User Manual

KILR® Daudi ADCP Bioassay Kit

For Direct Measurement of Antibody-Dependent Cellular Phagocytosis in a Co-culture Model

For Bioassay Kits:

97-1009Y025-00177: 2-Plate Kit

97-1009Y025-00178: 10-Plate Kit

Document Number 70-442 Revision 0

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Important: Please read this entire user manual before proceeding with the assay.

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1 Overview

The KILR Daudi ADCP Bioassay Kit is a simple, robust, non-radioactive, dye-free cell-based assay that specifically measures target cell death mediated by primary human macrophages. The bioassay kit contains all the materials needed to run the ADCP assay (except effector cells and the reference antibody) including ready-to-use cryopreserved target cells, assay plates and optimized reagents. The ready-to-use cryopreserved target cells have been manufactured to ensure assay reproducibility and faster implementation from screening to characterization and lot release. This bioassay has been optimized for a 96-well format.

2 Technology Principle

Eurofins DiscoverX has developed a number of KILR target cell models to evaluate effector cell mediated target cell death, utilizing our proprietary Enzyme Fragment Complementation (EFC) technology, which measures complementation between two fragments of the β-galactosidase (β-gal) enzyme. Briefly, target cells expressing the desired antigen are engineered to stably express a housekeeping protein (the KILR reporter) tagged with a small fragment of the β-gal enzyme (ePL). When used in an ADCP assay, the KILR target cells are incubated with the antibody of interest and polarized human macrophages as effectors. ADCP results in engulfment of the target cells by the macrophages, leading to dose-dependent degradation of the KILR reporter protein. To quantify ADCP, the target cell are lysed in the presence of detection reagent containing the complementing β-gal fragment, Enzyme Acceptor (EA). Complementation of the two enzyme fragments (EA and ePL; see Figure 1) produces an active enzyme, which hydrolyzes a chemiluminescent substrate to generate a light signal. The signal measured from the wells is inversely proportional to the number of phagocytosed KILR target cells. In the absence of antibody or effector cells, the KILR target cells produce high basal RLU values when lysed in the presence of EA. However, very low signal is observed from KILR target cells that are actively phagocytosed by macrophages as the KILR reporter in the target cells is degraded. The KILR platform measures direct target cell death by phagocytosis hence no secondary bridging assay is needed.

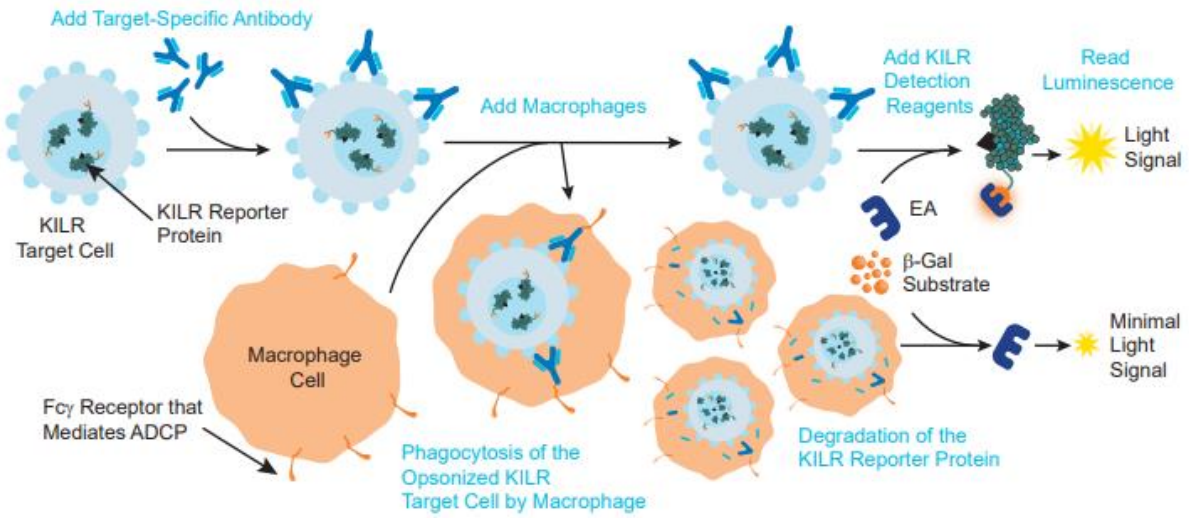


Figure 1: KILR Technology Principle and KILR ADCP Assay Principle

M0 macrophages from cryopreserved monocytes from a single healthy donor are polarized in a 96-well assay dish with M-CSF for 7 days. On the day of the assay, the KILR target cells and a dose response of the antibody of interest are added to the polarized macrophages, then co-cultured for 24h at 37°C. Since macrophages digest the target cells, rather than collecting the supernatant, we lyse all cells and add detection reagent containing the complementing EA to detect the remaining amount of KILR protein present. This leads to the formation of the active β-gal enzyme which hydrolyzes the substrate to give a chemiluminescent output, detected on any bench top luminescence reader. Higher signal correlates with less killing, while less signal correlates with more killing.

3 Application: ADCP

ADCP is the mechanism by which antibody-opsonized target cells activate the FcγRs on the surface of macrophages to induce phagocytosis, resulting in internalization and degradation of the target cell through phagosome acidification.

In the KILR Daudi ADCP Bioassay, ready-to-use KILR Daudi target cells expressing the antigens of interest are co-incubated with macrophages and an opsonizing therapeutic antibody for 24 hours. In this assay, KILR Daudi target cells endogenously expressing the desired antigen (e.g. CD19, CD20 or CD38) were engineered to stably express a housekeeping protein that is tagged with enhanced ProLabel® (ePL), a β-gal reporter fragment. These KILR Daudi target cells will be phagocytosed by macrophages during the process of ADCP. The remaining target cells in the co-culture are lysed in the presence of PathHunter® PL/PK Detection reagent kit which includes the EA fragment of β-gal enzyme, and enzyme substrate, resulting in complementation of any remaining KILR reporter protein in which produces a functional β-galactosidase enzyme, which generates a chemiluminescent signal that can be detected on any benchtop luminescence reader.

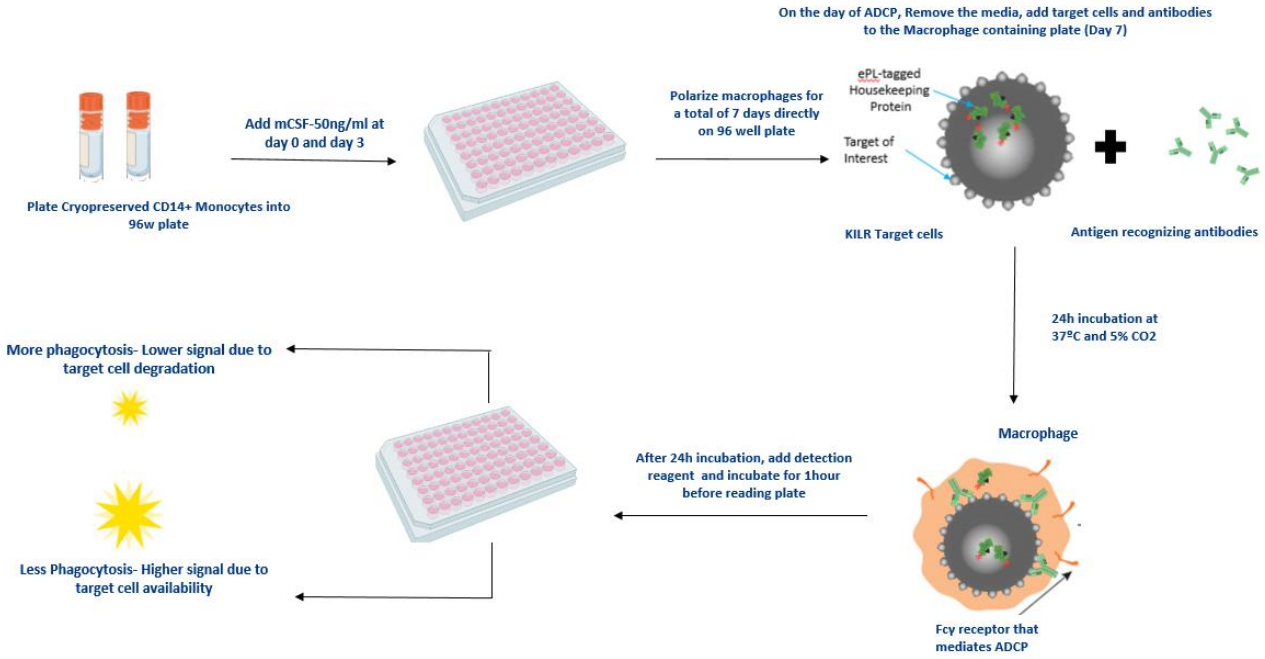


Figure 2 KILR ADCP Assay Workflow

The KILR Daudi ADCP bioassay has been developed to specifically quantify target cell phagocytosis mediated by target receptor recognizing antibody and Fc gamma receptors on the macrophages. When the stable KILR Daudi target cells are incubated with macrophages and the test antibody, effector-mediated phagocytosis of the target cell occurs. Phagocytosis of Daudi target cells by macrophages leads to a dose dependent decrease in chemiluminescent signal.

4 Materials Provided

List of Components	97-1009Y025-00177 (2 plate Kit)	97-1009Y025-00178 (10 plate Kit)
KILR Daudi Bioassay Cells (1 x 10^6 cells /100 µL /vial)	2 vials	10 vials
AssayComplete™ Cell Plating 39 Reagent (Bottle)	1 x 100 mL	1 x 500 mL
PathHunter® PL/PK Detection Kit EA reagent (Bottle) Lysis Buffer Bottle) Substrate Reagent (Bottle) Positive Control (1 vial)	1 x 20 mL 1 x 20mL 1 x 80 mL 1 x 0.2 mL	1 x 20 mL 1 x 20 mL 1 x 80 mL 1 x 0.2 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

5 Storage Conditions

KILR Daudi Bioassay Cells

Cells must arrive in a frozen state on dry ice and should be transferred to the vapor phase of liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage

Short-term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).

Long-term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Use tongs to remove cryovials from liquid nitrogen storage, and place the vials immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen that may be present inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

AssayComplete™ Cell Plating 39 Reagent (CP39)

Upon receipt, store at -20°C. Thaw contents at room temperature and mix well by gently inverting the bottle prior to use. Once thawed, store at 4°C for up to 12 weeks or until the expiration date on the product label, whichever comes first. Do not refreeze. Once L-glutamine has been added, the medium is stable for 10 days at 4°C.

PathHunter PL/PK Detection Kit

Upon arrival, store reagents at -20°C. The detection kit is stable until the expiry date indicated on the kit box outer label. Thaw frozen detection reagents at room temperature before use. After thawing, store detection reagents for up to 1 month at 2-8°C. For long-term storage, aliquots of all the detection components may be re-frozen in opaque containers at -20°C. The detection reagents can be thawed and frozen for a total of 3 times without loss in performance.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

6 Additional Materials Recommended for Assay

The following equipment and additional materials are recommended to perform the assays:

Material	Ordering Information
96-well Clear V-Bottom 500 µL Polypropylene Deep Well Plate, 10 per Pack, Sterile	Axygen®; (Cat#: P-96-450V-C-S)
Cryopreserved Human Monocytes	HemaCare/Charles River Labs; (Cat#: PB14C-1), or similar
AIM-V-medium, liquid (research grade)	ThermoFisher; (Cat #: 12055091), or similar
Fetal Bovine Serum (FBS)	Sigma Aldrich (Cat# 16140-071), or similar
Recombinant Human M-CSF (rh M-CSF)	Peprotech (Cat #: 300-25), or similar
Rituximab (Afucosylated hIgG1 anti-CD20 antibody)	Invivogen; Cat # hcd20-mab13
15 mL Polypropylene Tubes and 1.5 mL Microtubes	
Single and Multichannel Micro-pipettes and Pipette Tips (10 µL – 100 µL)	
2 mL Wide-bore Pipettes	
Hemocytometer	
Luminescence Reader	discoverx.com/instrument-compatibility
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	

7 Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.1 mL of AssayComplete™ Freezing Reagent (refer to the CoA for cell number provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.


Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

1. KILR Daudi Bioassay Cells must arrive in a frozen state on dry ice.

   
   Contact technical support immediately, if cells received were already thawed.

2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage,

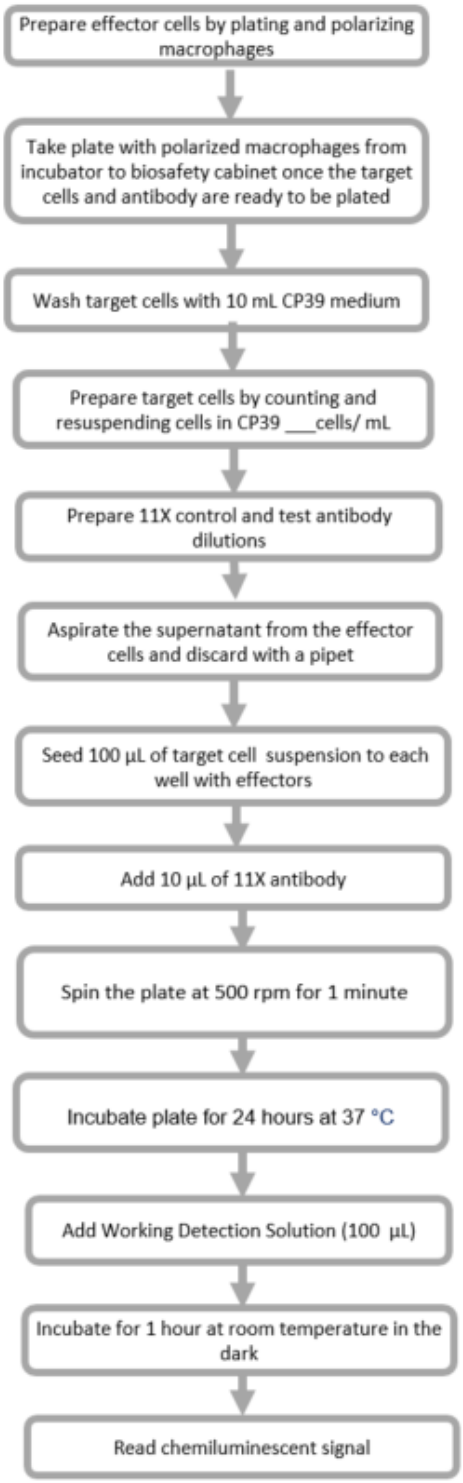
place vials in the vapor phase of liquid nitrogen storage.

 Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

3. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.
4. Proceed with the thawing and propagation protocols in the following section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

8 Protocol Schematic

Quick-start Procedure: For a 96-well plate format, perform the following steps.



*Room temperature refers to a range of 23-25°C

9 Detailed Assay Protocol

This user manual provides a protocol for quantifying target cell death by ADCP in a 96-well format. The following detailed protocol is specific to the detection of antibody-dependent cellular phagocytosis (ADCP). A 96-well tissue culture plate with pre-plated macrophages is used for the ADCP assay. To this plate, target cells and ADCP-mediating antibody are added.

9.1 Effector Cell Preparation

Note: Begin polarization of macrophages (from cryopreserved primary human monocytes) 7 days prior to running the ADCP assay.

Differentiate the cryopreserved primary human monocytes 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

1. Thaw cryopreserved monocytes from liquid nitrogen (LN2) tank and resuspend in 5 mL of 10% AIM-V media (AIM-V media containing 10% FBS) in a conical tube. Count the cells using a Nexcelom T4, Hemocytometer or any other cell counting device.
2. Determine the density of viable cells.
    a. Remove 50 µL or less of the resuspended cells and stain with trypan blue according to manufacturer's recommendation.
    b. Transfer an appropriate portion of this fraction of stained cells to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device. Count cells.
3. Centrifuge remaining cells at 300 x g for 3 minutes.
4. Adjust the cell suspension 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: Effector: Target (E:T) ratio may require optimization by user with their particular effector cells. For Daudi cells, we typically find an E:T of 5:1 is sufficient for generation of rank order and characterization data.

5. Add Macrophage polarizing agent (rh M-CSF) to the above prepared cell suspension to a final concentration of 50 ng/mL.
6. Plate the cells directly in a 96-well white-walled clear bottom plate at 25,000 cells / well in a volume of 100 µL, in the inner 60 wells of the plate, as shown in Figure 4, Representative Assay Plate Map (e.g. Rows B, C, D, E, F and G, columns 2 through 11). Fill edge wells with an equivalent volume of AIM-V media to prevent evaporation. Incubate the plates in a humidified chamber (37°C, 5% CO_2) for 3 days.

Day 3

1. Add 100 µL of fresh 10% AIM-V medium supplemented with 100 ng/mL M-CSF 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 is 50 ng/mL.
2. Incubate the assay plates at 37°C, 5% CO_2 for 4 additional days (7 days in total).

Day 7

On the day of the assay, follow the steps below:

1. Assay plate containing polarized macrophages will be processed as detailed in Perform ADCP Assay (Per Section 9.4).

9.2 Target Cell Preparation

The following protocol is for thawing and plating cryopreserved KILR Daudi bioassay target cells from cryovials (1 cryovial of target cell per assay plate), on the day of running the ADCP assay.

Note: It is ideal to have good viability of target cells to reduce background.

1. Before thawing the cells, ensure that all the materials required are set up in the tissue culture hood. These include:
   - One sterile 25 mL reagent reservoir
   - One sterile 15 mL conical tube (per cryovial thawed)
   - A micropipette (P1000) set to dispense 1 mL
   - A multichannel pipette and tips set to dispense 50 µL
   - Sterile 10 mL filter pipets
   - A bottle of AssayComplete™ Cell Plating 39 Reagent, pre-warmed in a 37°C water bath for 15 minutes and equilibrated to room temperature
   - A 96-Well Green, V-Bottom, Sterile Dilution Plate, to be used as the master dilution plate
2. Remove the cryovial from vapor phase of liquid nitrogen and immediately place it in dry ice.
3. On the day of use, prepare Cell Plating 39 (CP39) medium by adding fresh L-glutamine to AssayComplete Cell Plating 39 Reagent to a final concentration of 2 mM.
   a. **Note:** CP39 medium is comprised of the AssayComplete Cell Plating 39 Reagent supplemented with L-glutamine (provided in the AssayComplete Cell Plating 39 Reagent Pack within the KILR ADCP bioassay kit). CP39 medium is light-sensitive, thus protect it from light while working with it in the culture hood.
   b. Since L-glutamine is labile, prepare only the amount of CP39 medium required for the experiment. In this protocol, CP39 medium is used for preparation and plating of bioassay cells, effector cells and antibody dilutions.

Preparation of CP39 Medium		
Components	**2 Plates**	**10 Plates**
Cell Plating 39 Reagent (mL)	80	400
L-glutamine (100X) (mL)	0.8	4
Total Volume (mL)	**80.8**	**404**

4. For each vial of cells to be thawed, transfer 10 mL of room temperature CP39 to a fresh 15 mL conical tube.
5. Remove cryovial(s) of KILR Daudi Bioassay cells from liquid nitrogen tank immediately before setting up the assay. Keep the cell vials on dry ice while transporting to laboratory.
6. Remove the cryovial from dry ice and ensure cap is tightened. Holding the vial by the cap, immediately thaw vial in 37°C water bath for 30 seconds (± 5 seconds), gently agitating the vial to thaw cells. **DO NOT LEAVE**

VIAL SITTING IN WATER BATH.

7. Visually inspect bottom of vial after 20 seconds. If pellet has thawed, remove the vial from water bath, wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood. If ice is still visible, return vial to water bath for additional 10-15 seconds.
8. Add 1 mL of room temperature CP39 media from the 15 mL conical tube to the cryovial. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet up and down 3 times to uniformly resuspend the cells.
9. Transfer the resuspended cells from the cryovial to the 15 mL conical tube containing the remaining 9 mL of room temperature CP39.
10. Repeat the process 1-2 times to ensure all cells are completely transferred from the cryovial into the conical tube.
11. Replace the cap on the conical tube and mix by gentle inversion 2-3 times to ensure that the cells are properly resuspended in the reagent.
12. Centrifuge the cells at room temperature for 4 min @ 200-300g.
13. Using a sterile pipet, carefully aspirate the supernatant and discard.
14. Resuspend the cell pellet in 5 mL of fresh CP39 medium.
15. Mix cells suspension by gently pipeting up and down 2-3 times with a sterile 10 mL pipet. Use 10µL -100µL of cell suspension for counting.
16. Count cells and determine viability. For the purpose of determining the concentration of cells in the suspension:
    a. Set aside a small fraction of the suspended cells in a separate tube.
    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.
    c. Count number of viable cells and calculate the concentration of viable cells in the suspension. Then calculate the total number of viable cells remaining in the 15 mL conical tube.
    d. Adjust concentration of cell suspension to 0.05 x 10^6 viable cells / mL
17. Mix KILR Daudi bioassay cell suspension (from Step 16d) by gently inverting the tube 2-3 times, without creating any froth. Set the cell suspension aside in biosafety cabinet, until antibody preparation is complete.

9.3 Antibody Preparation

1. Follow the procedure below to set up an antibody dose-response dilution (for a Rituximab control). Prepare serial dilutions for the control antibody in a master dilution plate (V-bottom dilution plate) in a 9-point series of 4-fold dilutions of antibody in CP39 medium. The concentration of each dilution should be prepared at 11X the final concentration in the assay.
    a. Label the wells of a master dilution plate and proceed with antibody dilution as follows
    b. Prepare a working stock of Rituximab at 11 µg/mL, which is 11X the desired final top concentration of 1 µg/mL, in CP39.
    c. Add 60 µL of Step 1b solution (i.e CP39 with antibody) to well 2 column 2 of master dilution plate.
    d. Add 45 µL of CP39 medium alone without antibody into wells 3 to 10 (of column 3 to Column 10 of master

dilution plate)

e. With a clean tip, transfer 15 µL of antibody from Well 2 into Well 3 and mix by gently pipetting up/down 2-3 times.

f. Transfer 15 µL from Well 3 into Well 4 and so on using a fresh tip when moving to each new well. Mix by gently pipetting up/down 2-3 times.

g. Repeat this process until Well No. 9 is reached. Do not add antibody to Well No. 10 since this is the control (vehicle only) well.

Note: The top concentration in the antibody preparation step may need to be optimized depending on macrophage donor source

2. Set up serial dilutions for any additional antibodies in a similar manner (one row per antibody), using the desired dilution series.

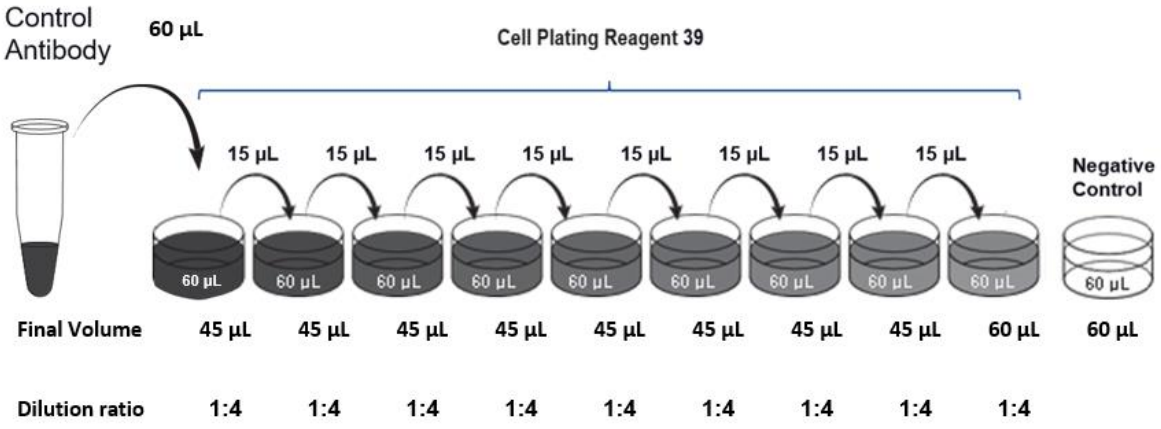


Figure 3 Antibody Serial Dilutions

In a master dilution plate, prepare a 10-pt dose response curve for each antibody (one antibody per row) with nine serial dilutions of 1:4, where the last dose is vehicle only.

9.4 Perform ADCP Assay

1. On day of assay, transfer assay plate containing the polarized macrophages (e.g. after 7 days of M-CSF treatment) from incubator to biosafety cabinet.
2. Using a sterile pipet, carefully aspirate the supernatant from each well in the assay plate and discard.
3. Mix KILR Daudi bioassay cell suspension (from Section 9.2, Step 16d) by gently inverting the tube 2-3 times, without creating any froth. Pour the cell suspension into a sterile 25 mL reagent reservoir .

   Note: The target cells are to be added to each well of the plate only once antibody preparation is complete.

4. Using a sterile multichannel pipette, plate 100 µL/well (5,000 cells/well) of prepared KILR Daudi Bioassay cell suspension in each well of assay plate containing macrophages.

5. Add 10 µL of each 11X antibody dilution series from the Master Dilution Plate in triplicate to the designated rows in the assay plate (e.g. Test Antibody 1 (Sample 1) in Rows B, D and F and Test Antibody 2 (Sample 2) in Rows C, E, and G as shown in the Representative Assay Plate Map (Figure 4).

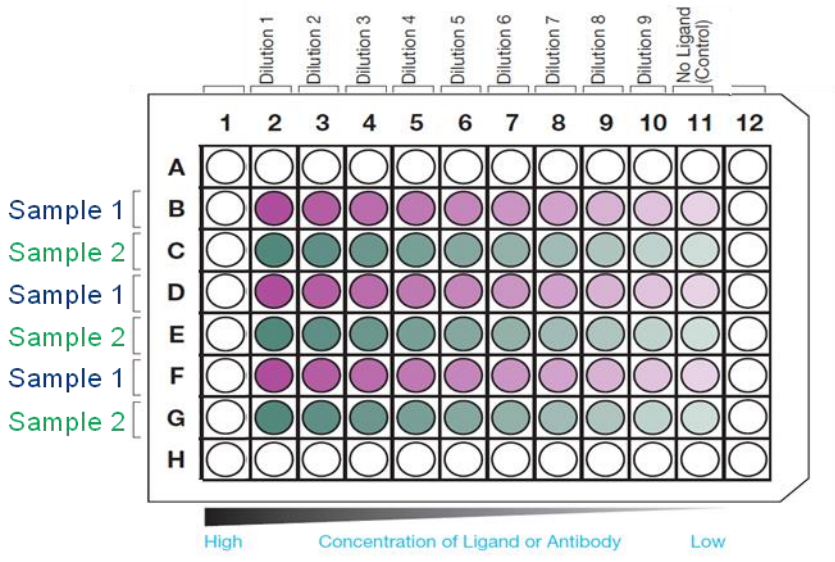


Figure 4 Representative Assay Plate Map

This plate map shows two interdigitated 10-point dose curves, with three replicates per dose point, for 1 test sample and 1 reference sample tested using the same dilution scheme **(Reference sample or control antibody is Rituximab)**

6. Spin the assay plate at **500 rpm** for 1 minute to bring the effector cells in contact with opsonized target cells.
7. Incubate the assay plate in a humidified tissue culture incubator at 37°C, 5% CO_2 for 24 hours.

9.5 Addition of Detection Reagent

At this point, the ADCP reaction has reached completion. The following section details the steps for preparing and adding the PathHunter PL/PK Detection Reagent to the assay plate and reading the assay plate on a luminescence reader.

PathHunter PL/PK Detection Working Solution Volume Guide		
Components	**Volume Ratio**	**Volume per Plate (mL)**
EA reagent	1	2
Lysis Buffer	1	2
Substrate Reagent	4	8
Total Volume		**12**

1. Prepare PathHunter PL/PK Detection Reagent working solution in a tube or reservoir by mixing 1-part of EA Reagent, 1-part of Lysis Buffer, and 4-parts of Substrate Reagent (these calculations take into account excess

volume needed to ensure accurate pipetting). Mix reagents by gently inverting the tube 2-3 times prior to addition to the reagent reservoir. Refer to the user manual for the PathHunter PL/PK Detection for more complete information on the detection reagents.

2. Using a multichannel pipet, add 100 µL of PathHunter PL/PK Detection working solution to all wells containing cells in the assay plate.

   NOTE: DO NOT mix the solution in the wells using a pipet or vortex the assay plate.

3. Gently mix the contents in assay plate by slowly moving the plate back and forth 2-3 times in a crisscross pattern on the surface of the tissue culture hood.
4. Place lid back on assay plate and incubate for at least 1 hour at room temperature in the dark. Read samples on a standard luminescence plate reader at 0.2 seconds/well for standard luminescence plate readers (e.g. Perkin Emer Envision or Molecular Devices iD3 or similar) or 5 to 10 seconds for imager based reader.
   a. A luminescence reader usually collects signal from all wavelengths. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.
5. Data analysis and graph plotting can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.)
6. Data may be plotted as raw RLU or transformed to % ADCP as described below:

   a. Calculate the mean of the RLU values of control sample for each well from Row B to Row G in column 11. This generates the 'Mean RLU Control sample' used to calculate % ADCP.
   b. The RLU value from each individual well (RLU test sample) is used to calculate %ADCP values for each well (e.g. from wells in column 2 through column 10) in Row B to Row G. will be used to calculate the ADCP from each well.
   c. Calculate the % ADCP value for each data point (well) using the following formula:

$$\%ADCP = \frac{(Mean\ RLU\ Control\ sample - RLU\ Test\ Sample)}{(\ Mean\ RLU\ Control\ Sample)} x100\text{d}.$$

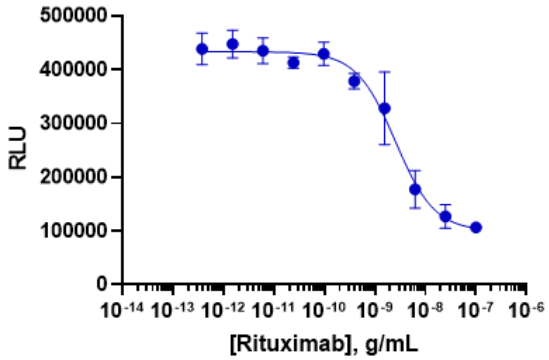
7. Final % ADCP dose curves are plotted by averaging the % ADCP values from wells for the same dose in relevant rows as per the Representative Plate Layout (for example, by calculating average data from same the well position in rows B, D and F for Sample 1, and from Rows C, E and G for Sample 2)

10 Typical Results

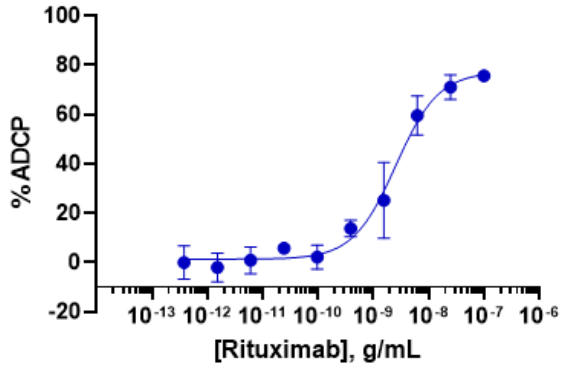
The following graphs are examples of a typical dose-response curve for the KILR Daudi ADCP Bioassay generated using the protocol outlined in this manual, when plotting either raw RLU signal (A) or data normalized to % ADCP (B). The data demonstrate a dose dependent increase in target cell phagocytosis with the control antibody, Rituximab in co-culture with effector cells.

The plate was read on the EnVision® Multimode Plate Reader and data analysis was performed using GraphPad Prism.

A



B



C

S/B	Hill Slope	IC_{50}, ng/mL	Emax, %
4.1	1.123	2.6	77

Figure 5: Typical Results

Representative **A**, raw RLU data for Rituximab dose-response curve **B**, ADCP data normalized to no antibody control and plotted as % ADCP **C,** Assay window (S/B), obtained from graph in (A), and Hillslope, IC_{50} and Max % killing (Emax) obtained from graph in (B) for KILR Daudi ADCP Bioassay, as measured using the procedure in this user manual.

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