User Manual

KILR® Raji ADCC Bioassay Kit

For Direct Measurement of Antibody-mediated Cell Cytotoxicity in a Co-culture Model

For Bioassay Kits:

97-1012Y026-00169: 2-Plate Kit

97-1012Y026-00170: 10-Plate Kit

Document Number 70-438 Revision 0

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

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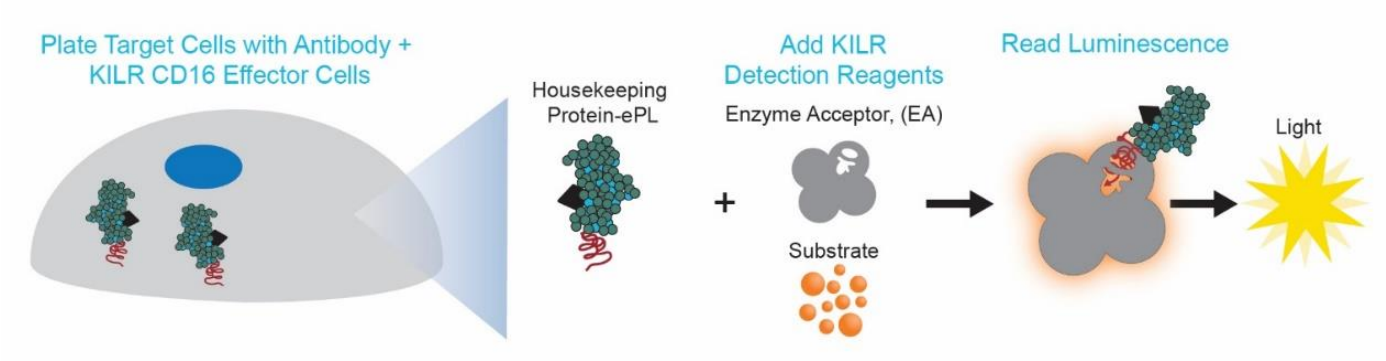
1 Overview

The KILR Raji ADCC Bioassay Kit is a simple, robust, non-radioactive, dye free cytotoxicity assay to specifically measure direct target cell death in a co-culture. The bioassay kit contains all the materials needed to run the ADCC assay (except effector cells) including ready-to-use cryopreserved target cells, assay plates and optimized reagents. The ready-to-use cryopreserved 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 KILR assay cell model expressing a KILR Reporter Protein, a housekeeping protein tagged with Enhanced ProLabel (ePL), a β-galactosidase (β-gal) reporter fragment. Cell death leads to rupture of the cell membrane, which results in the release of the KILR Reporter Protein into the medium. Addition of the KILR detection reagent, containing the complementing β-gal reporter fragment, Enzyme Acceptor (EA), results in complementation of the two enzyme fragments (EA and ePL; see Figure 1). The resulting active enzyme hydrolyzes a chemiluminescent substrate to generate a light signal. Very low signal is observed from healthy, intact cells, as the KILR Reporter Protein remains sequestered within the cells and cannot leak out of intact cell membranes. The signal measured from the well is directly proportional to the number of dead KILR target cells that have a compromised cell membrane, because only the target cells express the β-gal reporter fragment. The KILR platform measures direct target cell death hence no secondary bridging assay needed.



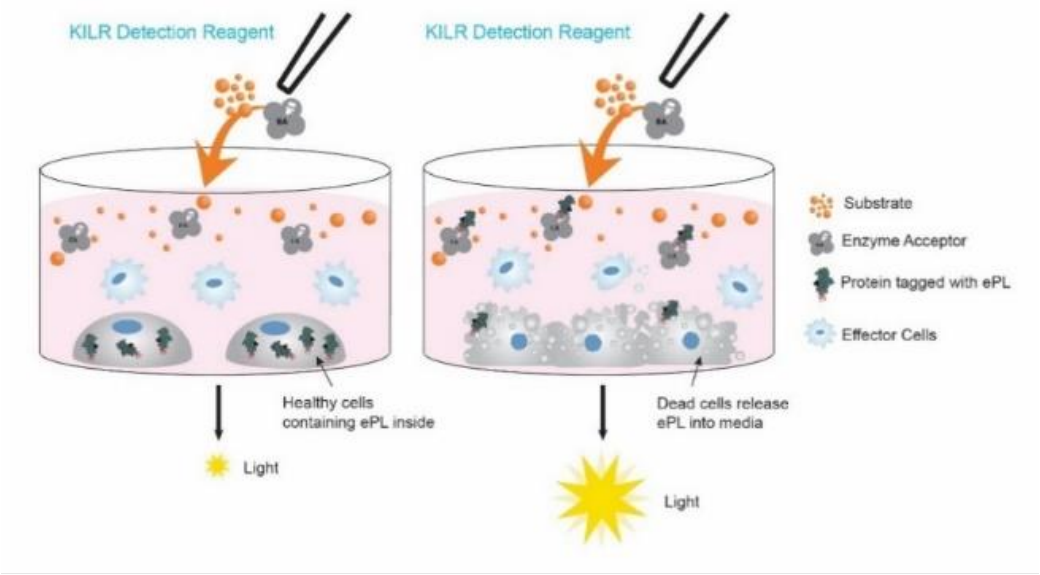


Figure 1: KILR Technology Principle

Target cells expressing the receptor antigen are engineered to stably express a housekeeping protein that is tagged with enhanced ProLabel® (ePL), a β-gal reporter fragment. When the stable target cells are used in a cytotoxicity assay, and target cell membranes are compromised due to cell death, the target cells will release the tagged protein into the media. We can detect this KILR Reporter Protein in the media by the addition of detection reagents containing the enzyme acceptor (EA) fragment of the β-gal reporter. 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.

3 Application: ADCC

In the KILR Raji ADCC Bioassay, ready-to-use KILR Raji target cells are opsonized with a therapeutic antibody targeting the relevant antigen endogenously expressed in the target cells, then co-incubated with immune effector cells (e.g. KILR CD16 Effector Cells). When the opsonized target Raji cells are used in a cytotoxicity assay, and its membrane is compromised due to Fc effector function during ADCC, it releases the tagged reporter protein into the media. We can detect this reporter protein by the addition of KILR detection reagents containing the enzyme acceptor (EA) fragment of the β-gal enzyme. The ePL, a β-gal enzyme fragment and Enzyme Acceptor (EA) complement through protein-protein interactions, leading to the formation of the active β-gal enzyme, which hydrolyzes the substrate to give a chemiluminescent output, detected on any benchtop luminescence reader. The KILR Raji ADCC Bioassay Kit measures target cell death using the gain of signal based on Enzyme Fragment complementation.

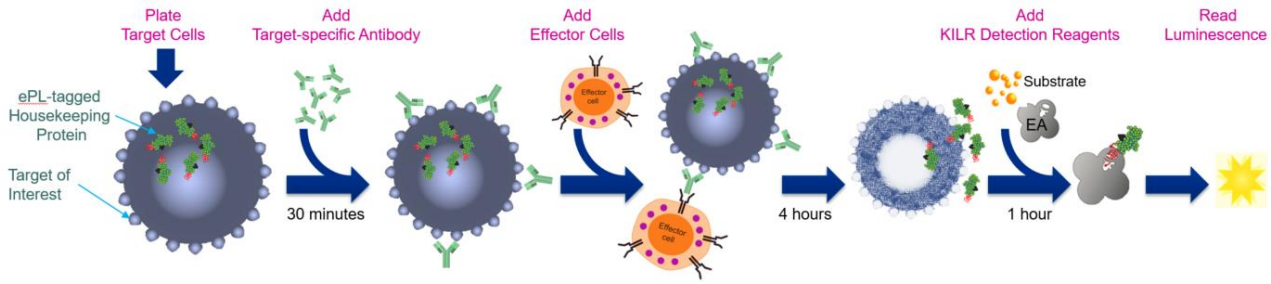


Figure 2 KILR ADCC Assay Principle

The KILR Raji ADCC bioassay has been developed to specifically quantify target cell death mediated by ADCC. When the stable target Raji cells are incubated with appropriate immune effector cells and the test antibody, effector-mediated killing releases the tagged protein into the media. We can detect this KILR Reporter Protein in the media by the addition of detection reagent containing the EA fragment

4 Materials Provided

List of Components	97-1012Y026-00169 (2 plate Kit)	97-1012Y026-00170 (10 plate Kit)
KILR Raji 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
KILR Detection Kit		
Detection Reagent 1 (Bottle)	1 x 17 mL	1 x 85 mL
Detection Reagent 2 (Bottle)	1 x 5 mL	1 x 25 mL
Detection Reagent 3 (Bottle)	1 x 5 mL	1 x 25 mL
Total Lysis Control (Bottle)	1 x 0.5 mL	1 x 2.5 mL
96-Well White, Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

5 Storage Conditions

KILR Raji 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.

KILR Detection Kit for Bioassays

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
KILR CD16 Effector Cells	97-0007
AssayComplete™ Cell Culture Kit-117	92-3117
Recombinant Human IL-2, Cell Culture Grade	92-1331
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 Raji Bioassay Cells must arrive in a frozen state on dry ice.

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

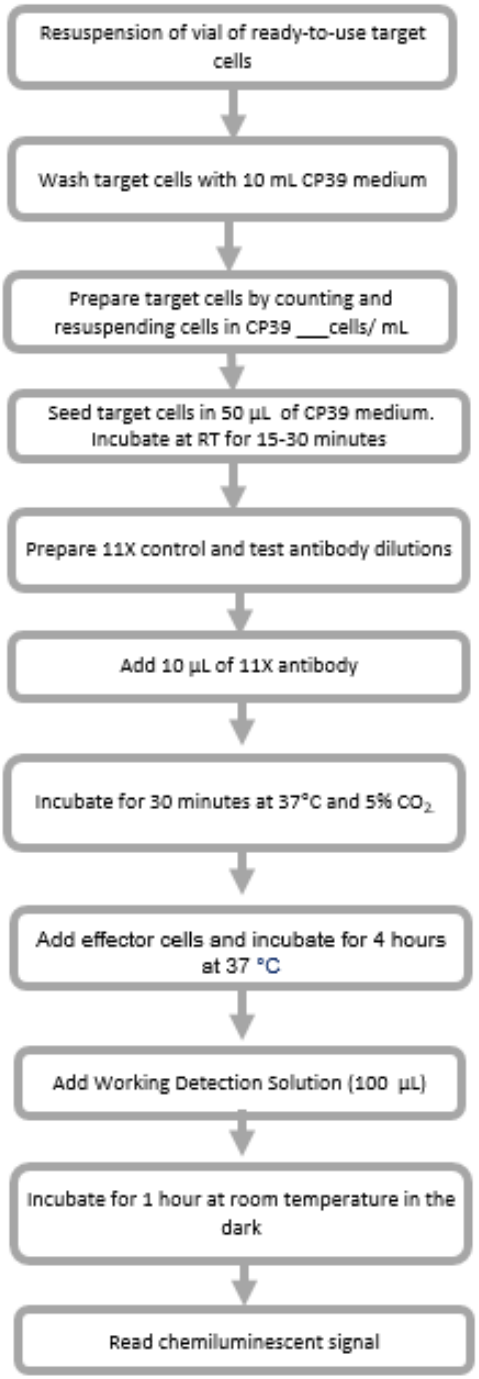
2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, place vials in the vapor phase of liquid nitrogen storage.

   
   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: In a 96-well plate, 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 in a 96-well format. The following detailed protocol is specific to detecting antibody-dependent cell-mediated cytotoxicity (ADCC). Target cells are opsonized with an ADCC-inducing antibody then co-incubated with effector cells in a 96-well tissue culture plate.

Note: Effector cell preparation must be initiated (i.e. thawing of primary PBMCs) the day before the opsonization step, as described in Supplemental Information, Effector Cell Preparation section. If using KILR® CD16 effector cells, thaw effector cells 7 days prior to setting up the ADCC assay. Refer to this user manual's Supplemental section 11.2 Effector Cell Preparation: KILR CD16 Effector Cells or the User Manual for KILR CD16 Effector Cells (Document# 70-388) for detailed handling instructions.

Note: Place the KILR Detection Kit at 4°C the day prior to running the assay.

9.1 Target Cell Preparation and Plating

The following protocol is for thawing and plating cryopreserved KILR Raji bioassay cells from cryovials.

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 White, Flat-Bottom, Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
   - A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, to be used as the master dilution plate
2. Remove the cryovial from the liquid nitrogen vapor 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 2mM.
   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 ADCC 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 Raji 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 10 mL of fresh CP39 medium.
15. Mix cells suspension by gently pipeting up and down 2-3 times with a sterile 10 mL pipet.
16. Count cells and determine viability. For the purpose of determining the concentration of cells in the suspension:
    a. Set aside a small fraction (0.5 mL or less) 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 1 x 10^5 viable cells / mL
17. Mix KILR Raji bioassay cell suspension (from Step 16d) by gently inverting the tube 2-3 times, without creating any froth. Immediately pour the cell suspension into a sterile 25 mL reagent reservoir.
18. Using a multichannel pipette, plate 50 µL/well (5,000 cells/well) of KILR Raji Bioassay cells into appropriate wells of a white, opaque bottom 96-well assay plate.
19. Incubate plate at room temperature, in the bioasafety cabinet, for 15 minutes to minimize the potential for edge effects.

Proceed with the KILR ADCC assay protocol below.

Note: If test sample preparation will take longer than 30 minutes, assay plate should be placed in humidified tissue culture incubator at 37°C, 5% CO_2, for up to 2 hours, before proceeding with assay.

9.2 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 an 11-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 dilution plate (for example: Row A) as control or label individual polypropylene tubes No. 1 through No. 12.
   b. Prepare a working stock of Rituximab at 1100 ng/mL, which is 11X the desired final top concentration of 100 ng/mL, in CP39.
   c. Add 45 µL of CP39 to wells 2 to 12 of Row A in master dilution plate. Do not add CP39 medium media to well 1.
   d. With a clean tip add 45 µL of the 1100 ng/mL stock of Rituximab to Well 1.
   e. With a clean tip, transfer 15 µL of antibody from Well 1 into Well 2 and mix by gently pipetting up/down 2-3 times.
   f. Transfer 15 µL from Well 2 into Well 3 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. 11 is reached. Do not add antibody to Well No. 12 since this is the negative control well.

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

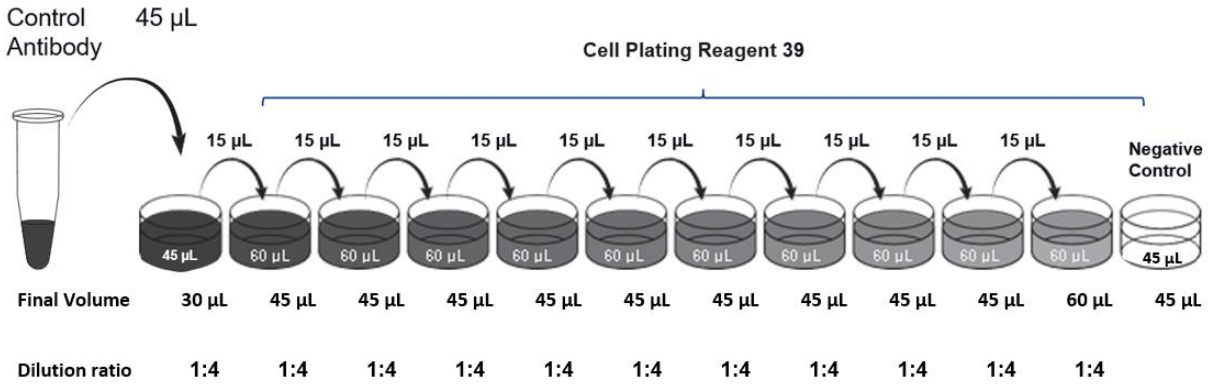


Figure 3 Antibody Serial Dilutions

Make eleven serial dilutions of 1:4 for each antibody in a separate master dilution plate

9.3 Antibody Addition

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

This plate map shows three interdigitated 11-point dose curves, with two replicates per dose point, for two test samples and reference sample tested using the same 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 D, Test Antibody 1 in Rows B and E, and Test Antibody 3 in Rows C and F as shown in the Assay Plate Map example below).
   Warning: Do not add antibody to the Spontaneous Release Control or to the Total Lysis Control. Spontaneous release control and total lysis control reagents should be added to the respective wells after the effector cell addition step.

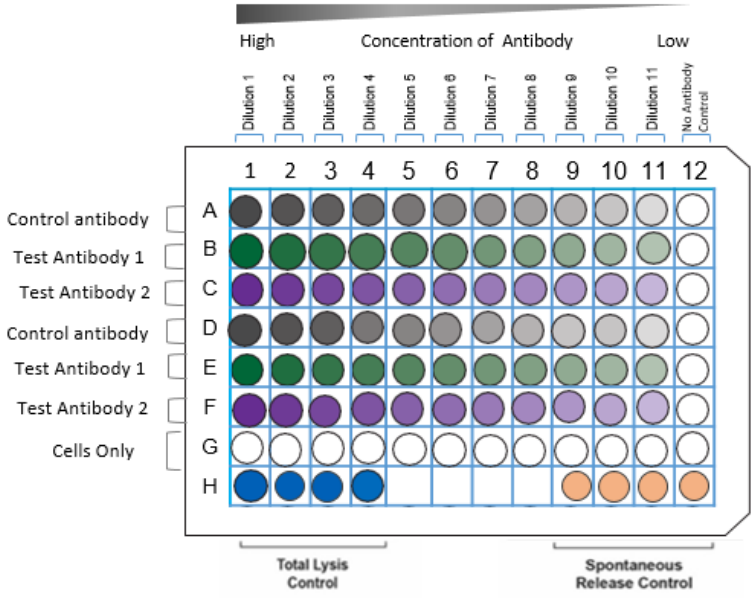


Figure 4 Representative Assay Plate Map

2. Gently mix the contents by slowly moving the plate back and forth 3-4 times in a criss-cross pattern on the surface of the tissue culture hood.
3. Incubate assay plate for opsonization at 30 minutes at 37ºC, 5% CO_2.

9.4 Effector Cell Preparation

The following is a procedure for preparing the effector cells for the ADCC assay.

Note: It is recommended to empirically establish the optimal effector to target cell (E:T) ratio for each effector cell type. Killing activity of effector cells depends on type, preparation, purity, and donor variability of effector cells. For peripheral blood mononuclear cells (PBMCs), we recommend an E:T ratio of 25:1 for optimal results using KILR Raji Bioassay Cells.

1. Thaw the effector cells according to manufacturer's protocol.

If using primary human PBMCs

The PBMCs are prepared one day prior to running the assay. Refer to the Supplementary information section 11.1 for the protocol.

   a. Thaw cryopreserved primary PBMCs and resuspend in pre-warmed CP39 medium as per Supplemental Instructions section below. Allow cells to recover in CP39 medium overnight in a humidified incubator at 37°C, 5% CO_2

b. On day of assay, count number of viable cells, pellet at 300 x g for 10 minutes and resuspend cell pellet in appropriate volume of CP39 medium to achieve desired density (e.g. 2.5 x 10^6 viable cells/mL for an E:T of 25:1).

If using KILR CD16 Effector Cells

The KILR effector Cells are thawed 7 days prior to running the assay. Refer to the Supplementary information section 11.2 for the protocol.

a. Thaw and rest effector cells for at least 7 days.

b. Follow the recommended protocol in supplemental section 11.2 on KILR CD16 Effector Cell Preparation in this user Manual or follow KILR CD16 Effector Cell User Manual Doc # 70-388 for instructions on Effector Cell Preparation.

2. On day of 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.

3. Determine the density of viable cells.

   a. **For best results, it is strongly recommended to use manual counting rather than automated counting devices. Automated cell counters are unable to discern the population of low trypan blue staining cells that should not be counted as live cells, as well as are often prone to miscounting clumped cells, leading to unreliable determination of viable cell numbers.**

   b. Take 50 µL or less of the suspended cell 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 (typically 10 µL of cell suspension) or another cell counting device.

5. Count the viable cells and prepare effector cells in CP39 medium at 1 x 10^6 viable cells/mL, reflecting the recommended E:T of 10:1 for KILR Raji Bioassay cells.

9.5 Effector Cell Addition

At this point, the opsonization step is complete. The following is a procedure for adding the prepared effector cells to the target cells in the assay plate.

1. Using a multichannel pipet and a reagent reservoir, add 50 µL of effector cells (prepared in Step 9.4) into each row of the 96-well assay plate containing opsonized target cells. **Do not** pipet up and down to mix.
2. Do not add effector cells to Spontaneous Release Control wells H9 through H12 and Total Lysis Control wells H1 through H4 as shown in the representative assay plate map.
3. Gently mix the contents by slowly moving the plate back and forth 3-4 times in a criss-cross pattern on the surface of the tissue culture hood.
4. Incubate effector cells with the opsonized KILR Bioassay target cells for 4 hours at 37°C, 5% CO_2 in a humidified tissue culture incubator.

Note: At the time of addition of effector cells to opsonized target cells, add reagents to control wells as described below. 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 (≥4 hours)

9.6 Preparation of Total Lysis and Spontaneous Release Controls

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

Cells in the **Total Lysis Control** wells are chemically 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.

Note: Refer to the Assay plate map for the recommended location of the control wells. Since Total Lysis Control wells will produce high signal, it is recommended to place these wells away from wells expected to produce low signal (for example: separated from wells containing low antibody concentrations) to prevent potential for signal bleed over.

1. For Spontaneous Release Controls (wells H9 through H12), add 50 µL of CP39 reagent.
2. For Total Lysis Control Wells (wells H1 through H4), add 47.5 µL of CP39 reagent and 2.5 µL of KILR Total Lysis Control Reagent (included in the KILR Detection kit; Cat. No. 97-0001 Series) **only** to the Total Lysis Control wells.

9.7 Addition of Detection Reagent

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

Working KILR Detection Solution Volume Guide		
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**

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 (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 KILR Detection Kit for more complete information on the detection reagents.
2. Using a multichannel pipet, add 100 µL of working KILR detection solution to 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 criss-cross 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 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager.
   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 plotting graph 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 % ADCC as described below:
   a. 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.
   b. 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.
   c. Calculate the % ADCC value for each data point using the following formula:

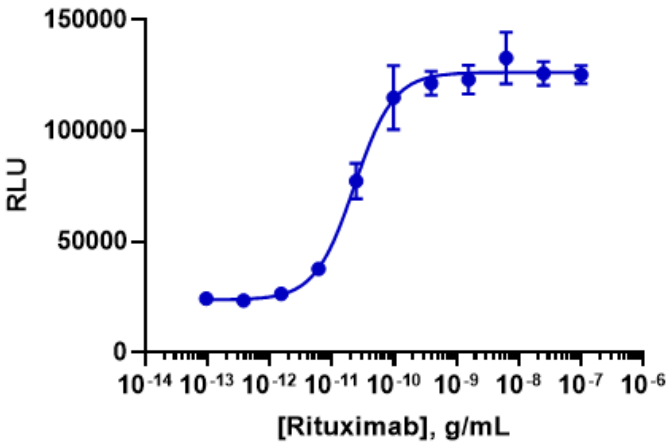
$$\%ADCC = \frac{(Test\ antibody\ value - mean\ spontaneous\ release\ value)}{(Mean\ total\ lysis - mean\ spontaneous\ release)} x100$$

10 Typical Results

The following graph is an example of a typical dose-response curve for the KILR Raji ADCC Bioassay generated using the protocol outlined in this manual. The data shows a potent, dose-dependent increase in target cell death when treated with Antibody drug and effector cells.

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

A



B

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

Figure 5: Typical Results

Representative **A**, dose-response curve and **B**, the EC_{50}, HillSlope and assay window for KILR Raji ADCC Bioassay, as measured in this bioassay.

11 Supplemental Information

11.1 Effector Cell Preparation: Isolated PBMCs

The following sections include two separate protocols for preparing effector cells: (1) PBMCs (peripheral blood mononuclear cells) and (2) KILR CD16 Effector Cells

11.1.1 Preparation of PBMCs Day 1: Thawing PBMCs

The following is a procedure for preparing rested PBMCs from frozen stocks and should be initiated the day before the opsonization step of the ADCC assay

1. Pre-warm AssayComplete® Cell Plating 39 Reagent (cell plating 39 medium, or CP39), in a clean 37°C water bath, for at least 20 minutes. Add fresh L-glutamine to the CP39 Reagent to a final concentration of 2 mM. Use only L-glutamine supplemented in CP39 medium when using the following protocol.
2. Remove the cryovial(s) of PBMCs from -80°C or liquid nitrogen vapor storage and immediately place them on dry ice prior to thawing.

   Safety Warning: When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate.
3. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellet is almost completely thawed.
4. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.
5. With a 1 mL filter pipette, carefully transfer the thawed cells to a sterile 15 mL conical tube.
6. Slowly add 10 mL pre-warmed CP39 medium to the conical tube.
7. Add an additional 2 mL of CP39 medium to the vial and rinse to collect the remaining cells. Transfer the additional volume to the 15 mL conical tube.
8. For the purpose of determining the total number of cells in the 15 mL tube:
   a. Set aside a small fraction (0.5 mL or less) 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 viable cells and calculate the concentration of viable cells in the suspension.
   d. Calculate the total number of viable cells remaining in the 15 mL conical tube.
9. Centrifuge at 300 X g for 15 minutes at room temperature to pellet cells.
10. Using a sterile pipet, to carefully aspirate the supernatant and discard.

PBMC's must be suspended in CP39 medium to a density of 1 x 10^6 cells/mL

11. Add the required volume of CP39 medium to a T25 or T75 flask. The table below provides recommendations for selection of appropriate flask size:

Total PBMCs per Vial	Suggested Flask Size
< 10 x 10^6	T25
≥ 10 x 10^6	T75

12. Transfer 10 mL of CP39 medium from the flask to the 15 mL tube containing the pellet of PBMCs.
13. Using the pipette, break up the pellet of PBMCs by gently pipetting up and down.
14. Transfer the entire volume of the PBMC suspension back to the tissue culture flask.
15. Incubate overnight at 37°C and 5% CO_2 with the flask standing on end. This resting period allows the PBMCs to

recover functionality, and it is essential for allowing cells that may appear to be alive after initial thaw to die (so they don't affect the final cell count).

In general, 16-24 hours in culture is a sufficient recovery period; PBMCs can be left in culture for up to 3 days with minimal loss of killing capability. Monocytes in PBMCs will attach to the plastic in about 2-3 hours when incubated at 37°C. Longer incubation times will result in firm attachment. As lymphocytes are not adherent cell types, they will mostly be in suspension, and can be easily removed by mildly flushing the wells with medium.

11.1.2 Preparation of PBMCs Day 2: Final Preparation of PBMCs

The following is a procedure for the final preparation of the effector PBMCs on the day of the ADCC assay.

1. On the day of the ADCC assay, determine density of viable cells,
    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    b. Stain cells with trypan blue.
    c. Transfer an appropriate portion of this fraction of stained cells to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.
    d. Count the viable cells and calculate the concentration of cells in the suspension. Then calculate the total number of viable cells remaining in the flask.
2. Determine the number of PBMCs required for the assay. This number is dependent on the following factors:
    a. Effector cell to target cell ratio (E:T ratio) determined for the target cell
    b. Total number of desired assay wells with target cells that will be used for the experiment.
    c. Total number of target cells that were plated in each assay well of the 96-well assay plate.
    d. Density of PBMCs in culture flask.
    e. The table below is an example calculation, as well as a column to use for calculating the volume of PBMC suspension required for running the assay:

Determination of Number of PBMCs		
Variable	**Example**	**Experiment**
E:T ratio*	25:1	
Number of assay wells	96 wells	
Number of target cells per well	5,000 cells	
Density of PBMCs in culture	1 x 10^6/mL	
PBMCs Needed per well	25 x 5,000 cells/well = 125,000 cells/well	
Overage Factor**	0.25	
Total PBMCs needed per assay (accounting for overage factor)	[96 wells + (96 wells x 0.25)] x 125,000 cells = 1.5 x 10^7 cells	
Volume of PBMCs suspension (including overage)	(1.5 x 10^7 cells) / (1 x 10^6/mL) = 1.2 mL	

*Typically an E:T of 25:1 is adequate for PBMCs with the KILR Raji Bioassay cells, but we recommend determining empirically for each donor.

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

3. Transfer the required volume of PBMC suspension (calculated in the table above) from the tissue culture flask to a sterile 50 mL conical tube.
4. To wash cells, fill the conical tube to 50 mL with pre-warmed CP39 medium. Gently invert the tube several times to mix.
5. Centrifuge at 300 X g for 15 minutes at room temperature to pellet cells.
6. Decant supernatant or carefully remove media with a 10 mL pipette without disturbing cell pellet.
7. Resuspend cell pellet in pre-warmed CP39 medium. Suspend the PBMCs in a volume that produces the desired PBMC density for the assay. Gently invert the tube several times to mix. The table below includes a sample calculation for determining suspension volume, and space to use for calculating the volume of PBMC suspension required for the assay

Preparation of PBMC Suspension for Assay		
Variable	**Example**	**Experiment**
Number of assay wells	96 wells (plus overage)	
Volume of PBMCs per assay well	50 µL (0.05 mL)	50 µL (0.05 mL)
Number of PBMCs in pellet, including overage	1.5×10^7	
PBMCs needed per well	25 x 5,000 cells/well = 125,000 cells/well	
Required PBMC density	125,000 cells/0.05 mL = 2.5×10^6 cells/mL	
Volume added to PBMC pellet, including overage*	(1.5×10^7 cells) / (1×10^6 /mL) = 1.2 mL	

*CP39 medium is added to the suspension to reach the required final volume.

8. Leave cells in a 37ºC incubator until needed for the Effector Cell Addition in Section 9.5.

11.2 Effector Cell Preparation: KILR CD16 Effector Cells

11.2.1 Preparation of KILR CD16 Effector Cells Day 1: Thawing Cells

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

1. Prepare fresh cell culture medium by supplementing the Cell Culture Reagent with L-glutamine to a final concentration of 2 mM. Both reagents are provided in the AssayComplete™ Cell Culture Kit 117. For example, for a final cell culture medium volume of 50 mL, add 0.5 mL of 200 mM (100X) L-glutamine to 50 mL of Cell Culture Reagent. Store unused medium (minus L-glutamine) at 4°C for up to 4 weeks.
2. Pre-warm prepared cell culture medium and aliquot 19 mL of medium into a 50 mL conical tube. 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 cell viability and recovery time will be seriously compromised.
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 worn at all times 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 cryovial of effector cells rapidly with gentle manual agitation in a 37°C water bath, immersing the vial as close as possible to the cap edge, until only a small piece of ice is visible (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 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. Homogenize the cell suspension by gently pipetting up and down twice and transfer the entire volume of the thawed cells into a T75 flask.

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 flask.

8. Add IL-2 to a final concentration of 600 IU/mL to each T75 flask

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

9. Incubate the flask(s) by standing it 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.
10. After 24 hours, 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, carefully pipet the cell suspension up and down several times using a 10 mL disposable pipet to dissociate cell clumps. Avoid creating any froth or foam during this step. Count viable cells in phase contrast and add the necessary amount of fresh culture medium (supplemented with IL-2 to a final concentration of 600 IU/mL) to achieve a density of $0.8x10^6$ viable cells/mL.
11. Determine the total number of viable cells
    a. Carefully pipet the cell suspension up and down several 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 dispense onto a hemocytometer or Malassez slide (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.
    c. Count number of viable cells and calculate the concentration of viable cells in the suspension. Note: It is recommended to use trypan blue to distinguish non-refractile, dead cells from round, refractile living cells.

Note: To maintain cells beyond day 7, KILR CD16 Effector Cells should be maintained at a density not to exceed 1.5×10^6 cells/mL. Adjust cell density to $0.8x10^6$ viable cells/mL by adding the appropriate volume of fresh culture medium supplemented with IL-2 to a final concentration of 600 IU/mL.

12. Optimal recovery is observed when effector cells are maintained in culture for seven days to maximize recovery. The cells are now ready to be used in the assay.

13. 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.

11.2.2 Preparation of KILR CD16 Effector Cells Day 2

The following is the procedure for preparation of the KILR CD16 Effector Cells on the day of the ADCC assay. For KILR Raji Bioassay Cells, the optimal E:T ratio is 10:1.

Assay Reagents (Volume per Well)	**96-Well Plate**
Number of Target Cells	5,000
Effector Cell to Target Cell (E:T) Ratio	10:1
Volume of Target Cells (µL)	50
Antibody Solution (µL)	10
Volume of KILR CD16 Effector Cells (µL)	50
Working Detection Solution (µL)	100
Total Assay Volume (µL)	**210**

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.
   a. Take 50 µL or less of the suspended cell, prepared in the previous step in a 500 µL tube. **For best results, it is highly recommended to use manual counting rather than automated counting devices. Automated cell counters are unable to discern the population of low trypan blue staining cells that should not be counted as live cells, as well as are often prone to miscounting clumped cells, leading to unreliable determination of viable cell numbers.**
   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:
   b. Effector cell to target cell ratio (E:T ratio) determined for the target cell
   c. The total number of desired assay wells containing target cells that will be used for the experiment
   d. The total number of target cells that were plated in each assay well of the 96-well assay plate
   e. The density of KILR CD16 Effector Cells in culture flask

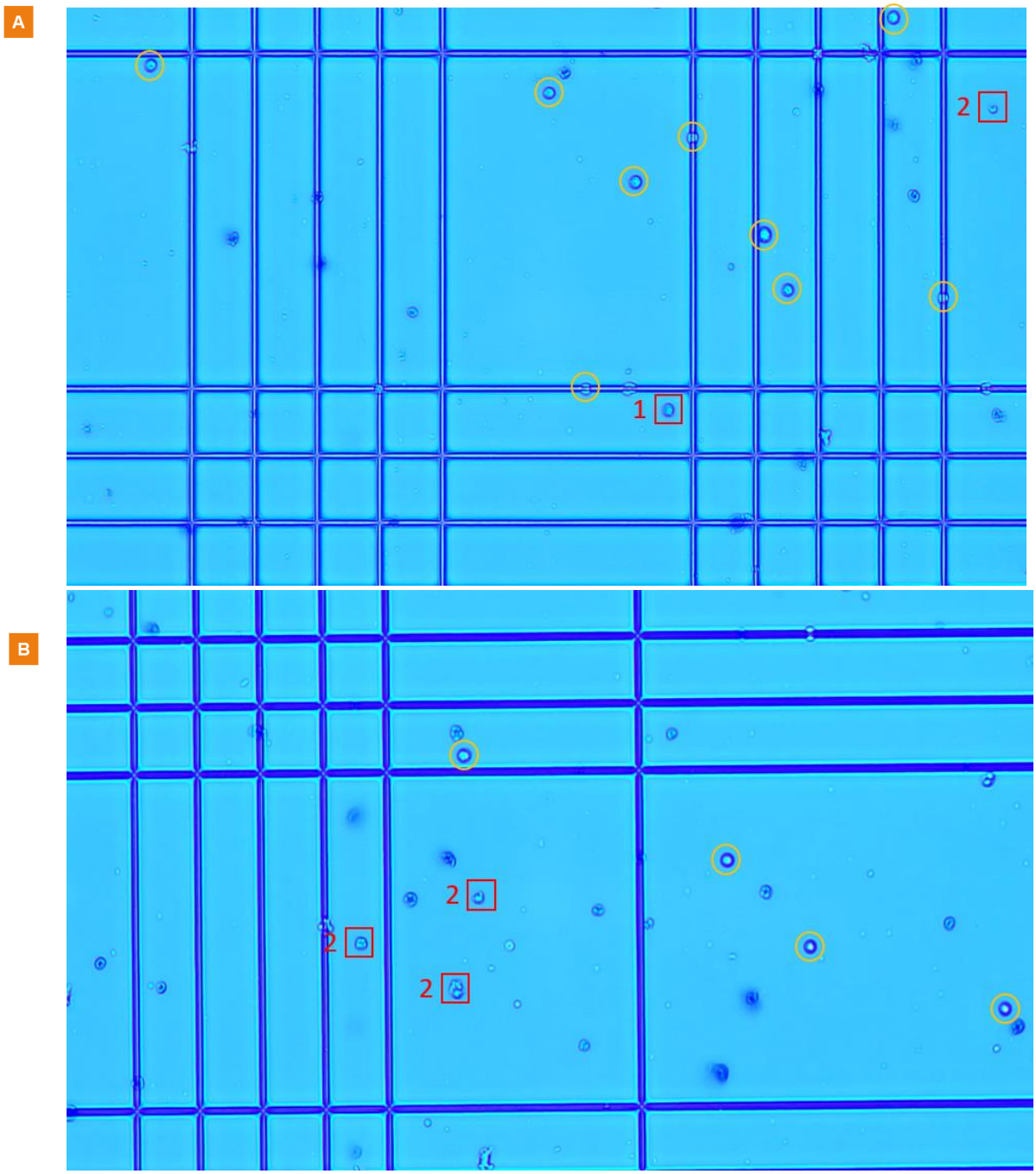


Figure 6 Recommendations for counting of Trypan Blue-stained KILR CD16 Effector Cells

The images A and B above indicate 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, and space 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 cells = 6 x 10^6 cells	
Volume of effector cells suspension (including overage)	(6 x 10^6 cells) / (1 x 10^6 cells/mL) = 6 mL	

4. After counting the cells, calculate the volume needed for the 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 needed number of cells for the assay and keep 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 remove media with a 10 mL pipette without disturbing cell pellet.
7. Prepare fresh CP39 medium by supplementing the CP39 medium 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 pre-warmed CP39 medium. Suspend the KILR CD16 Effector Cells in a volume that produces the desired KILR CD16 Effector Cell density for the assay. The table below includes a sample calculation for determining suspension volume, and space to use 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	50 µL
Number of effector cells in pellet, including overage	6 x 10^6 cells	
Effector cells needed per well	10 X 5,000 target cells/well = 50,000 cells/well	
Required effector cells density	50,000 cells / 0.05 mL = 1 x 10^6 cells /mL	
Volume added to effector cells pellet, including overage*	(6 x 10^6 cells) / (1 x 10^6 cells /mL) = 6 mL	

9. Gently invert the tube several times to mix. For best results, use prepared effector cells in assay within 1 hour of preparation. Prepared cells may be kept in a 37°C incubator until ready to add to target cells.

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