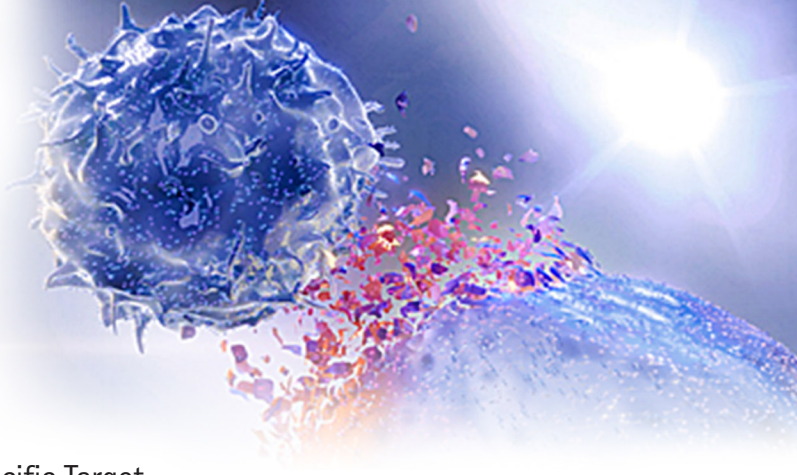

APPLICATION NOTE

A Simple and Powerful Cell-Based Assay to Quantify Specific Target Cell Death by Antibody-Dependent Cellular Phagocytosis (ADCP)

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INTRODUCTION

With the increasing industry focus on antibody drugs, there is an ever-greater need for functional bioassays that interrogate the therapeutic antibody's mechanisms-of-action (MOA). Typical formats for antibody therapeutics contains two antigen-binding Fab arms and an Fc region. The Fc domain can be comprised of different isotypes (IgG1, IgG2, IgG3 or IgG4) which have varying affinities for binding to Fcγ receptors expressed on immune effector cells. Engagement of Fcγ receptors mediates target cell death by various mechanisms such as Complement-Dependent Cytotoxicity (CDC), Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC), and Antibody-Dependent Cell-Mediated Phagocytosis (ADCP). Regulatory authorities now commonly require data on the impact of each of these Fc-mediated effector mechanisms for the submitted antibody therapeutic. There are several methods available to measure these Fc-mediated effector MOAs; however, getting a reproducible true measure of ADCP, in particular, has been quite challenging.

ANTIBODY-DEPENDENT CELL-MEDIATED PHAGOCYTOSIS

ADCP is a physiologically important MOA of therapeutic antibodies that can be mediated by various immune effector cells, namely: monocytes, macrophages, dendritic cells, and neutrophils through multiple FcγRs. The Fab region of the antibody binds to a specific antigen on the surface of target cells. In contrast, the Fc region of the antibody binds and activates various Fcγ receptors on immune effector cells. Activation of specific Fcγ receptors, like FcγRIIa, FcγRI, and FcγRIIIa, leads to the continued activation of a complex pathway that triggers phagocytosis and destruction of the target cells within the lysosomes of different effector cells.

Traditional methods for measuring ADCP require loading the target cells with a fluorescent dye or reporter protein (e.g. Green Fluorescent Protein) and then tracking the number of target cells that have been engulfed by macrophages using flow cytometry or confocal microscopy. These methods are laborious, time-consuming, and measure co-localization of macrophages and target cells rather than target cell death by phagocytosis. There are also reporter gene assays that measure Fc receptor engagement as a surrogate measure of ADCP. Unfortunately, these assays do not measure true ADCP or target cell engulfment, but rather measure antibody binding and subsequent activation of FcγRIIa, one of the multiple Fcγ receptors involved in ADCP. If reporter gene assays were implemented for ADCP, a secondary assay that measures direct cell death would also be needed as a bridging assay to be implemented for use in characterization and lot release of the biologic.

In this application note, Eurofins DiscoverX describes a simple and powerful plate-based method vs. flow-based format to measure ADCP using direct target cell death as a readout and demonstrates its application. In this cytotoxicity assay, target cells are transduced to stably express a Killing-Mediated Immune Lysis Reporter (KILR®) protein (see DiscoverX.com/KILR for details on how to transduce cell lines with the KILR reporter protein).

Primary human macrophages are differentiated from monocytes in a 96-well plate, isolated from cryopreserved peripheral blood mononuclear cell (PBMCs), and co-incubated with the KILR target cells. If an antibody promotes phagocytosis, the effector cells (macrophages) will engulf and destroy the target cells, resulting

in the degradation of the KILR® reporter protein as well. The KILR ADCP assay (Figure 1) measures the total amount of KILR reporter protein left in each well at the end of the experiment, thus providing a simple yet powerful way to measure ADCP-mediated target cell death. This information is used to determine the percentage of cells killed by ADCP relative to appropriate controls. The KILR assay is the first and only commercially available, plate-based, scalable, cell-death-endpoint assay to measure ADCP with an easy-to-use protocol.

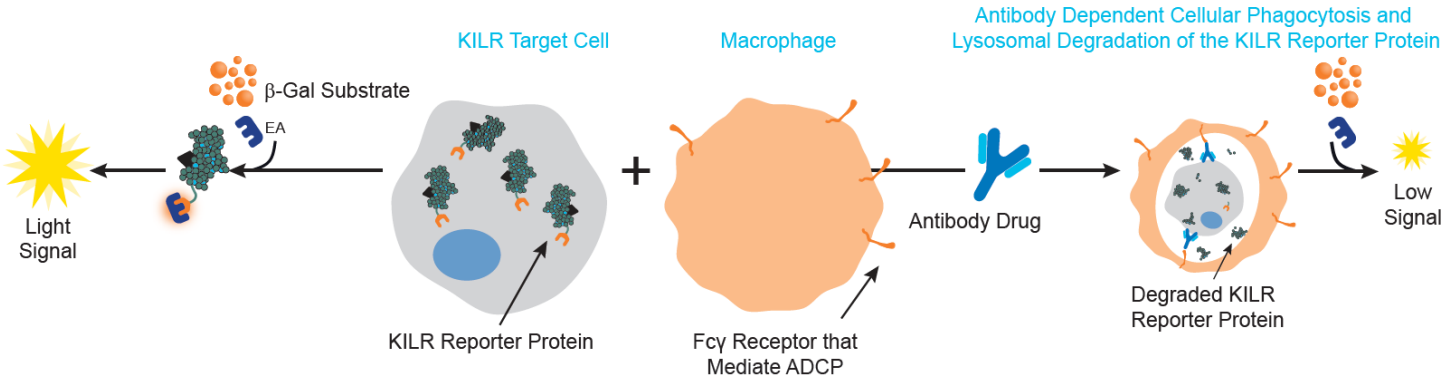


Figure 1. KILR ADCP assay principle. The KILR reporter protein is stably expressed in target cells. When these target cells undergo phagocytosis, the effector cells destroy the target cells including the KILR reporter protein inside the target cells. The amount of total KILR reporter protein in each well is measured through an enzymatic assay with a chemiluminescent output. Lower quantities of KILR reporter protein compared to controls indicate ADCP activity.

METHODS

The following flow-chart depicts an overview of the main steps (target cells maintenance, effector cells preparation, and priming target cells for phagocytosis followed by quantifying ADCP) involved in performing the ADCP assay. For the detailed protocol, please refer to the 'Assay Protocol' section in the Appendix.

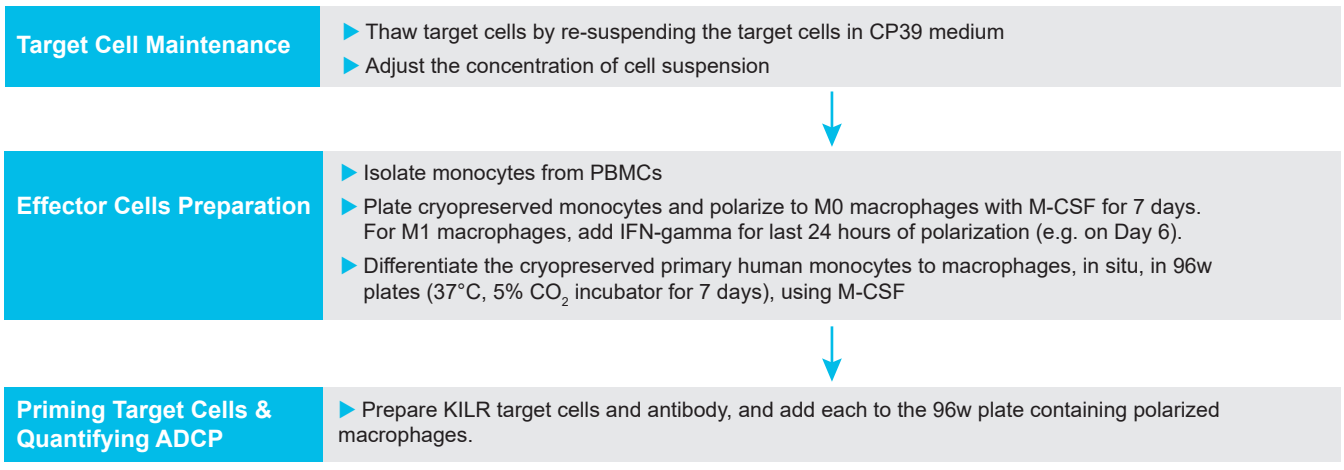
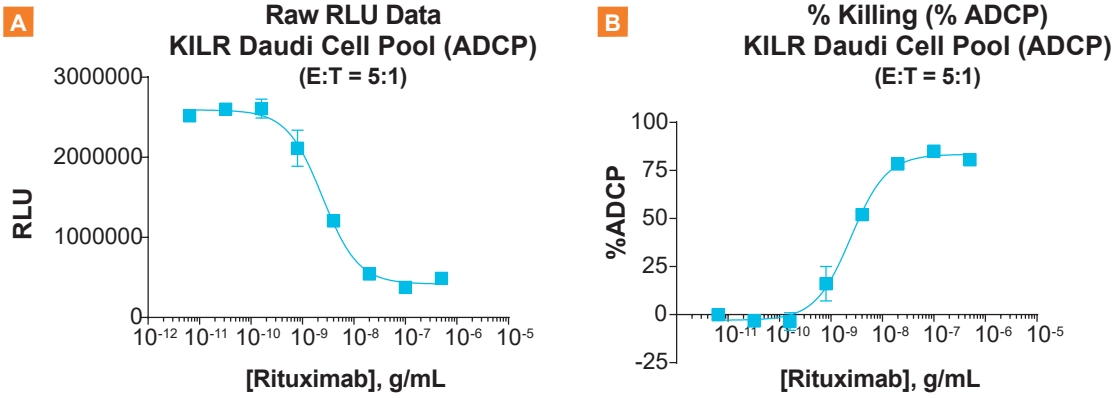


Figure 2. Flow-chart of ADCP Assay. A flow-chart depicting main steps involved in performing the ADCP assay. For the detailed protocol and materials used, please refer to the Appendix section.

RESULTS AND DISCUSSION

The KILR® model was developed to support cytotoxicity read-outs associated with CDC, ADCC, CAR-T, and other cell-mediated killing. This model has been further developed to support ADCP assays, and an example of this application using the KILR platform is illustrated in Figure 3, and described as follows: stably transduced CD20-positive Daudi cells with the KILR reporter protein were opsonized with the anti-CD20 antibody, rituximab, or an IgG1 isotype control. They were then incubated with primary M1 macrophages (differentiated from monocytes with M-CSF for 6 days, then stimulated with IFNγ for 1 day prior to use in assay) for 24 hours. The number of Daudi cells that were still alive in each well was quantified by adding detection reagent for 1 hour.

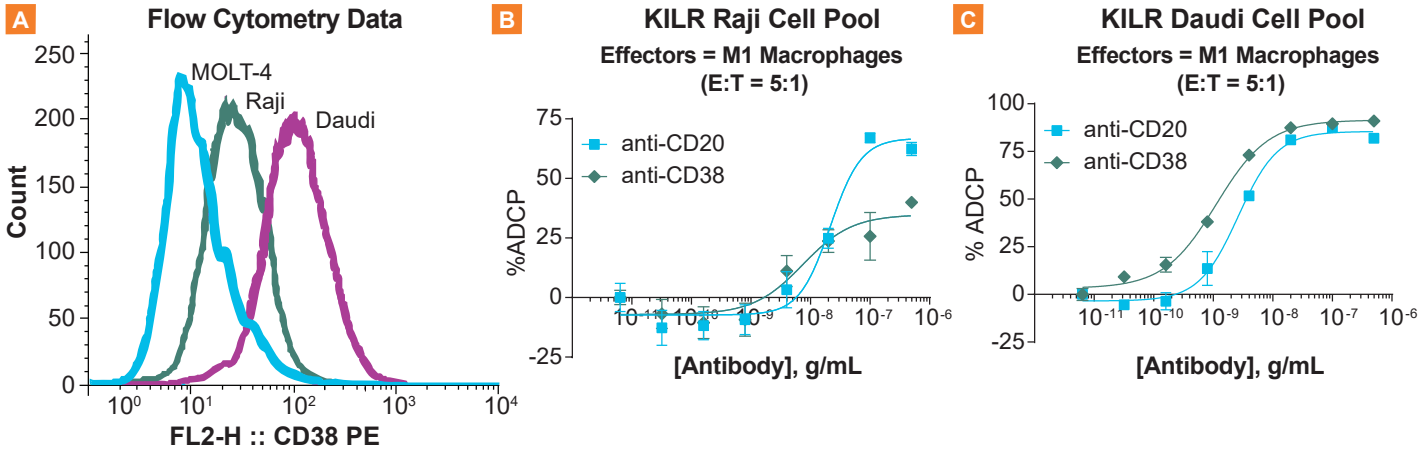
As shown in Figure 3. A., rituximab mediates dose-dependent phagocytosis with M1 macrophages as evidenced by a decrease in signal from labeled Daudi cells. The raw data from the assay wells was converted to % ADCP by comparing the assay readout to wells containing target cells and effectors but no antibody (Figure 3. B.). These results show the use of the KILR platform in measuring an antibody-mediated phagocytic killing by macrophages with maximum % ADCP observed at ~83% at EC_{50} of 2.4 ng/mL. These validation results demonstrate and confirm the KILR platform as a valuable platform for different types of cytotoxicity read-outs and proves that its compatible with different cell and antibody types including bispecifics.



Sample	S/B	E_{Max}	EC_{50}, ng/mL
Rituximab	6.2	83%	2.44

Figure 3. Representative ADCP data for rituximab in KILR Daudi cells. A. KILR Daudi cells were opsonized with an 8-point dose-response curve of rituximab and co-incubated with M1 macrophages at an E:T of 5:1 for 24 hours. The raw RLU data demonstrates a robust dose-dependent decrease in signal (S/B = 6.2) with increasing concentrations of rituximab. B. Data from panel A. plotted as % ADCP (each data point normalized to wells containing vehicle only). An excellent E_{MAX} value of 83% was obtained in this experiment with an EC_{50} of 2.4 ng/mL. S/B = signal-to-background. E:T = macrophage:target.

Next, we determined the extent to which ADCP activity of two antibodies correlates with receptor density of antigens (CD20 and CD38) as demonstrated in KILR® MOLT-4, Raji, and Daudi cells, shown in Figure 4.



Cell Model	CD20 (Receptors/Cell)	CD38 (Receptors/Cell)
MOLT-4	n. d.	833
Raji	132K	1898
Daudi	184K	7300

Sample	E_{Max}	EC_{50}, ng/mL
Anti-CD20	67%	21.6
Anti-CD38	35%	7.45

Sample	E_{Max}	EC_{50}, ng/mL
Anti-CD20	91%	1.13
Anti-CD38	86%	2.64

Figure 4. Efficacy of ADCP correlates with receptor density in two B-cell cancer models. A. Receptor density for CD38 and CD20, two cell surface receptors highly expressed in B-cell malignancies as determined by flow cytometry in KILR MOLT-4, Raji, and Daudi cell models (n.d. = not determined). B. An anti-CD20 antibody produces a robust ADCP response in KILR Raji cells with an E_{MAX} of 67%, while response to an anti-CD38 antibody is lower, consistent with lower expression of CD38 on these cells. C. Both anti-CD20 and anti-CD38 antibodies produced a robust ADCP response in KILR Daudi cells, which have high expression of both receptors. Note that the rank order of the two antibodies is the same in both cell models, but ADCP is more efficient in the KILR Daudi cell model.

Further, we investigated the phagocytic killing of HER2+ cancer cells by trastuzumab that produced a dose-dependent % ADCP roughly 50-fold higher than an IgG1 isotype control in two different HER2 expressing cell lines (Figure 5).

The results signify the assay's robustness and support the assay's use for screening and characterization of antibody drugs during both early phase and late-stage characterization.

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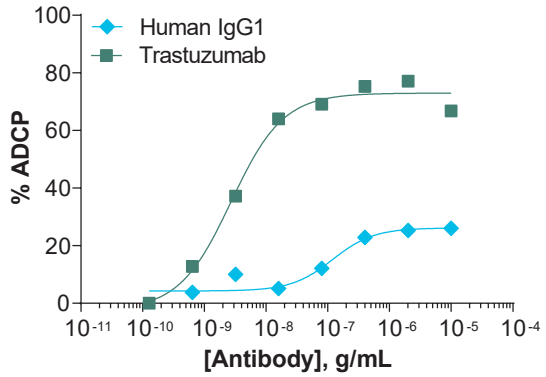

KILR SKBR3 Cell Pool M1 Macrophages (E:T = 5:1)			
Sample	S/B	E_{Max}	EC_{50}, ng/mL
Human IgG1	1.3	26%	120
Trastuzumab	3.8	73%	2.72

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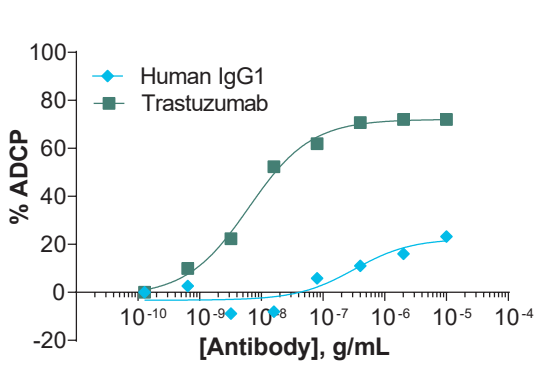

KILR NCI-N87 Cell Pool M1 Macrophages (E:T = 5:1)			
Sample	S/B	E_{Max}	EC_{50}, ng/mL
Human IgG1	1.3	22%	318
Trastuzumab	3.6	72%	6.24

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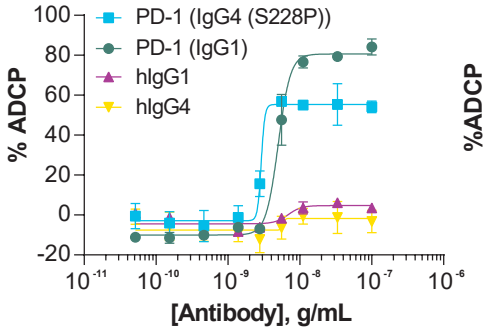

Sample	E_{Max}	EC_{50}, ng/mL
Anti-PD1 (IgG1)	81%	5
Human IgG1	4.7%	
Anti-PD1 (IgG4 (S228P))	55%	2.9
Human IgG4	-	-

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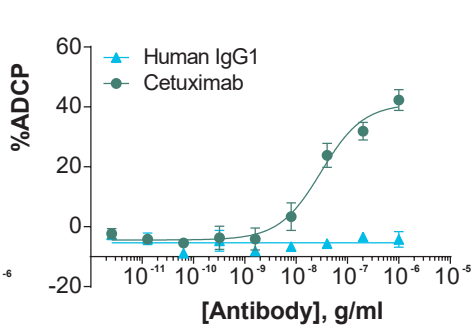
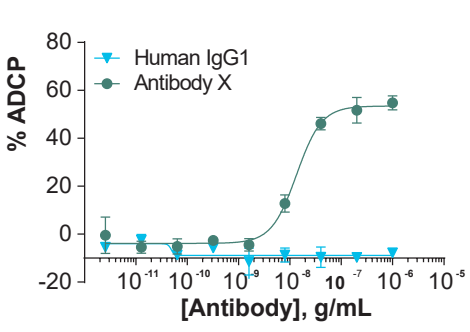


Sample	E_{Max}	EC_{50}, ng/mL
Cetuximab	44.7%	30.6
Human IgG1	-	-

E



Sample	E_{Max}	EC_{50}, ng/mL
Antibody X	53.5%	13.5
Human IgG1	-	-

Figure 5. Robust ADCP observed with trastuzumab in two HER2+ solid tumor models. Cell models, SKBR3 and NCI-N87, both have high expression of HER2 at 85K receptors/cell and 59K receptors/cell (receptor density), respectively. ADCP mediated by trastuzumab relative to an IgG1 control in A. KILR® SKBR3 cells, a breast cancer model, or in B. KILR NCI N87 cells, a gastric cancer model. Note the human IgG1 control of this particular macrophage donor shows some activity in both cell models, but it is roughly 50-fold less potent than trastuzumab. Additionally, we have shown that other IgG1-based antibodies such as cetuximab and a commercial anti-PD-L1 antibody mediate ADCP in the non-HER2-dependent breast cancer line, KILR MDA-MB-231 (data available upon request). C. ADCP in the KILR Jurkat PD-1 cell model with two commercial anti-PD-1 antibodies (IgG1 and IGG4 (S228P) formats) relative to the two isotype controls (human IgG1 and human IgG4) using M0 macrophages (E:T = 5:1). Note that as reported in the literature, the IgG4 (S228P) isotype is able to mediate ADCP nearly as effectively as the IgG1 formatted antibody, suggesting this assay may be useful for characterizing Pembrolizumab biosimilars. ADCP in KILR MDA-MB-231 breast cancer cells using D. Cetuximab or E. Antibody X, which recognizes a novel antigen expressed on MDA-MB-231 cells, using M0 macrophages (E:T = 8:1) as effectors.

Furthermore, the inter- and intra-assay reproducibility when running the same anti-HER2 antibody on KILR® SKBR3 cell pools was documented with minimal variability (Figure 6). These results demonstrate the highly reproducible nature of the assay platform as evident from the low variability of EC_{50} observed among different plates, complemented with low inter-day variation.

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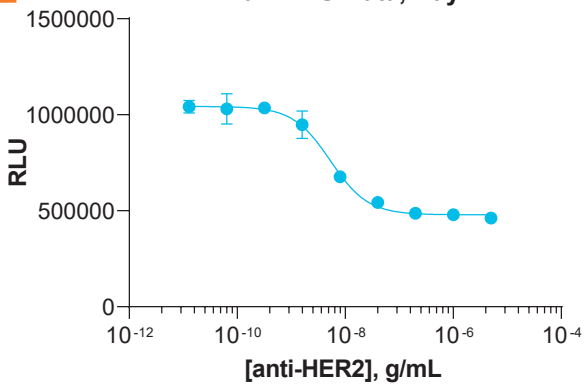

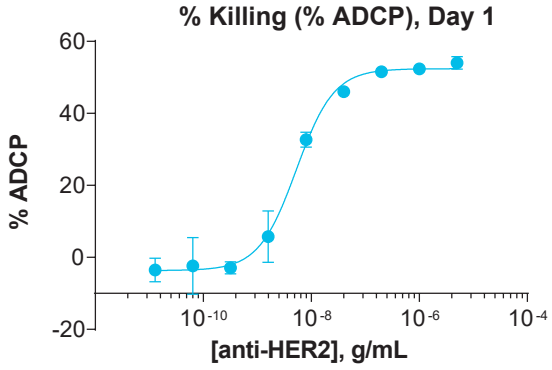

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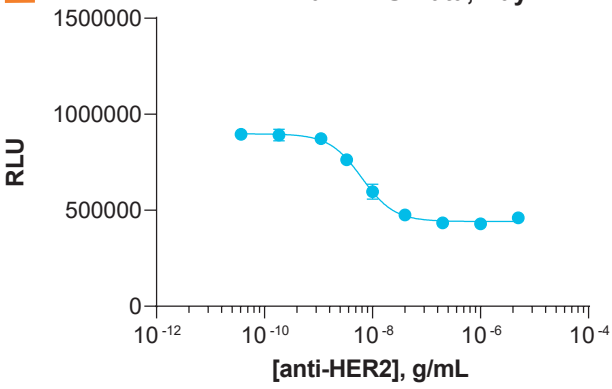
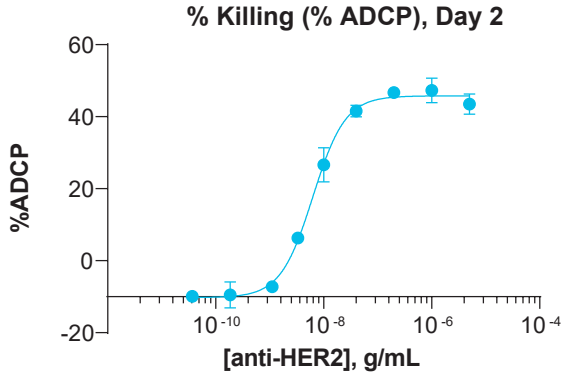




DAY 1 – KILR SKBR3 Cell Pool M0 Macrophages (E:T = 5:1)			
Sample	S/B	E_{Max}	EC_{50}, ng/mL
Anti-HER2	2.24	55.39%	5.3

DAY 2 – KILR SKBR3 Cell Pool M0 Macrophages (E:T = 5:1)			
Sample	S/B	E_{Max}	EC_{50}, ng/mL
Anti-HER2	2	45.79%	6.3

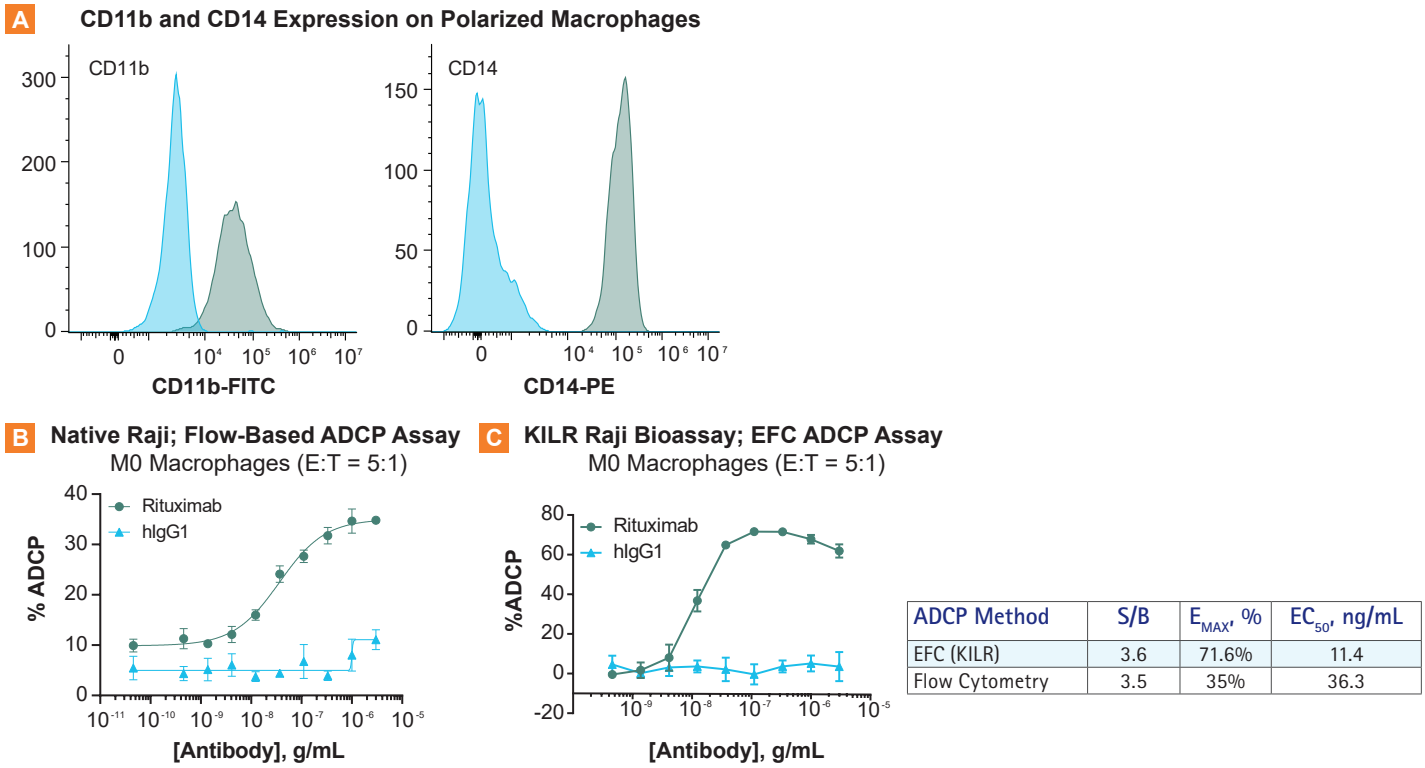
C

Inter-Plate Variability – Same Day (EC_{50}, ng/mL of Anti-HER2)				
Plate 1	Plate 2	Plate 3	Mean	%CV
7.99	6.12	8.95	7.69	18.76

Inter-Day Variability (EC_{50}, ng/mL of Anti-HER2)				
Day 1	Day 2	Day 3	Mean	%CV
*7.69	5.3	6.3	5.8	12.19

Figure 6. ADCP assay with KILR SKBR3 cell pools shows high reproducibility between experiments. The same antibody, anti-HER2, was run on three different plates on the same day (inter-plate experiment) and over the course of two days (A. day 1 and B. day 2; inter-day experiement) using M0 macrophages as effector cells. C. EC_{50} between plates varied by less than 20% CV, while inter-day %CV was only 12.2%. * Mean of plate 1, 2, and 3 data.

Rituximab-mediated ADCP was assessed in two different ADCP assay formats (flow cytometry vs EFC) using M0 macrophages differentiated from the same vial of cells to show the efficacy of the EFC format. The KILR assay format was demonstrated to be more effective at quantifying ADCP activity as shown by the better E_{MAX} value and more potent EC_{50} of ~11 ng/mL.



ADCP Method	S/B	E_{MAX}, %	EC_{50}, ng/mL
EFC (KILR)	3.6	71.6%	11.4
Flow Cytometry	3.5	35%	36.3

Figure 7. Comparison of Rituximab-mediated ADCP in two different ADCP assay formats (flow cytometry vs EFC). Monocytes from the same healthy donor were isolated and differentiated to M0 macrophages prior to use in each assay. A. Expression of CD11b and CD14 was assessed on the polarized macrophages by flow cytometry at time of harvest to confirm the differentiation state. B. For the flow-based assay, polarized macrophages were incubated with CFSE labeled Raji target cells (E:T = 5:1). Rituximab was added for 24 hours before quantifying co-localization of CD14+ effectors (with APC-labeled anti-CD14 antibody) and CFSE-stained target cells by flow cytometry. C. For the EFC-based assay, monocytes polarized to M0 macrophages *in situ* for 7 days were co-incubated with KILR Raji Bioassay target cells (E:T = 5:1). Rituximab was added for 24 hours before addition of PathHunter PK/PL Detection Reagents and detection of assay signal on a plate-based luminometer. Data from each assay were normalized to the vehicle treated cells to calculate % ADCP.

Note: This data has been generated using a short version of the assay protocol (shown in the appendix section).

CONCLUSION

Investigational new drug (IND) applications require submission of data that captures all effector MOAs of an antibody therapeutic, including ADCP activity. The utilization of *in vitro* ADCP assays is necessary for the characterization of novel molecules. We have developed a unique plate-based, easy-to-run assay using the validated KILR® cytotoxicity platform to measure target cell death as an endpoint for ADCP activity using primary human macrophages. The KILR platform provides a sensitive, target-specific assay format for quantifying ADCP in a homogeneous assay format. This approach eliminates laborious and expensive flow cytometry or imaging approaches. The measurement of ADCP activity with KILR cells is a simple and reproducible method that measures the physiologically relevant endpoint: target cell destruction inside the lysosomes of effector macrophages.

KILR ADCP assays are fast, robust, and reproducible, supporting screening and characterization of antibody drugs during early phase bio-comparability as well as in late-stage characterization work. The broad application of KILR assays along the drug development pipeline, together with the ability of each stable KILR model to support CDC, ADCC, and ADCP assays, allows this product to support the development of antibody therapeutics.

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

APPENDIX

MATERIALS

Materials Used for ADCP Assay	
Product Description	**Catalog Number**
KILR® MDA-MB-231 Cell Pool	97-1023P036 (Lot No. 16G0503)
KILR SKBR3 Cell Pool	97-1002P018 (Lot No. 19D0902)
KILR Raji Bioassay Cells	97-1012Y026-00169
AssayComplete™ Cell Culture Kit-105	92-3105G
AssayComplete Cell Culture Kit-102	92-3102G
AssayComplete Cell Plating 39 Reagent (CP39)	93-0563R39A
AssayComplete Cell Detachment Reagent	92-0009
KILR Bioassay Detection Kit	97-0001
PathHunter® PL/PK Detection Kit	93-0812
96-Well White, Flat-Bottom, TC-Treated, Sterile Plates (10 plates/pack)	92-0027
Frozen Primary Human PBMCs*	Astarte Biologics (Cat. No. 1001; Donor 369)
Monocyte Enrichment Kit (without CD16 depletion)*	STEMCELL Technologies (Cat. No. 19058)
X-VIVO™ 15 serum free hematopoietic cell medium, with L-glutamine, gentamicin, and phenol red*	Lonza (Cat. No. 04-318Q)
Cetuximab (Anti-Human EGFR Therapeutic Antibody)*	R&D Systems (Cat. No. MAB9577)
Trastuzumab*	Absolute Antibody (Cat. No. Ab00103-10.0)
Rituximab (Afucosylated hIgG1 anti-CD20 antibody)	Invivogen (Cat. No. hcd20-mab13)
Recombinant Human M-CSF	Peprotech (Cat. No. 300-25) or similar
Human IgG1 Isotype Control*	BioLegend (Cat. No. 403502)

*Additional Materials Used for ADCP Assay

The following assay protocols describe the step-by-step details for performing the KILR ADCP assay. The long assay protocol should be followed for most cases. However, to minimize handling of polarized macrophages and eliminating the challenging steps of lifting and counting of cells on the day of the assay, the shorter protocol can be followed. The shorter protocol also minimizes assay variability.

ASSAY PROTOCOL (Long)

Routine Maintenance of Cell Lines

- KILR MDA-MB-231 cells were maintained in AssayComplete Culture Kit-105 supplemented with 700 µg/mL G418. Cells were split 1:3 to 1:4 approximately twice per week or when cells reached approximately 80-85% confluency.
- KILR SKBR3 cells were maintained in AssayComplete Culture Kit-102 supplemented with 250 µg/mL G418. Cells were split 1:2 every 3 days or when cells reached 80-85% confluency.

ADCP Protocol

Effector Cell Preparation

- Monocytes were isolated from two vials of frozen primary human PBMCs (approximately 79-99M starting cells/vial) using a monocyte enrichment kit without CD16 depletion (STEMCELL Technologies) per the manufacturer's recommendations.
- Monocyte cell density was adjusted to 1 x 10^6 cells / mL in differentiation medium consisting of X-VIVO-15 + 10% FBS, 1X PSG and 50 ng/mL m-CSF. 5 mL (5 x10^6 cells total) of monocytes in differentiation medium were seeded into a T25 flask.
- Monocytes were differentiated to M0 macrophages in a humidified 37°C, 5% CO_2 incubator for 6 days. On Day 7, M0 macrophages were harvested by two rounds of cell detachment reagent, re-suspended in CP39, and counted prior to use in ADCP assay.

Running ADCP Assay

- KILR MDA-MB-231 or SKBR3 cells were detached using 0.05% trypsin/EDTA solution and re-suspended in fresh CP39, counted, and seeded at 5K/well (in 50 µL) in an opaque-bottom 96-well, white walled plate and incubated at 37°C, 5% CO_2 for 30 minutes while antibody dilutions were prepared.
- Serial dilutions (1:5) of cetuximab, trastuzumab, and relevant isotype control antibodies were prepared in CP39 as 11X stocks (final concentration top concentration = 1 µg/mL; 11X = 11 µg/mL).
- Target cells were opsonized with 10 µL of antibody serial dilutions for 30 minutes at 37°C.
- 50 µL of prepared M0 macrophages were incubated with the opsonized target cells at an effector: target ratio of 8:1 for 24 hours in a humidified incubator at 37°C, 5% CO_2.
- 100 µL of PathHunter PK/PL Detection Reagent (prepared as recommended by the manufacturer) was added to the cells and incubated at R/T for 0.5 to 1 hour (in the dark).
- Signal detection was performed on a PerkinElmer Envision instrument in luminescence mode (integration time 0.1 sec/well).

Calculating the Percentage ADCP

Data for each dose (average luminescence units (+/- standard deviation) was derived from duplicate wells, and normalized to appropriate controls to calculate % ADCP:

$$\% \text{ ADCP} = \{(RLU_{\text{Control Sample}} - RLU_{\text{Test Sample}}) / RLU_{\text{Control Sample}}\} \times 100$$

[Control Sample = Target cells + Effector Cells ONLY
(average of all wells containing no antibody);
Test Sample = Target cells + Effector Cells + Test Antibody]

ASSAY PROTOCOL (Short)

The following assay protocol is a modification of the long assay protocol shown above.

Effector Cell Preparation

Note: When using primary cryopreserved monocytes, polarization of macrophages was done 7 days prior to running the assay.

The cryopreserved primary human monocytes were differentiated to macrophages, *in situ*, in 96-well assay plates in a humidified 37°C, 5% CO_2 incubator for 7 days, using M-CSF at a final concentration of 50 ng/mL as follows.

Day 0

- The cryopreserved monocytes were thawed from liquid nitrogen tank and re-suspended in 5 mL of 10% AIM-V media (AIM-V media containing 10% FBS) in a conical tube. The cells were counted using a hemocytometer or other cell counting device.
- Cells were centrifuged at 300 x g for 3 minutes.
- The cell suspension was adjusted to the required cell density (2.5 x 10^5 cells/mL for an E:T of 5:1) with 10% AIM-V media.

Note: E:T ratio may require optimization by the user with their particular effectors. For Raji cells, we typically find an E:T of 5:1 is sufficient for generation of rank order and characterization data.

- Macrophage polarizing agent (rhM-CSF) was added to the above prepared cell suspension to a final concentration of 50 ng/mL.
- The cells were plated directly in a 96 well white-walled clear bottom plate at 25,000 cells/well in a volume of 100 µL within the inner 60 wells of the plate. The edge wells were filled up with an equivalent volume of AIM-V media to prevent evaporation. The plates were incubated in a humidified chamber (37°C, 5% CO_2) for 3 days.

Day 3

- 100 µL of fresh 10% AIM-V medium supplemented with 100 ng/mL M-CSF was added to each well containing macrophages (originally plated in 100 µL volume) to bring volume up to a total of 200 µL in each well. The final concentration of M-CSF, in each well, was 50 ng/mL.
- The assay plates were incubated at 37°C, 5% CO_2 for 4 additional days (7 days in total).

Day 7

On the day of the assay, following steps were followed:

Note: The assay plate containing polarized macrophages were brought into the biosafety cabinet once the target and antibody preparation steps were complete.

Target Cell Preparation

- On the day of the assay, cryopreserved KILR bioassay cells were thawed from cryovials, re-suspended in CP39 assay medium, counted, and concentration of cell suspension was adjusted to 0.05 x 10^6 viable cells/mL.

Note: The target cells were added to each well of the assay plate only after antibody preparation was complete.

Antibody Preparation

- Serial dilutions (1:4) of antibody including relevant control antibodies were prepared in CP39 as 11X stocks (final concentration top concentration = 1 µg/mL; 11X = 11 µg/mL).

Perform ADCP Assay

- The assay plate containing the polarized macrophages (e.g. on Day 7) was transferred from the incubator to biosafety cabinet.
- Spent media from the wells of the assay plate(s) containing polarized macrophages was removed.

Trouble shooting tips: Do not aspirate the macrophages with excessive pressure or high pressured vacuum aspirators as this may aspirate the macrophages from the wells.

- 100 µL/well (5,000 cells/well) of prepared KILR Raji bioassay target cell suspension was plated to each well of assay plate containing macrophages.
- 10 µL of each 11X antibody serial dilution in triplicate was added to the designated antibody rows.
- The assay plate was spun at 500 rpm for 1 minute to bring the effector cells in contact with opsonized target cells.
- The assay plate was incubated in a humidified tissue culture incubator at 37°C, 5% CO_2 for 24 hours.
- 100 µL of PathHunter PK/PL Detection Reagent (prepared as recommended by the user manual) was added to the cells and incubated at R/T for 0.5 to 1 hour (in the dark).
- Signal detection was recorded on a PerkinElmer Envision instrument in luminescence mode (integration time 0.2 second/well).
- The RLU value from each individual well (RLU test sample) was used to calculate % ADCP values for each well.

Calculating the Percentage ADCP

The % ADCP value for each data point (well) was calculated using the following formula:

$$\%\ ADCP = \{(RLU_{Control\ Sample} - RLU_{Test\ Sample}) / RLU_{Control\ Sample}\} \times 100$$

[Control Sample = Target cells + Effector Cells ONLY
(average of all wells containing no antibody);
Test Sample = Target cells + Effector Cells + Test Antibody]

The final % ADCP dose curves should be plotted by averaging the % ADCP values from wells for the same dose in relevant rows.