with Rituximab over a range of 50-150%. The qualification design is shown in Figure 9. A, and a summary of the observed relative potency values obtained for all tested NCs are shown in the table in Figure 9. C. The average relative potency (RP) for each NC is plotted relative to the expected value in the graph in Figure 9. B. to demonstrate dilutional linearity of the assay. A correlation efficient of 0.9926 was obtained, demonstrating excellent dilutional linearity of the method. The Figure 9. B. table summarizes the relevant assay characteristics from qualifications of the method.

A Qualification Study Design

Details of Study Design

Evaluated 5 nominal concentrations (NC) over a range of 50%-150% (n ≥ 4 for each NC)

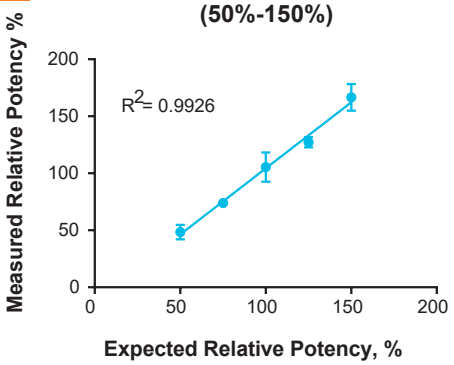
Repeatability: 4 Runs of 100% NC by single analyst

Intermediate precision incorporates:

- 2 Analysts
- Multiple days
- 2 Lots of Bioassay Target Cells
- 3 Lots of KILR CD16 Effectors

Day	Nominal Concentrations	Analyst
1	100% x 4 (Repeatability)	1
2	150%, 125%, 75%, 50%	1
2/3	150%, 100%, 50%	2
3	150%, 125%, 75%, 50%	1
4	125%, 75%	2
4	150%, 125%, 75%, 50%	1

B KILR Raji Bioassay Dilutional Linearity (50%-150%)



Parameter	Value	Specification
Accuracy (Average % Recovery)	103%	100% +/- 20%
Repeatability	14.2%	≤20%
Intermediate Precision	≤13.2%	≤20%
Linearity (R2)	0.9926	≥0.95

C

Nominal RP, %	Analyst	Observed RP, %	Average RP, %	% RSD	Average % Recovery
150	1	156	166.6	7.0	111.1%
	1	179.4			
	1	177.4			
	2	166			
	2	154.3			
125	1	130.6	127.3	3.6	101.8%
	1	131.2			
	1	126			
	2	121.4			
100	1	82	106.5	13.0	106.5%
	1	116			
	1	112.7			
	1	98.4			
	1	117.8			
	2	112.2			
75	1	70.4	73.9	4.9	98.5%
	1	76.6			
	1	77.3			
	2	71.1			
50	1	47.8	48.5	13.2	96.9%
	1	45.8			
	1	40.9			
	2	58.4			
	2	49.4			

Figure 9. Qualification study design, dilutional linearity of KILR® Raji Bioassay model, and observed relative potency values for NCs. A. Study design for qualification of KILR Raji ADCC Bioassay. Five nominal concentrations of Rituximab were evaluated over a range of 50-150%. Repeatability (four runs) was assessed at the 100% nominal concentration by a single analyst. Intermediate precision incorporated several sources of variability, including multiple analysts, multiple days, two lots of KILR Raji Bioassay target cells, and three different lots of KILR CD16 Effector Cells. B. Results obtained from this qualification study indicate very good accuracy, high repeatability, intermediate precision, and dilutional linearity for KILR Raji Bioassay. C. Table shows observed relative potency values obtained for all tested NCs.

Overall, the qualification study showed good accuracy, repeatability, intermediate precision, and dilutional linearity for the KILR® Raji bioassay model. Intermediate precision of 13.2% is excellent, especially considering that engineered primary effector cells derived from multiple lots were used in the assay. These data demonstrate the suitability of the KILR Raji Bioassay for use in relative potency assays for CD20-, CD19- or CD38-targeted therapeutics that mediate ADCC.

In this application note, we have demonstrated that the KILR ADCC bioassay is a robust, reproducible and fit-for-purpose assay for screening and relative potency applications in lot-release testing. The datasets demonstrate that this KILR ADCC bioassay meets the requirement of a cell-based assay that can evaluate and quantify ADCC MOA for antibody therapeutics.

CONCLUSION

IND applications require proof of data that captures all effector MOAs of an antibody therapeutic including ADCC activity. The availability of a functionally relevant and standardized bioassay format for evaluating the cytotoxicity of therapeutic antibodies and, more specifically, the ADCC activity is a key challenge in fulfilling regulatory requirements.

The development and availability of a KILR cytotoxicity assay platform enabling the measurement of target cell death mediated by a wide range of effector cell types, and compatible with a wide range of applications (ADCP, ADCC, CAR-T, T-cell redirection) offers advantages and addresses the gaps for implementing and development of antibody therapeutics from screening to lot release. The RTU bioassay format for a subset of cell models within Eurofins DiscoverX's KILR portfolio has been developed with demonstrated data on the suitability for antibody rank ordering as well as for use in characterization and relative potency and lot-release testing applications.

The broad application of KILR platform along the drug development pipeline, together with the ability of each stable KILR model to support KILR CDC, ADCC, and ADCP assays, allows these KILR products to support the development of antibody therapeutics. The KILR ADCC assays are easy-to implement and contain most required reagents needed for ADCC (except effector cells and client specific antibody). These assays have short assay performance times, and are robust and reproducible, all supporting the screening and characterization of antibody drugs during early phase as well as for measuring potency in lot-release programs.

Learn more about the Eurofins DiscoverX cytotoxicity KILR assays at DiscoverX.com/KILR.

APPENDIX

MATERIALS

Eurofins DiscoverX Materials Used for ADCC Assay	
Product Description	**DiscoverX Catalog Number**
KILR® Raji Bioassay Cells	97-1012Y026
AssayComplete™ Cell Plating 39 Reagent (CP39)	93-0563R39A
KILR Detection Kit	97-0001
96-Well White, Flat-Bottom, TC-Treated, Sterile Plates, (10 plates/pack)	92-0027

Additional Materials Recommended for Assay (But Not Included in Kit)	
Product Description	**DiscoverX Catalog Number**
KILR CD16 Effector Cells	97-1007
AssayComplete Cell Culture Kit-117	92-3117
Recombinant Human IL-2, Cell Culture Grade	92-1331

ASSAY PROTOCOLS

Handling of KILR Bioassay Cells

- On day of use, add fresh L-Glutamine to CP39 to a final concentration of 2 mM. Pre-warm CP39 at 37°C, then equilibrate to room temperature (R/T) before setting up the assay.
- For each vial of cells to be thawed, transfer 10 mL of R/T CP39 to a fresh 15 mL conical tube.
- Remove cryovial of KILR Raji or Daudi Bioassay Cells from LN tank immediately before setting up the assay. Keep on dry ice while transporting to lab.
- Wipe outside of cryovial with 70% Ethanol, carefully remove cap and slowly add 1 mL of R/T CP39 from the conical tube to the cryovial.
- Using a 1 mL pipette, gently mix the cells by pipetting up and down several times to completely dissolve the cell pellet in the CP39.
- Transfer the re-suspended cells into the 15 mL conical tube containing the remaining 9 mL of R/T CP39 and mix gently to disperse.
- Repeat the process 2-3 times to make sure all cells are completely transferred from the cryovial into the conical tube.
- Spin down the cells at R/T for 4 minutes at 200-300g.
- Discard the supernatant and re-suspend the cell pellet in 10 mL of fresh CP39.
- Count cells and determine viability. Adjust concentration of cells to 1 x 10^5 cells/ mL.
- Pour the cell suspension into a sterile 25 mL reagent reservoir.
- Using a multichannel pipette, plate 50 µL/well (5K/well) of KILR Raji or Daudi Bioassay Cells into appropriate wells of a white, opaque bottom 96-well assay plate. Incubate plate at R/T in the bioasafety cabinet for 15 minutes to minimize the potential for edge effects. Proceed with KILR ADCC assay protocol below.
- Note: If the sample preparation takes longer than 30 minutes, the assay plate may be placed in humidified tissue culture incubator at 37°C, 5% CO_2 for up to 2 hours before proceeding with assay.

ADCC Assay

Prepare Antibody Solutions

- Prepare antibody serial dilutions (e.g. Rituximab or test samples) in CP39 as 11X stocks in 10-pt dose response.
- **Note**: We recommend using a wide range of test sample dilutions initially (e.g. 1 µg/mL top dose; 1:4 dilutions), until the optimal dose range for a given antibody is identified.

- Add 10 µL/well of antibody serial dilutions to appropriate wells containing KILR® Bioassay target cells and opsonize for 30 minutes at 37°C, 5% CO_2.

Prepare Effector Cells

- If using primary PBMCs, thaw primary frozen PBMCs 1 day before running the assay and re-suspend in pre-warmed CP39 as per Appendix in KILR Suspension Cell Line User Manual. Allow cells to recover in CP39 overnight in humidified 37°C, 5% CO_2 incubator
    - On the day of performing the assay, count the number of viable cells, pellet at 300 x g for 10 minutes and re-suspend cell pellet in appropriate volume of CP39 to achieve desired density (2.5 x 10^6 viable cells/mL for an E:T of 25:1).
    - Seed 50 L of prepared effector cells per well into appropriate wells of assay plate containing opsonized target cells.
- If using the Eurofins DiscoverX KILR CD16 Effector Cells, thaw and rest these effector cells for at least 7 days prior to running the assay using the protocol recommended in KILR CD16 Effector Cell User Manual.
    - On the day of assay, count number of viable cells and prepare effectors in CP39 at the concentration recommended for the KILR target cell model to be used. For KILR Raji and Daudi models, where an E:T of 10:1 is recommended, prepare effector cells at a concentration of 1 x 10^6 viable cells/mL.
    - Consult the KILR CD16 Effector Cell User Manual for proper handling of the KILR CD16 Effector Cells.
    - Seed 50 µL of prepared KILR CD16 Effector Cells into the appropriate wells in the assay plate.
- Incubate effector cells with the opsonized KILR Bioassay target cells for 4 hours at 37°C, 5% CO_2.
- For each plate, include 3-4 wells of target cells with no antibody or effectors that are treated with lysis buffer (total lysis controls), and 3-4 wells with target cells and no antibody or effectors that are not lysed (spontaneous release controls).
- At time of addition of effector cells to samples, add 2.5 µL/well of KILR Total Lysis Control Reagent to only the total lysis control wells. Since these wells will produce a high signal, it is recommend to place these wells away from wells expected to produce a low signal (e.g. wells containing low antibody concentrations) to prevent potential for bleed-over.
- At the end of the incubation time, prepare the KILR detection reagent per manufacturer's protocol. Add 100 µL/well of KILR working solution to all wells and incubate at room temperature in the dark for 1 hour without shaking.
- Read plate on a standard luminescence plate reader (0.1 to 1 second/well). For Perkin Elmer Envision, use a 0.2 sec/well integration time.

  Calculate % ADCC using the following calculation:

  % ADCC = (test antibody value – mean spontaneous release value) / (mean total lysis – mean spontaneous release) x 100
- Plot the dose response data (both Raw RLU and % ADCC) using GraphPad Prism and calculate EC_{50} values using a sigmoidal dose response curve fit with a variable slope (four parameter) with no constraints; fit method = least squares (normal fit).