User Manual

KILR® CD16 Effector Cells

Effector Cells for Use in Immune-Mediated Cytotoxicity Applications

Part numbers:

97-0007-01: 1 Vial

97-0007-05: 5 Vials

Document Number 70-388 Rev 7

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1. Overview

KILR CD16 Effector Cells are purified human cytotoxic T lymphocytes stably expressing CD16 (FcγRIIIa; V158 variant), produced in a standardized batch. The cells were developed for Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and T Cell Redirection (TCR) applications to reproducibly mediate target cell death.

The KILR cytotoxicity target cell line enables the development of a simple, non-radioactive and dye-free method to specifically measure target cell death in a co-culture. The KILR assay has several applications in Cancer Immunotherapy drug development including measuring target cell death through ADCC, Complement-Dependent Cytotoxicity (CDC), Antibody-Dependent Cell Phagocytosis (ADCP), Cytotoxic T Cell Lymphocyte-Mediated Death (CTL), Bi-specific Antibody Mediated T Cell Redirection, Chimeric Antigen Receptor T Cell (CAR-T), and adoptive T cell therapies. This assay can drive all aspects of the drug discovery process, from screening to characterization, and the development of a QC lot-release assay for the immunotherapy drugs.

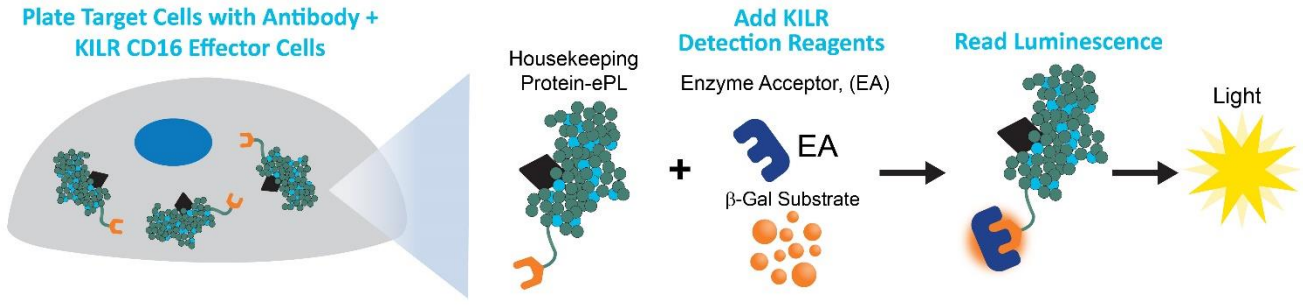


Figure 1: Assay Principle

The KILR target cells, expressing the receptor antigen of interest, have been engineered to stably express the KILR reporter protein which is a housekeeping protein tagged with enhanced ProLabel® (ePL), a β-gal enzyme fragment. Treatment of KILR target cells with a test antibody leads to opsonization. Following this, addition of KILR CD16 Effector cells results in engagement of the effector cells with the antibody Fc region that leads to effector cell-mediated lysis of the target cells. This results in release of the KILR reporter protein into the assay media. Addition of detection reagent (containing the EA fragment and substrate) leads to formation of the active β-gal enzyme which hydrolyses the substrate to generate a chemiluminescent signal.

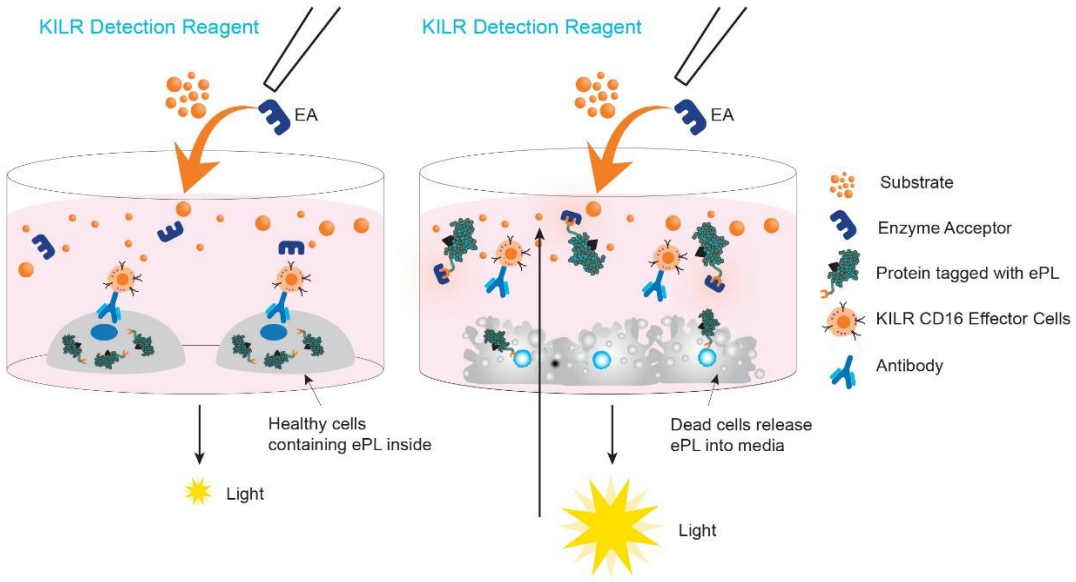


Figure 2: KILR Detection

In the figure, the well on the left contains intact target cells that are alive in the presence of immune effector cells. When detection reagents are added to the well, we cannot detect chemiluminescence as the reporter protein does not leak out through an intact cell membrane into the media. In contrast, in the well on the right, after addition of antigen-specific antibody, the target cells are killed by the KILR CD16 Effector Cells, releasing the reporter protein into the media. Addition of the detection reagents leads to the recognition of the reporter protein and generation of a chemiluminescent signal that is proportional to the number of dead cells. Death of any other cell type, including the KILR CD16 Effector Cells present within the co-culture will not affect the assay output, giving the KILR cytotoxicity assay an unparalleled specificity to detect target cell death within a co-culture system.

2. Suggested Applications

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism of cell-mediated immune defense whereby the immune effector cells actively lyse the target cells whose membrane-surface antigens have been coated with antigen-specific antibodies that harbor an active Fc region.

KILR cell lines have been successfully used to quantify T cell redirection-mediated killing via engineered bi-specific antibodies or other means. This class of biologics (antibodies and antibody-like proteins) are designed to simultaneously recognize a target antigen presented on the membrane of target KILR cells, and an activating receptor on the surface of T cells, leading to T cells activation and subsequent lysis of the KILR target cells.

3. Materials Provided

KILR CD16 Effector Cells are used to measure target cell death in the KILR cytotoxicity assays as well as other established assays, such as Chromium 51 release assays, substituting KILR CD16 Effector Cells for primary human PBMCs or NK cells.

Components	Ordering Information
KILR CD16 Effector Cells, 1 Vial	97-0007-01
KILR CD16 Effector Cells, 5 Vials	97-0007-05

3.1 Storage Conditions

KILR CD16 Effector Cells

Cells must arrive in a frozen state on dry ice. The cell vials should be transferred to the vapor phase of liquid nitrogen storage immediately upon arrival. Storage at temperatures greater than -150°C is not recommended and should be minimized as much as possible.

- Short-term storage for less than 24 hours: If storage in liquid nitrogen upon receipt of cells is not possible, store vials at -80°C immediately upon arrival. DO NOT store at -80°C for longer than 24 hours.
- Long-term storage for longer than 24 hours: Vials should ONLY be stored in vapor phase of liquid nitrogen only or in ultra-low temperature (-150°C) freezers.
- For one-time use only. Do not refreeze.

Cell Culture Medium

- Store at -20°C. Thaw contents at room temperature and mix well by gently inverting the bottle prior to use. Once thawed and mixed, store the final product at 4°C for up to 4 weeks. Avoid multiple freeze/thaw cycles.
- Make small aliquots and store media for longer term storage (>4-week storage) to avoid multiple freeze/thaw cycles.

4. Materials Needed

Required Materials	Ordering Information
AssayComplete™ Cell Culture Kit 117	92-3117G
Recombinant Human IL-2, Cell Culture Grade	92-1331

The following materials are also required when using the KILR CD16 Effector Cells with KILR target cell lines or cell pools. For using these effector cells in other cytotoxicity assays, follow the manufacturer's recommendations.

Recommended Materials	Ordering Information
KILR Target cell pools or cell lines	discoverx.com/ KILR
AssayComplete Cell Plating 39 Reagent Pack*	93-0563R39 Series**
AssayComplete Protein Dilution Buffer***	92-0023 Series
KILR Detection Reagent	97-0001 Series
96-well White Bottom TC Treated Plates, Sterile with Lid, 10/ pack	92-0027
V-bottom PP Dilution Plates, 10/pack (for antibody dilutions)	92-0011
T75 Flask with vented cap	Corning, Cat. No. CLS430641U or similar
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
15 mL Polypropylene Tubes and 1.5 mL Microtubes	
Single and Multichannel Micro-pipettes and Pipette Tips (10 µL – 1000 µL)	
2 mL Wide-bore Pipettes	
Hemocytometer	
Luminescence Reader	discoverx.com/instrument-compatibility
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	

* It is not recommended to substitute the Cell Plating (CP) Reagent that has been specified in the datasheet for the cell line with another reagent.

** Series refer to the different sizes available for that reagent or kit.

*** Refer to the respective KILR Target cell line or cell pool datasheet for the recommended dilution buffer.

5. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and each vial contains 15 x 10^6 cells (±15%) in 1 mL of freezing media. Upon receipt, the vials should be transferred to vapor phase of liquid nitrogen for long-term storage (longer than 24 hours).

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

The following procedures are for placing in, handling and removal of cryovials from the vapor phase of liquid nitrogen storage.

1. KILR CD16 Effector Cells must arrive in a frozen state on dry ice.
2. Frozen cell cryovials 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 for a short period at -80°C. For long-term storage, cryovials must be stored in the vapor phase of liquid nitrogen.

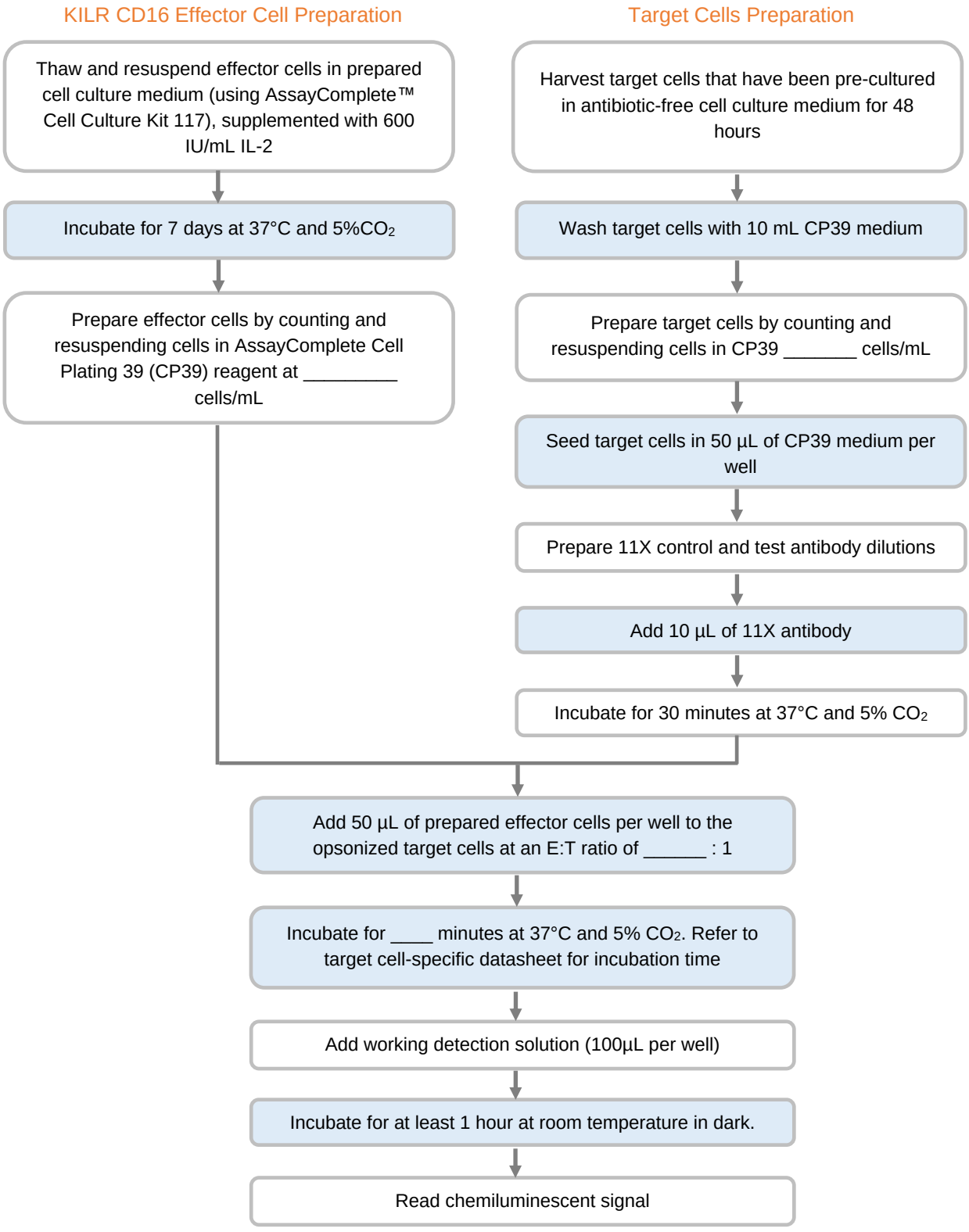
 Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

 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.
4. Wait for at least one minute for any liquid nitrogen that may be present inside the vial to evaporate, before handling the vials.
5. Proceed with the Cell Preparation Protocol in Section 7.3 below for **KILR CD16 Effector Cells Preparation**.

6. Protocol Schematic

In a 96-well tissue culture-treated plate, perform the following steps. Refer to the respective KILR target cell pool (or cell line) datasheet for appropriate seeding density and incubation time.



7. Detailed Assay Protocol

The following detailed protocol is specific for detecting antibody-dependent cell-mediated cytotoxicity (ADCC). Target cells are opsonized with an ADCC-inducing antibody, and then co-incubated with the KILR CD16 Effector Cells in a 96-well tissue culture plate.

KILR CD16 Effector Cells Preparation

KILR CD16 Effector Cells are single-donor derived primary human effector cells. These are not an immortalized cell line and, once thawed, these cells recover for a limited period. For most applications, including ADCC, T cell redirection, etc., it is recommended to use the cells after maintaining the cells in culture for a period of seven days in cell culture medium [supplemented with L-glutamine (Component G) and IL-2]. This 7-day resting period helps in maximizing recovery of cells that will be available for the assay. Any remaining KILR CD16 Effector Cells can be maintained in culture for a maximum of 14 days without a significant loss of killing capacity. It is not recommended that these effector cells be used after 14 days in culture, because there is no selection marker to maintain expression of the CD16 receptor, so some reduction of CD16-mediated killing capacity may occur.

7.1 Preparing Cell Culture Medium

AssayComplete™ Cell Culture Kit 117 includes cell culture reagent (base medium), serum (Component B) and L-glutamine (Component G). Fresh culture medium is prepared by supplementing the Cell Culture Reagent with serum to a final concentration of 10%, and L-glutamine to a final concentration of 2 mM. **Note:** L-glutamine must be added fresh each day prior to running the assay.

Note: It is not recommended to prepare and store the media supplement with Component G

1. Using aseptic techniques, prepare complete medium by adding the entire contents of Component B to the 500 mL of AssayComplete Cell Culture Reagent. Place a checkmark next to the component on the bottle label.
   a. Medium supplemented with Component B can now be aliquoted and stored at -20°C.
   b. Component G should be added to medium supplemented with component B immediately prior to running the assay.

Note: Component G can be aliquoted and stored independently at -20°C. Equilibrate to room temperature prior to addition to the medium. Once aliquoted avoid multiple free-thaw cycles.

   c. For example, for a final cell culture medium volume of 50 mL, add 0.5 mL of 200 mM (100X) Component G to 50 mL of complete medium.

7.2 Thawing KILR CD16 Effector Cells

The following is a procedure for preparing rested KILR CD16 Effector Cells from frozen stocks. This procedure should be initiated 7 days before the opsonization step of the ADCC assay.

1. **Equilibrate a water bath to 37°C.**
2. Aliquot 19 mL of complete medium supplemented with Component G into a 50 mL conical tube and pre-warm in water bath.

Note: If thawing more than one vial, cells can be pooled at thawing (e.g. 2 vials in 38 mL of pre-warmed medium). It is not recommended to thaw more than two vials at a time, as it may seriously compromise cell viability and recovery time.

3. Remove the cryovials from -80°C or liquid nitrogen vapor storage and immediately place them in dry ice.

Safety Warning: A face shield, gloves, and a lab coat should be always worn when handling frozen vials. When removing cryovials from liquid nitrogen vapor storage, use tongs to place them immediately in dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time, to avoid inadvertent thawing of the cells.

4. Thaw the effector cells rapidly by immersing the cryovials into the pre-warmed water bath and gently agitating it until only a small piece of ice is visible (may take up to 2 minutes). **DO NOT centrifuge or vortex freshly thawed cells.**

Do not leave the frozen cell vials in the water bath for longer than 2 minutes. Prolonged thawing at 37ºC may result in cell death.

5. Decontaminate the external surface of the vials by spraying and wiping them with 70% ethanol. Transfer them to a tissue culture hood.
6. Using a 1000 µL micropipette, gently transfer the contents of each cryovial (about 1 mL) dropwise into 19 mL of pre-warmed cell culture medium in the 50 mL conical tube.
7. Using a 1000 µL micropipette, add 1 mL of medium from conical tube to rinse the cryovial to increase cell recovery.
8. After rinsing is complete, transfer the 1 mL of medium back into conical tube using a micropipette.
9. Homogenize the cell suspension by gently pipetting up and down twice with a 25 mL serological pipet and transfer the entire volume of the thawed cells into a T75 flask with vented cap.

Note: If thawing 2 vials at once, cells can be pooled at thaw (e.g. dispense 2 mL of cells from the 2 vials into 38 mL of pre-warmed medium), then 20 mL of cell suspension can be dispensed into each of two T75 flasks with vented cap.

10. Add IL-2 to inside of the flask to a final concentration of 600 IU/mL to each T75 flask with vented cap and mix by gently rotating flask.

Note: Refer to the datasheet provided with the IL-2 to for information on the specific activity of the supplied lot.

11. Incubate the flask(s) upright in a humidified incubator at 37°C and 5% CO_2.

Note: In the first 24-48 hours after thawing, about 40% decrease in the number of viable cells is expected and normal.

12. After 24 hours of recovery, the KILR CD16 Effector Cells will begin to slowly recover. Performance is best if cells are left untouched for approximately 3 days. After 3-4 days, gently pipet the cell suspension up and down at least 6-8 times using a 10 mL disposable pipet to dissociate cell clumps. Avoid creating any froth or foam during this step. Count viable cells under phase contrast. **Refer to example image in figure (3A) or with trypan blue exclusion, as described below.**
13. Determine the total number of viable cells:

a. Gently pipet the cell suspension up and down at least 6-8 times using a 10 mL disposable pipet to dissociate cell clumps. Avoid creating any froth or foam during this step.

b. Remove an aliquot (typically 10 µL of cell suspension) and stain cells with trypan blue according to manufacturer's recommendation.

c. Transfer an appropriate portion of this fraction of stained cells to a hemocytometer or Malassez slide (typically 10 µL of cell suspension) or a similar cell-counting device. For best results, allow the cells to settle on the slide surface for at least 5 minutes before counting.

Note: For best results, it is strongly recommended to use manual counting rather than automated counting devices. Before implementing the use of automated cell counters, appropriate calibration studies including bridging with manual counting must be performed**.**

d. Count number of viable cells and calculate the concentration of viable cells in the suspension.

Note: If use of phase contrast for counting is not possible, it is recommended to use trypan blue to distinguish non-refractile, dead cells from round, refractile living cells. **Refer to the example images in figure 3 (B) & (C) of the cells visualized with trypan blue.**

14. Adjust cell density to 0.8 x 10^6 viable cells/mL by adding the appropriate volume of fresh culture medium supplemented with IL-2 to a final concentration of 600 IU/mL. In order to maintain cells beyond day 7, KILR CD16 Effector Cells should be maintained at a density not to exceed 1.5 x 10^6 cells/mL.

    a. Optimal recovery is observed when effector cells are maintained in culture for seven days. This ensures sufficient number of effector cells are available to be used in the assay.

    b. Cells can be maintained in culture for up to 14 days with no adverse effect on killing capacity. No significant differences have been observed in the killing capacity of KILR CD16 Effector Cells at Day 7 (post-thaw recovery) through to Day 14.

Note: Counting the effector cells every day is **not** recommended. This may adversely affect cell recovery.

7.3 Preparation of KILR CD16 Effector Cells for ADCC Assay

The following is a procedure for final preparation of the KILR CD16 Effector Cells on the day of the ADCC assay. For optimal ADCC results, it is recommended to empirically establish the appropriate Effector cell to Target cell (E: T) ratio for each new target cell line. For most target cell types, we recommend testing E:T ratios from 5:1 to 10:1 to determine optimal ADCC assay performance. The below protocol uses manual counting of target and effector cells to determine cell seeding density.

Note: Refer to the KILR cell line-specific datasheet for the recommended E:T ratio (with primary PBMCs) and target cells/well. KILR CD16 Effector Cells are about 2-4 fold more potent than PBMCs, therefore, the E:T ratios required for KILR CD16 Effector Cells are typically lower than the ratios used for isolated PBMCs.

For more killing-resistant target cell types (e.g. SKOV-3), a higher E:T may be necessary for optimal results.

Assay Reagents (Volume per Well)	96-Well Plate
Number of Target Cells	Refer to cell line-specific datasheet
Effector Cell to Target Cell (E:T) Ratio	5:1 to 10:1; determined empirically
Cell Plating (CP) 39 medium (µL)	50
Antibody Solution (µL)	10
KILR CD16 Effector Cells suspension (µL)	50
Working Detection Solution (µL)	100
Total Assay Volume (µL)	**210**

Note: KILR CD16 Effector Cells preparation should be initiated at least 7 days before the opsonization step in section 7.5 Antibody Preparation

1. On the day of running the ADCC assay, check the morphology of the cells in the flask under a phase-contrast microscope. The cells should resemble normal human lymphocytes, i.e., round and small to medium in size.
2. Determine the density of viable cells.

   For best results, it is strongly recommended to use manual counting rather than automated counting devices. Before implementing the use of automated cell counters, appropriate calibration studies including bridging with manual counting must be performed.

   a. Take 50 µL or less of the suspended cell, prepared as in step 7.2.12.

   b. Stain cells with trypan blue according to manufacturer's recommendation.

   c. Transfer an appropriate portion of this fraction of stained cells to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.

   d. Count the viable cells and calculate the concentration of the viable cells in the suspension. Then calculate the total number of viable cells remaining in the flask.

3. Only round and refractile living cells should be counted and taken into account for the E:T cell ratio calculation. Determine the number of KILR CD16 Effector Cells required for the assay. This number is dependent on the following factors:

   a. The effector cell to target cell ratio (E:T ratio) determined for the target cell.

   b. The total number of desired assay wells containing target cells that will be used for the experiment.

   c. The total number of target cells that were plated in each assay well of the 96-well assay plate.

   d. The density of KILR CD16 Effector Cells in culture flask

A

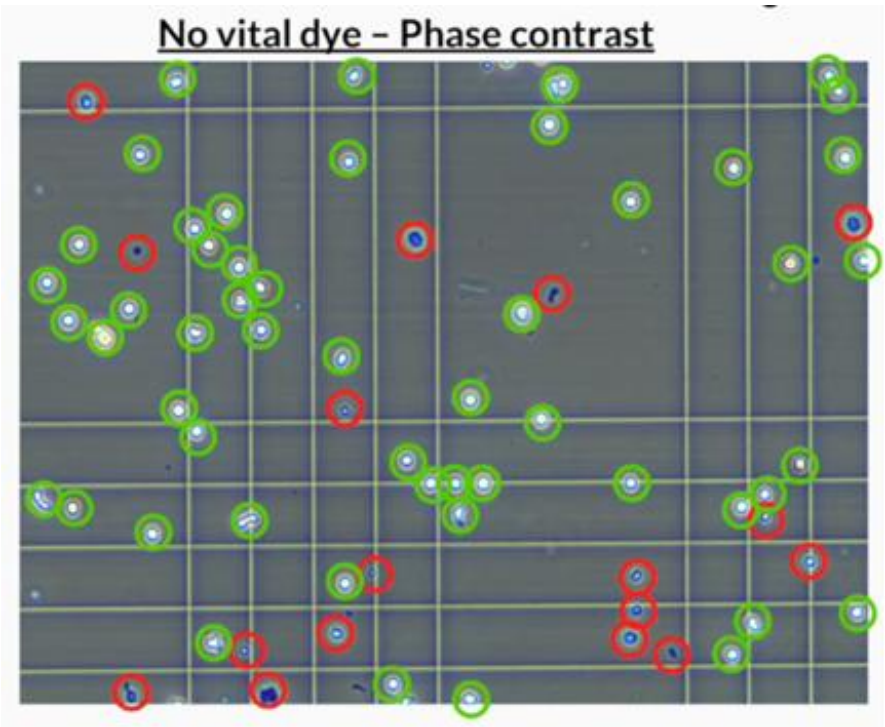
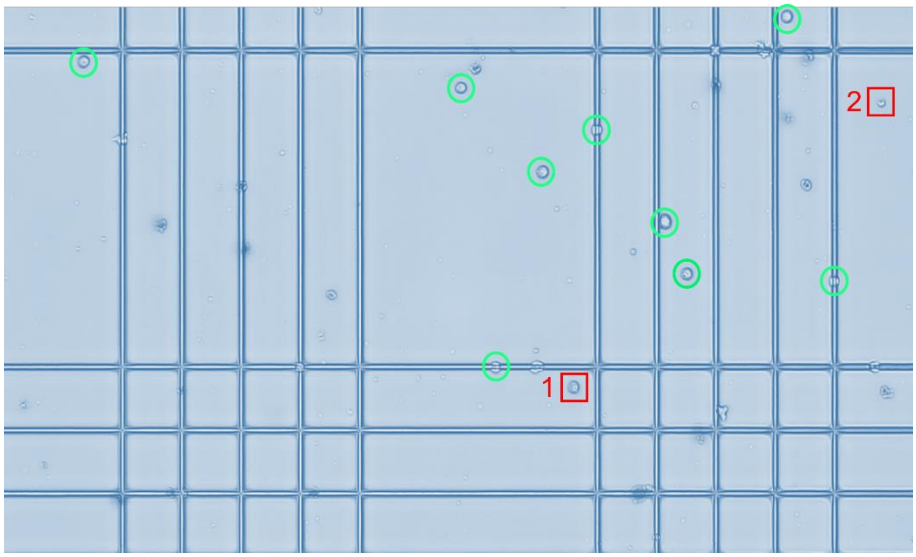


Figure 3A: Representative image of KILR CD16 Effector Cells under phase contrast.

- Viable KILR CD16 Effector Cells (to be counted)
- Cells that should NOT be counted as live cells (Not viable or active as effector cells)

B



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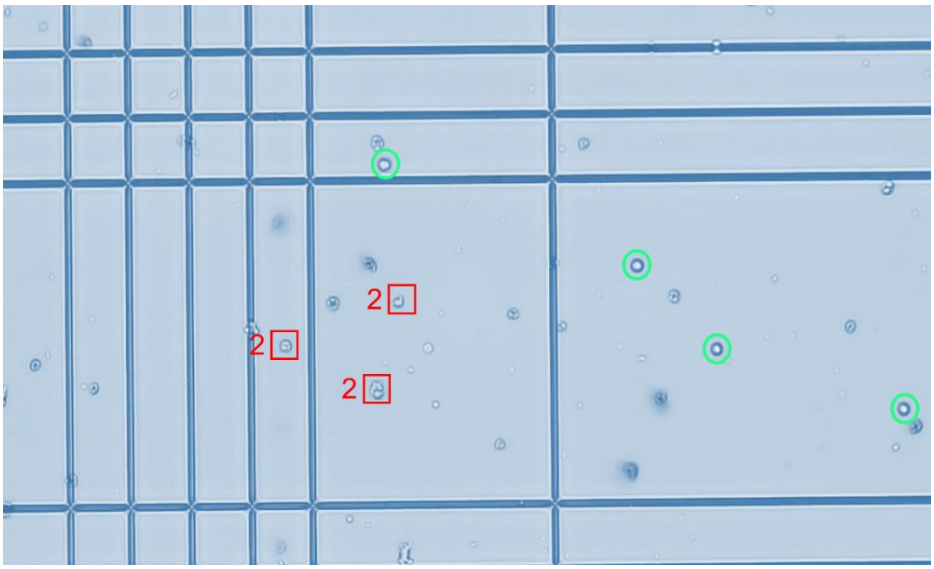


Figure 3B and 3C: Representative images of Trypan Blue-stained KILR CD16 Effector Cells.

The images B and C above highlight examples of viable KILR CD16 Effector Cells vs non-viable "Traps"

- Viable KILR CD16 Effector Cells (to be counted)
- Cells that should NOT be counted as live cells (Not viable or active as effector cells)
  - 1 – Round but not very refractive and just weakly blue
  - 2 – Not blue, but low refractive, with abnormal / irregular cytoplasm aspect

The table below includes an example calculation to use for calculating the volume of KILR CD16 Effector Cells suspension required for the assay.

Determination of Number of KILR CD16 Effector Cells		
Variable	**Example**	**Experiment**
E:T ratio*	10:1	
Number of assay wells	96 wells	
Number of Target Cells per well*	5,000 cells	
Density of Effector Cells in culture	1 x 10^6 cells/mL	
Effector Cells needed per well	10 X 5,000 cells/well = 50,000 cells/well	
Overage Factor**	0.25	
Total Effector Cells needed per assay (accounting for overage factor)	[96 wells + (96 wells X 0.25)] X 50,000 Effector Cells = 6 x 10^6 Effector Cells	
Volume of Effector Cells suspension (including overage)	(6 x 10^6 Effector Cells) / (1 x 10^6 cells/mL) = 6 mL	

* Refer to the KILR cell line-specific datasheet for the recommended E:T ratio (with primary PBMCs) and target cells/well. KILR CD16 Effector Cells are about 2-4 fold more potent than PBMCs.

** It is advisable to factor in at least 25% more effector cells (and therefore a greater volume of effector cells suspension) than would be required for the assay, to account for pipetting losses while transferring cells to the assay plate

4. After counting the cells, calculate the volume needed for assay. Take the required volume of the KILR CD16 Effector Cells suspension (calculated in the table above) out of the culture flask and transfer into a new sterile 15 mL tube (or 50 mL, as appropriate, depending on culture volume).

Note: Centrifuge only the required number of cells for the assay and leave the remaining cells in culture.

5. Centrifuge at 300 X g for 10 minutes to pellet cells, preferably in a refrigerated centrifuge, at 20°C.
6. Decant supernatant or carefully aspirate media with a 10 mL pipette without disturbing cell pellet.
7. Prepare fresh CP39 medium by supplementing the CP39 Reagent with L-glutamine (both provided in the AssayComplete™ Cell Plating 39 Reagent Pack) to a final concentration of 2 mM.
8. Resuspend cell pellet in appropriate volume of pre-warmed CP39 medium to obtain the desired density needed. The table below includes a sample calculation for determining suspension volume for calculating the volume of KILR CD16 Effector Cells suspension required for the assay.

Preparation of KILR CD16 Effector Cells Suspension for Assay		
Variable	**Example**	**Experiment**
Number of Assay Wells	96 wells (plus overage)	
Volume of Effector Cells per Assay Well	50 µL (0.05 mL)	50 µL (0.05 mL)
Number of Effector Cells in Pellet, including overage	6 x 10^6 Effector Cells	
Effector Cells Needed per Well	10 X 5,000 target cells/well = 50,000 Effector Cells/well	
Required Effector Cells Density	50,000 Effector Cells / 0.05 mL = 1 x 10^6 Effector Cells /mL	
Volume Added to Effector Cells Pellet, including overage*	(6 x 10^6 Effector Cells) / (1 x 10^6 Effector Cells /mL) = 6 mL	

9. Gently invert the tube several times to mix. Leave prepared cells in 37°C incubator until ready to add to target cells.

It is recommended to empirically determine the optimal E:T ratio for the desired target cells and application. Based on our observations, KILR CD16 Effector Cells are at least 2-4-fold more potent than primary PBMCs, and roughly equipotent to primary NKs, providing a starting point for experimentation of E:T for ADCC with KILR target cells. The KILR CD16 Effector Cells can also be used in other ADCC assay formats (e.g. Chromium 51 or Europium release assays) that measure cell killing. Replace the PBMCs or other effector cells with the KILR CD16 Effector Cells and determine the optimal E:T ratio for the target cells, then continue to run the assays as per the manufacturer's guidelines.

7.4 Target Cell Preparation and Plating

The following protocols provide guidance for using target cells (e.g. KILR Cytotoxicity Cell assays) in continuous culture format. For detailed protocols for preparing target cells, please refer to the KILR Assay for Suspension Cell Lines User Manual (Doc# 70-338) and KILR Assay for Adherent Cell Lines User Manual (Doc# 70-352). If using the ready-to-use cryopreserved target cells, refer to the User Manual provided in the KILR ADCC Bioassay kits.

Target cells should be prepared on the day of the assay. These cells should have been maintained in cell culture media without any selection antibiotics in a 37°C and 5% CO_2 humidified incubator for 48 hours, before using them in the KILR ADCC assay. This helps in delivering optimal assay performance.

To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

1. After harvesting the cells, gently re-suspend the cell pellet in L-glutamine supplemented CP39 medium and dilute the resuspended cells to the desired concentration (e.g. if 0.6 x 10^6 cells then add 6 mL to obtain 100,000 cells/mL or 5,000 cells/50 µL).

Cell culture media is prepared by mixing the components provided in the cell line-specific AssayComplete™ Cell Culture Kit, and appropriate selection antibiotics. Refer to the Cell Culture Kit's datasheet for instructions on using its components.

2. Return the flask to a tissue culture incubator, and then proceed to Step 3.

3. Transfer 50 µL per well of the cell suspension to a 96-well assay plate. Include wells for the Control and Test Antibody dose-response curves, and wells for the Spontaneous Release Control and Total Lysis Control. Refer to the plate map in the section 7.6 Antibody Addition.

7.5 Antibody Preparation

The following is a procedure for setting up an antibody dose-response dilution.

1. Prepare antibody serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of antibody in Protein Dilution Buffer. The concentration of each dilution should be prepared at 11X of the final screening concentration. For each antibody, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
2. Add 60 µL of Protein Dilution Buffer to dilution wells No. 2 through No. 12. This is enough volume required for over 4 replicates for each concentration. The dilution volume may be adjusted according to the number of replicate wells desired.
   a. Prepare the highest concentration of antibody in Protein Dilution Buffer. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the antibody. Therefore, prepare a working concentration that is 2,750X the expected EC_{50} per well to get an 11X working antibody concentration. For example, for an expected EC_{50} of 10 ng/mL, prepare the highest working concentration at 27,500 ng/mL (i.e. 27.5 µg/mL). This is 11X the screening or final top concentration of 2,500 ng/mL (i.e. 2.5 µg/mL) and the expected EC_{50} will lie near the center of the dose-response curve.
   b. Add 90 µL of the highest concentration of antibody to well No. 1 (see figure: Antibody Serial Dilutions).
   c. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.
   d. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.
3. Repeat this process until well No. 11 is reached. Do not add antibody to well No. 12 since this is the negative control.
4. Set up serial dilutions for any additional antibodies in a similar manner.

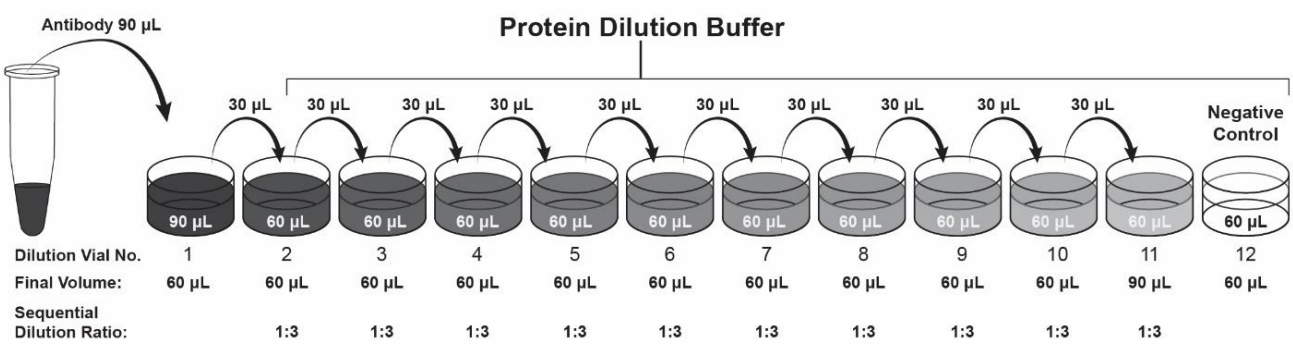


Figure 4: Reference Compound Serial Dilutions
Make eleven 3-fold serial dilutions of the reference compound in a master dilution plate.

7.6 Antibody Addition

The following is a procedure for adding the antibody dilutions to the assay plate to generate a dose-response curve.

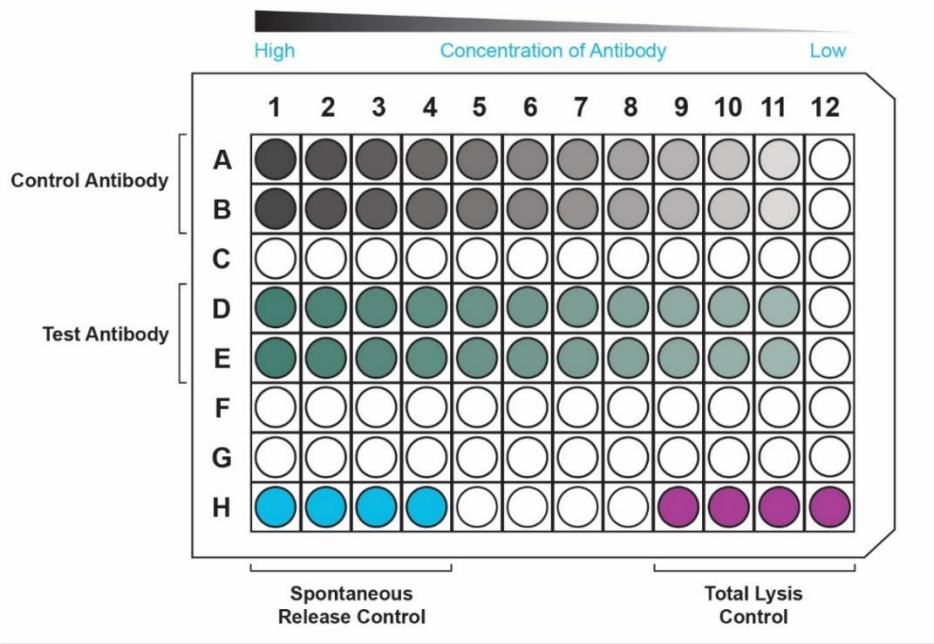


Figure 5 Assay Plate Map
This plate map shows an 11-point dose curve, with 4 data points at each concentration for one reference and seven test samples per plate with a 1:3 serial dilution scheme.

1. Add 10 µL of each 11X antibody serial dilution in duplicate to the designated antibody rows (e.g. Control Antibody in Rows A and B, Test Antibody in Rows D and E as shown in the figure above). Do not add antibody to the “Spontaneous Release Control” and “Total Lysis Control” wells.
2. Gently tap plate on all sides to ensure antibody and cells are mixed well.
3. Incubate assay plate for opsonization at the indicated time and temperature for the cell line (please refer to the cell line-specific datasheet for conditions). For several KILR cell lines, no reference control antibody is available. Datasheets for these cell lines indicate an incubation time and temperature using a non-antibody control reagent (e.g. Triton X-100 or Digitoxin) that provides a reference for potential assay signal. For many of our cell lines validated with an antibody, we opsonize for 30 minutes at 37ºC. For the best results, the optimal incubation time and temperature should be empirically determined.

7.7 Effector Cell Addition

1. Using a multichannel pipette and reagent reservoir, add 50 µL of KILR CD16 Effector Cells (prepared as described in the Section 0 above) into each well of the 96-well assay plate.

   Do not add the effector cells to Spontaneous Release Control wells H1 through H4 or Total Lysis Control wells H9 through H12 as shown in Figure 5 Assay Plate Map.

2. Prepare Spontaneous Release and Total Lysis Controls, as described in the next section 7.8 Preparation of Total Lysis and Spontaneous Release Controls. Incubate assay plate for optimal incubation time for the relevant KILR cell model (and application) as indicated on their respective datasheet) in a 37°C and 5% CO_2 humidified tissue culture incubator. Note: a typical incubation time for ADCC is 3-4 hours.

7.8 Preparation of Total Lysis and Spontaneous Release Controls

The following is a procedure for preparing Total Lysis Controls and Spontaneous Release Controls.

Cells in the Total Lysis Control wells are artificially lysed to release the KILR Reporter Protein into the supernatant to enable detection of a maximum potential signal. The Spontaneous Release Control wells contain untreated cells to enable detection of basal activity levels.

1. Set-up of Total Lysis Control (TLC) in wells H9 through H12 of the tissue culture plate:
   a. Add 48 µL of CP39 medium to the wells.
   b. Add 2 µL of KILR Total Lysis Control solution to these wells. The KILR Total Lysis Control solution is included in the KILR Detection Kit (Cat. No. 97-0001 Series).
2. Set-up of Spontaneous Release Control (SRC) in the wells H1 through H4 of the tissue culture plate:
3. Add 50 µL of CP39 medium to these wells.

We recommend adding Total Lysis Control reagent to the Total Lysis Control wells at the same time as effector cells are added to the sample wells, especially for assays requiring long incubation times (>3 hours).

7.9 Addition of Detection Reagent

At this point, the opsonization and effector cell treatment steps have been completed. The following section contains procedures for adding the KILR Detection Reagent and reading the assay plate on a luminescence reader. Refer to the user manual for the KILR Detection Kit for complete information on the detection reagents.

1. Prepare working KILR detection solution in a tube or reservoir by mixing 4-parts of KILR Detection Reagent 1, 1-part of KILR Detection Reagent 2 and 1-part of KILR Detection Reagent 3 (including excess volume for accurate pipetting). Mix reagents by gently inverting the tube twice or swirling the reagent reservoir.
2. Refer to the KILR Detection Kit User Manual (Doc# 70-337) for more complete information on the detection reagents.

Working KILR Detection Solution		
Components	**Volume Ratio**	**Volume per Plate (mL)**
KILR Detection Reagent 1	4	8
KILR Detection Reagent 2	1	2
KILR Detection Reagent 3	1	2
Total Volume		**12**

3. Add 100 µL of working KILR detection solution to wells containing cells in the assay plate. **Do Not mix solution by pipetting up and down or use an orbital shaker to shake the assay plate.**

4. Place lid back on plate and incubate assay plate for at least 1 hour at room temperature in the dark.
5. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. In general, the signal continues to increase.

   The plate can be incubated overnight (16 hours), and the signal measured on the next day. This is useful as a longer incubation with detection reagent can sometimes improve assay window and sensitivity in cell models with lower receptor density. Once an optimal read time has been established, continue to use this incubation time to maintain consistency between assays. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturers may include a cut-off filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.
6. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.).

7.10 Calculation of % ADCC

The following are instructions for calculating % ADCC values. The resulting calculated % ADCC value for each data point (Antibody Value) will then be plotted.

1. Calculate the mean of the RLU values generated by the four replicate Total Lysis Control wells (wells H9 through H12). The result is the Mean TLC Value.
2. Calculate the mean of the RLU values generated by the four replicate Spontaneous Release Control wells (wells H1 through H4). The result is the Mean SRC Value.
3. Calculate the % ADCC value for each data point using the following formula:

$$\%\ ADCC = \frac{(\text{Antibody Value} - \text{Mean SRC Value})}{(\text{Mean TLC Value} - \text{Mean SRC Value})}\ X\ 100$$

4. Plot the resulting %ADCC for each data point versus each concentration of antibody using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.).

   Example Values and Calculations for % ADCC

   The following is an example of % ADCC calculation for data from a single concentration of antibody. Repeat the Steps 3 and 4 in calculations for % ADCC Section for each antibody concentration. The following are hypothetical values (RLU) created for this example.

   SRC Values (4 replicates): 210; 220; 208; 216

   Antibody Value (1 data point): 65,000

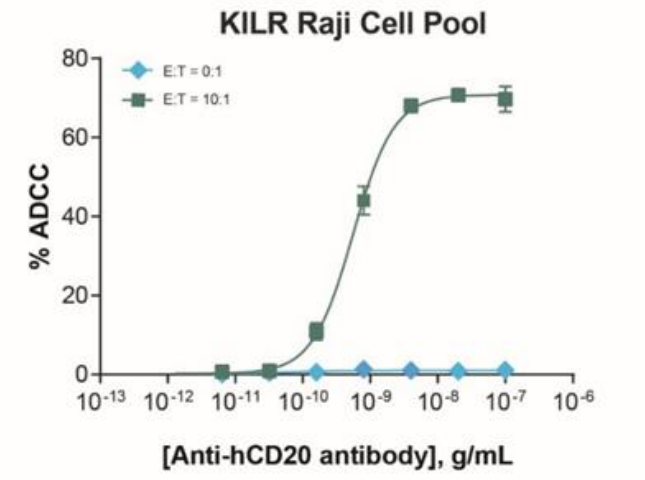
   TLC Values (4 replicates): 307,000; 300,000; 310,000; 305,000

Example of a % ADCC Calculation	
Step 1: Mean SRC Value (RLU)	(210 + 220 + 208 + 216) / 4 = 213.5
Step 2: Mean TLC Value (RLU)	(307,000 + 300,000 + 310,000 + 305,000) / 4 = 305,500
Step 3: Antibody Value (RLU)	65,000
Step 4: Final % ADCC calculation	100 X (65,000 – 213.5) / (305,287- 213.5) = 21%
Step 5: Repeat Steps 3 and 4 for the next data point	
Step 6: Plot all % ADCC values versus each antibody concentration	

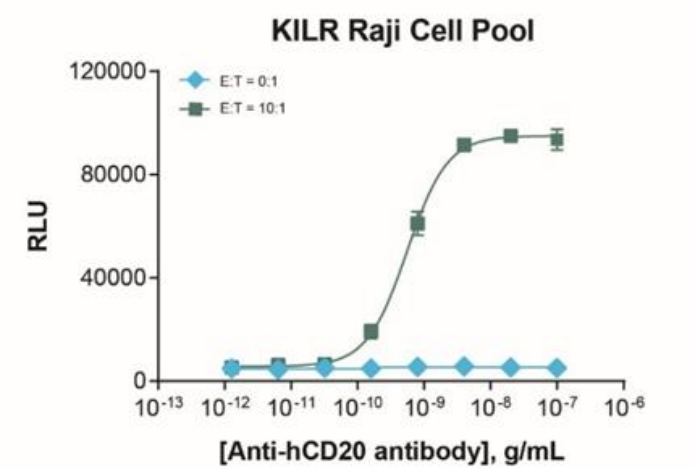
8. Typical Results

Shown below are an example of typical results from an ADCC assay using KILR CD16 Effector Cells for anti-CD20 antibody-mediated direct killing of KILR Raji target cells.

(A)



(B)



(C)

Effector to Target Cells (E:T) ratio	EC_{50} (ng/mL)	Max Lysis (%)	S/B
0:1	–	0.34	–
10:1	0.81	71	16.9

Figure 6: Typical results for anti-CD20 antibody-mediated ADCC
KILR Raji cells stably expressing the ePL-labeled housekeeping protein were washed and resuspended in CP39 medium, opsonized with a titration of anti-CD20 antibody, then incubated with KILR CD16 Effector cells. An E:T ratio of 10:1 or an equivalent volume of vehicle E:T ratio of 0:1 was used. After 4 hours, KILR Detection Reagent was added to the medium and incubated for one hour prior to detection of luminescence signal on a luminescence plate reader (Envision, PE). (A) %ADCC; (B) Total Lysis, and (C) Summary of ADCC results

9. Frequently Asked Questions

What types of assays can use the KILR Target Cells and CD16 Effector Cells?

The KILR stable cell line/pool can be used for a variety of cytotoxicity applications, including ADCC and ADCP assays. The KILR CD16 Effector Cells can be used for the ADCC and TCR applications.

At what passage number do you freeze down the KILR CD16 Effector Cells?

The KILR CD16 Effector Cells are single donor-derived primary cells and not an immortalized cell line. These cells are frozen at single passage after isolation and purification.

Do the regulatory authorities (FDA, EMA, etc.) accept the KILR assays?

The KILR assay is a simple way to measure direct target cell death. If the drug molecule causes target cell death, this assay will be able to measure it. KILR Assays have been implemented in drug development programs and KILR CD16 Effectors have been implemented in lot- release of approved biologics. The results have been published in the scientific literature. In addition, the KILR assay has the advantage of reading the same assay endpoint but with greater simplicity, specificity, and sensitivity than alternative assay formats.

We use another non-radioactive assay format, which is simple, and cost-effective. Why should we use a new assay format?

KILR ADCC platform has the benefit of being robust, highly sensitive and measures only death of the target cells, and not the effector cells. Other assay formats usually cannot differentiate between effector cell death and target cell death or use a dye-based method to label their target cells, which can lead to elevated background in the assay due to leakiness of the dye. In addition, our assay will be able to simplify your assay protocol and increase the speed of data generation, resulting in greater efficiency and cost savings for your lab.

What is the sensitivity of the KILR assay? What is the minimal number of target cells needed per well?

We have observed that the absolute % lysis induced by ADCC varies greatly between cell lines and is likely dependent on the target and the cell line. For most cell lines, we have tested a minimum of 1,500-2,000/well in a 96-well format. We robustly detect as low as 5% cell death (% lysis), which would equate to the signal from 75-100 cells. To increase maximum % lysis, you can optimize several parameters such as the number of target cells, the number of effector cells and increasing the incubation time with the effector cells or the detection reagent.

What is the serum tolerance of the KILR assay for use in ADCC?

In general, serum raises the background in the ADCC assay, which is why our current plating medium is completely serum–free. However, we have tested several of our cell lines in the presence of serum (typically low IgG serum, in the 2-5% range), and can produce good results. Increasing serum does tend to shrink the assay window, but you will still typically get a response.

Can you recommend isotype controls for an ADCC assay?

The appropriate isotype control will depend on the specific constant region used for the antibody being tested. Typically for antibodies that mediate ADCC it will be either human IgG1 or IgG3. An appropriate control will have a variable region from an unrelated antigen, e.g. a receptor not expressed on the surface of your target cells.

For questions on using this product, please contact Technical Support at discoverx.com/support/

Document Revision History

Revision Number	Date Released	Revision Details
7	December 2023	Protocol updates • Section 7.2: Thawing KILR CD16 Effector Cells • Section 7.3: Preparation of KILR CD16 Effector Cells for ADCC Assay
6	June 2022	Protocol updates in • Section 7.3: Preparation of KILR CD16 Effector Cells for ADCC Assay
5	February 2022	Protocol update: • Section Thawing KILR CD16 Effector Cells (Page 8) • Section Preparation of KILR CD16 Effector Cells for ADCC Assay (Page 9)
4	January 2019	Protocol update: • Clarified cell vials storage under Vapor phase of Liquid Nitrogen • Updated expected cells recovery to 80%
3	May 2018	Protocol Update • Updated IL-2 final concentration to 600 IU/mL • Updated media preparation example • Update document branding
2	August 2017	Protocol Update • Incubation time post-thaw updated to 7 days prior to using the cells to run the ADCC assay. • Updated example calculations for “Determination of Number of KILR CD16 Effector Cells” and “Preparation of KILR CD16 Effector Cells Suspension for Assay” and graphs in “Typical Results” section for E:T ratio of 10:1
1	May 2017	Protocol Update • Day 1: Updated thawing time & adjustment of cell density for incubation
0	April 2017	New Document

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