DiscoverX

User Manual

KILR® Cytotoxicity Assay – Suspension Target Cell Lines

Chemiluminescent Assay for Immune-Mediated Cytotoxicity

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

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Overview

The KILR cytotoxicity 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 iOnc drug development including measuring target cell death through Antibody Dependent Cell-mediated Cytotoxicity (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.

Each KILR Assay target clonal cell line or stable pool has been characterized for its stability in culture, and has been found to be stable for at least 15 passages. Assays should be run using fresh, low-passage cells that are seeded and rapidly followed by opsonization with antigen-specific antibodies where appropriate.

Technology Principle

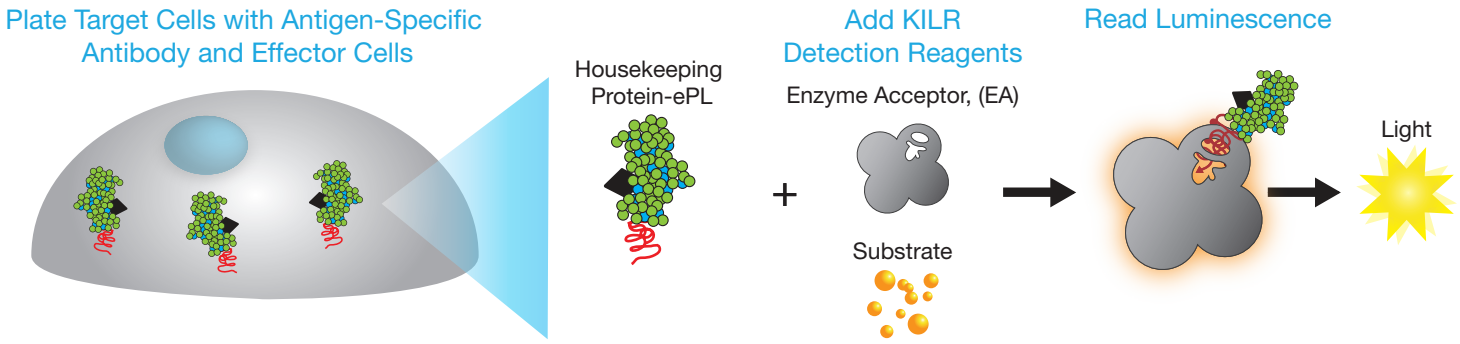


Figure 1. Target cells expressing the receptor antigen can be engineered to stably express a housekeeping protein that is tagged with enhanced ProLabel® (ePL), a β-gal reporter fragment using the KILR Retroparticles. When the stable target cell line is used in a cytotoxicity assay, and its membrane is compromised due to cell death, it 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 luminometer.

DiscoverX has developed a panel of KILR assay cell models expressing a KILR Reporter Protein, a housekeeping protein tagged with Enhanced ProLabel (ePL), a β-galactosidase (β-gal) reporter fragment. Death of the cells leads to rupture of the cell membrane and the KILR Reporter Protein is released into the medium. Addition of 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 and Figure 2). The resulting active enzyme hydrolyzes substrate to generate a chemiluminescent signal. Very little signal is observed from healthy, intact cells, as the KILR Reporter Protein is contained inside the cells and cannot leak out of intact cell membranes. The signal 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.

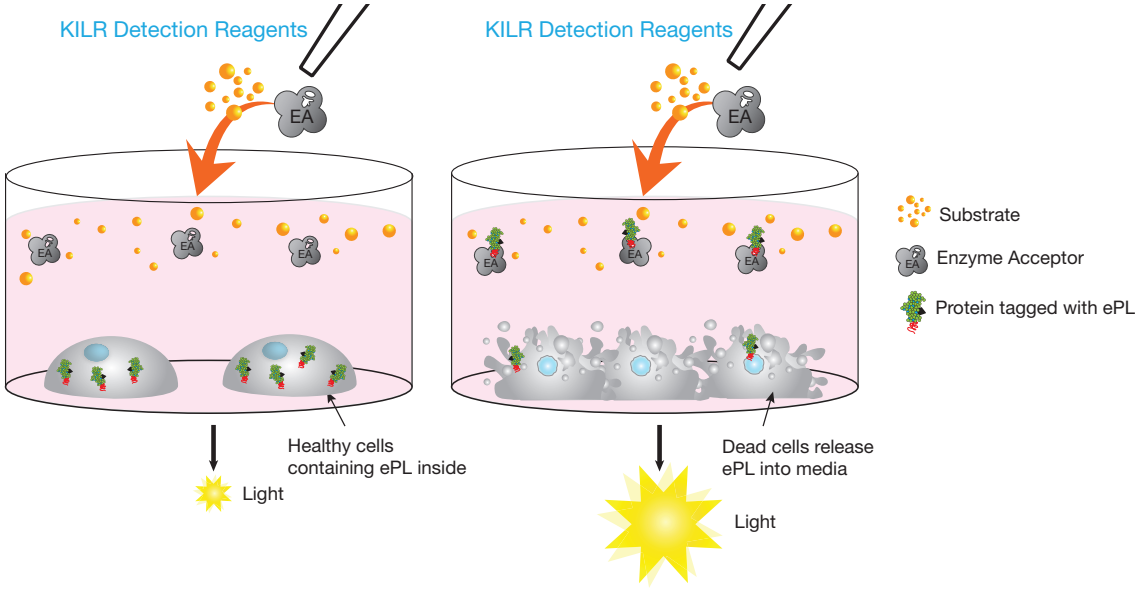


Figure 2. The target cells are engineered to stably express the KILR Reporter Protein. In the figure, the well on the left contains healthy, intact target cells that are alive in the presence of immune effector cells. When KILR Detection Reagent is added, we cannot detect chemiluminescence as the KILR Reporter Protein does not leak out into the media. Alternatively, in the well on the right, the target KILR cells are killed by the immune effector cells, releasing the KILR Reporter Protein into the media. Addition of the KILR Detection Reagent leads to the recognition of this Reporter Protein and the generation of a chemiluminescent signal that is proportional to the number of dead cells. Death of any other cell type, including immune effector cells present within the co-culture will not affect the assay output, giving the KILR assay unparalleled specificity to detect target cell death within a co-culture assay.

Suggested Applications

Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) is a mechanism of cell-mediated immune defense whereby the immune system (effector cells) actively lyses target cells whose membrane-surface antigens have been coated with antigen-specific antibodies that harbor an active Fc region. See protocol in this user manual to determine how to use the KILR cells for ADCC with the appropriate effector cells.

Antibody Dependent Cell-Mediated Phagocytosis (ADCP) is another mechanism of cell-mediated immune defense where the immune system actively kills target cells via phagocytosis. The target cells have membrane-surface antigens that are coated with antigen-specific antibodies, that have an active Fc region, which in turn activates multiple FcγRs. Monocytes, macrophages, dendritic cells, and neutrophils that get activated, envelope and then digest the target cells. A protocol for using KILR cell lines for ADCP can be found in an application note entitled *An Easy-to-Use Assay to Measure Target Cell Death by Antibody Dependent Cellular Phagocytosis (ADCP)*, available at discoverx.com/ADCP.

Complement-Dependent Cytotoxicity (CDC) takes advantage of the complement immune response system to trigger formation of a membrane attack complex to kill cells. Complement proteins bind to antigen-specific antibodies coating the target cells. The subsequent triggering of the complement cascade induces lysis and death of the target cells. The KILR cells have been used to measure CDC similar to other cell lines that measure target cell cytotoxicity.

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 a target KILR cell line, and an activating receptor on the surface of T cell, leading to T cell activation and subsequent lysis of the KILR target cell line.

KILR cell lines can also be used to evaluate Adoptive T cell Therapies (ACTs) such as Chimeric Antigen Receptor T cells (CAR-T) for their ability to drive target cell death. KILR target cells serving as tumor-specific antigen presenting cells (APC) can be used to detect the cytotoxicity of various passive immune effector cells (e.g. engineered CAR-T cells, tumor infiltrating lymphocytes [TILs], and tumor-specific T cell clones).

Materials Provided

Components	Configuration
2 vials of cells	Refer to cell line-specific datasheet

Storage Conditions

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.

Additional Materials Required

Refer to the cell line-specific datasheet to determine catalog numbers for the media and reagent requirements for the specific KILR cell line you are testing. Each KILR cell line or pool has been validated for optimal assay performance using the recommended Cell Plating 39 Reagent and control antibody indicated on the cell line-specific datasheet.

It is highly recommended to not substitute CP Reagent.

Required Materials	Ordering Information
Control Antibody	Refer to cell line-specific datasheet
AssayComplete™ Cell Culture Kit	Refer to cell line-specific datasheet
AssayComplete Cell Plating 39 Reagent Pack	93-0563R39 Series*
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Protein Dilution Buffer	92-0023 Series*
AssayComplete Thawing Reagent	Refer to cell line-specific datasheet
AssayComplete Freezing Reagent	Refer to cell line-specific datasheet
KILR CD16 Effector Cells**	97-0007 Series*
Recombinant Human IL-2, Cell Culture Grade	92-1331
96-well White Bottom TC Treated Plates, Sterile w/Lid, 10/ pack	92-0027
V-bottom PP Dilution Plates, 10/pack (for antibody dilutions)	92-0011
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Multimode or Luminescence Reader	discoverx.com/instrument-compatibility
Single and Multichannel Micro-pipettors and Pipette Tips (10 µL – 1000 µL)	
50 mL and 15 mL Polypropylene Tubes	
1.5 mL Microtubes	
Tissue Culture Disposable Pipettes (1 mL – 25 mL) and Tissue Culture Flasks (T25 and T75 flasks, etc.)	
Cryovials for Freezing Cells	
Hemocytometer	
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	

* Series refer to the different sizes available for that product.

** Fresh or frozen peripheral blood mononuclear cells (e.g. PBMCs; Hemacare, Cat. No. PB009C-50) or isolated natural killer (NK) cells (e.g. AllCells Cat. No. PB012F or similar) can also be used as effector cells in the KILR Cytotoxicity assays. Refer to the Supplemental Information section for Effector Cell Preparation.

Unpacking Cell Cryovials

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells 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.

1. Cells must arrive in a frozen state on dry ice.
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 longer storage, place vials in the vapor phase of liquid nitrogen storage.
3. When removing cryovials from liquid nitrogen storage, use appropriate personal protective equipment and place the cryovials immediately in dry ice in a covered container. Wait 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 cell line product datasheet for the appropriate AssayComplete products mentioned in the protocol below.

Contact technical support immediately if cells are thawed upon arrival.

A face shield, gloves, and a lab coat should be worn during these procedures.

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

Cell Culture Protocol

The following procedures are for thawing cells in cryovials, seeding and expanding the cells, maintaining the cell cultures, and preparing them for assay.

Care should be taken while handling cells to avoid contamination.

Cell Thawing

The following is a protocol for thawing cells in a T25 flask.

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.
2. Add 9 mL of the AssayComplete Thawing Reagent into a 15 mL conical tube in a sterile tissue culture hood. Set aside for Step 6 below. DO NOT add selection antibiotics to the Thawing Reagent.
3. Remove the cell cryovials from vapor phase of liquid nitrogen and immediately place them in dry ice prior to thawing.

Prolonged thawing at 37°C may result in cell death.

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 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. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

4. Place the cryovials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until the cell pellet is almost thawed with only small ice crystals remaining. DO NOT vortex freshly thawed cells.
5. Decontaminate the outside surface of the vial by spraying and wiping with 70% ethanol. Transfer it to a tissue culture hood.
6. With a pipette, gently transfer the thawed cells to the pre-filled 15 mL conical tube.

7. Centrifuge the conical tube at 300 X g for 5 minutes at room temperature to pellet cells.
8. Decant the supernatant, or carefully remove the media with a pipette without disturbing the cell pellet.
9. Add 5 mL of fresh pre-warmed AssayComplete™ Thawing Reagent to the tube.
10. Using the pipette, gently resuspend cells by slowly pipetting up and down.
11. With a pipette, gently transfer the entire volume of cell suspension to a T25 flask and incubate at 37°C and 5% CO_2.
12. Maintain the cells in a T25 flask, until the cell density reaches the maximum recommended density for the indicated KILR Cell Line, as shown in the Recommended Cell Densities table in the Supplemental Information section. The flask should be kept upright in the incubator to encourage recovery. Once the cells have reached the desired density, proceed to the Cell Propagation section. Do not split cells, if density is below the recommended density or growth issues may occur. To determine 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 another cell counting device.
    c. Count cells and calculate the concentration of cells in the suspension.

Cell Propagation

Refer to the Recommended Cell Densities table in the Supplemental Information section for recommended starting densities of various KILR Cell Lines. For cell lines not included in the Recommended Cell Densities table, refer to the cell line-specific datasheet for the recommended cell densities.

Follow the protocol below for propagating cells once they have reached an appropriate density in a T25 flask. It is recommended that suspension cells in culture reach an optimal density before diluting the culture into a fresh T75 flask; 1 x 10^6 cells/mL is a commonly used seeding density for many cell lines. However, refer to the Recommended Cell Densities table in the Supplemental Information section for recommended maximum densities of various KILR Cell Lines. Once in culture, it is also important to feed suspension cells with fresh medium every 2-3 days, and not allow them to exceed the recommended maximum cell density.

For optimal KILR ADCC assay performance, target cells should be grown in AssayComplete Cell Culture Reagent in the absence of selection antibiotics for at least 48 hours prior to use in the ADCC assay. However, it is important to always maintain a stock culture of KILR target cells under antibiotic selection pressure to ensure stable expression of the KILR Reporter Protein. Therefore, set up two culture flasks, one used for preparing cells for the assay, which will be in culture medium without selection antibiotics; and a second flask to maintain the stock culture of cells in AssayComplete Cell Culture Reagent supplemented with appropriate selection antibiotics. Transfer the flask to a tissue culture incubator at 37°C and 5% CO_2.

The cell culture medium is comprised of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotics.

1. Pre-warm cell culture medium in a 37°C water bath for 15 minutes.
2. Remove the T25 flask from the tissue culture incubator and place in a sterile tissue culture hood.
3. Once cells have grown to their maximum density, transfer the cells from the T25 flask to a fresh tissue culture flask containing a volume of fresh cell culture medium sufficient for diluting the cells to their optimal starting density. The Volumes of Medium for Propagating Cells table lists recommended maximum culture volumes for various tissue culture flasks and plates. Avoid overdiluting the cells to a density lower than the recommended starting density, which could lead to very slow growth.

Volumes of Medium for Propagating Cells			
Flask Size	**T25**	**T75**	**10 cm Plate**
AssayComplete™ Cell Culture Medium (mL)	10	50	10

4. Transfer the flask to a tissue culture incubator at 37°C and 5% CO_2.
5. Maintain the cell culture by diluting the cells every 2-3 days (or when the maximum cell density is reached) in a fresh tissue culture flask, using cell culture medium supplemented with the recommended concentration of selection antibiotics. If preparing cells for the assay, they should be cultured in a separate flask using AssayComplete Cell Culture Reagent without selection antibiotics.

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 flasks. This protocol assumes that cells have reached an optimal density and in sufficient numbers to produce the desired number of vials of frozen cells, in 1 mL per vial aliquots, with the desired number of cells per vial. Refer to the cell line-specific datasheet for recommended density for freezing (most suspension cell lines are frozen at ≥ 2 x 10^6 cells per vial).

It is recommended to freeze cells at a low passage number (2 to 3 passages) for optimal assay performance.

1. Remove T75 flasks from incubator and place in a sterile tissue culture hood.

Care should be taken in handling to avoid contamination.

2. Remove the entire volume of cell suspension from the T75 flask and transfer to a 50 mL conical tube. Gently pipette up and down several times to ensure a single cell suspension with no cell clumps.
3. 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 another cell counting device.
   c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 50 mL conical tube.
4. Centrifuge the collected cells at 300 X g for 4 minutes.
5. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.

6. Based on the total cell number calculated in Step 3 above, resuspend cells to the desired concentration (e.g. ≥ 2 x 10^6 cells/mL) with ice-cold AssayComplete™ Freezing Reagent.

7. Make aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.

Keep cells on ice during this process to maintain cell viability.

8. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight.
   This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short-term storage, vials can be stored in the -80°C freezer for a maximum of two days.

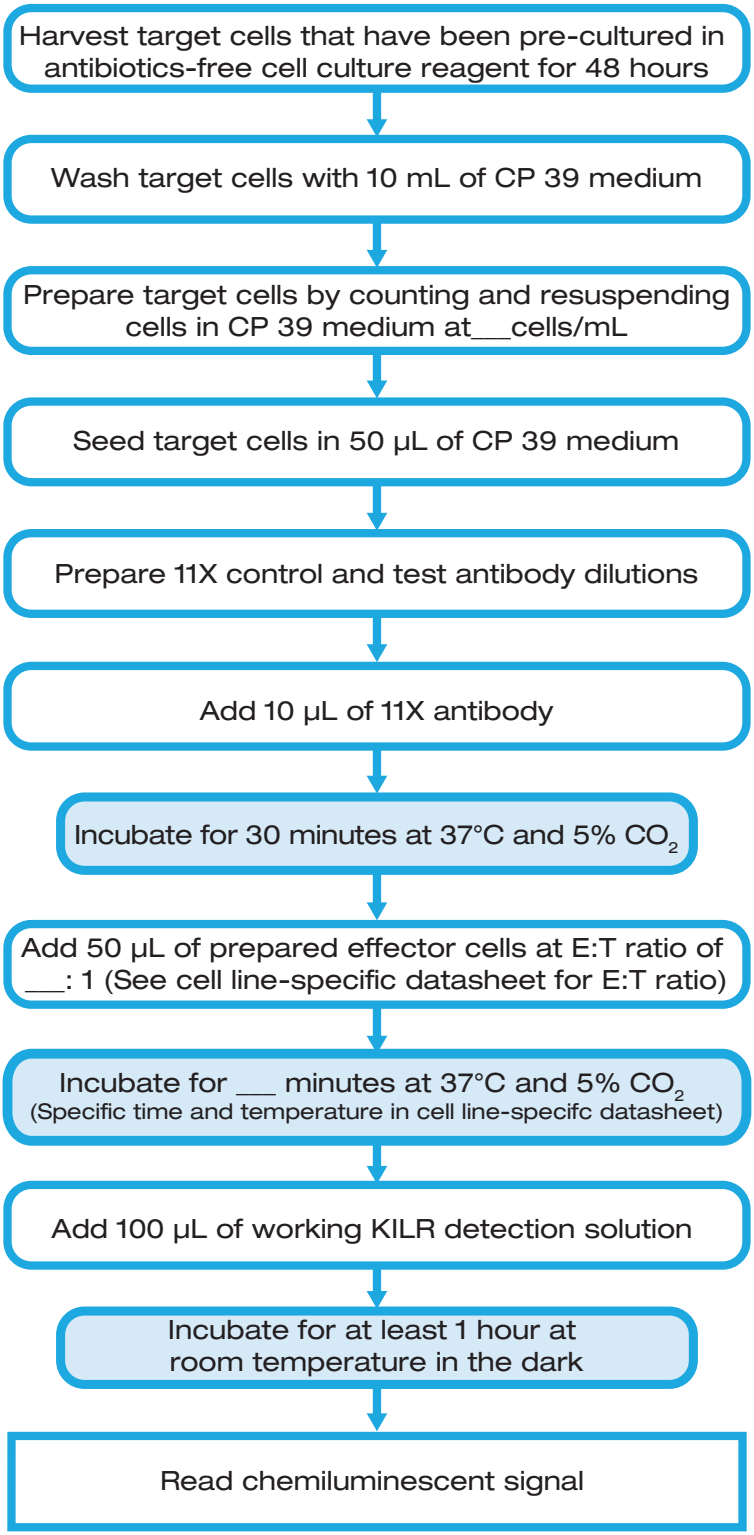
Protocol Schematic

Tip: *Use this sheet to note your assay-specific conditions. Post it on your bench to use as a quick reference guide.*

Assay Name:_____ Date: _____

Product Details: _____

Quick-Start Procedure: In a 96-well tissue culture treated plate, perform the following steps:



Detailed Assay Protocol

The following detailed protocol is specific to detecting antibody dependent cell-mediated cytotoxicity (ADCC).

Target cells are exposed to an ADCC-inducing antibody then co-incubated with an effector cell in a 96-well tissue culture plate.

Reagents	96-Well Plate Volume per Well
Number of Target Cells	Refer to cell line-specific datasheet
Effector Cell to Target Cell Ratio (E:T)	Refer to cell line-specific datasheet
AssayComplete™ Cell Plating 39 Reagent (µL)	50
Antibody (µL)	10
Effector Cells (µL)	50
Working Detection Solution (µL)	100
Total Assay Volume (µL)	**210**

Note: Effector cell preparation must be initiated (i.e. thawing of PBMCs or NK cells) the day before the opsonization step, as described in Effector Cell Preparation section (Section IV).

Section I: Target Cell Preparation and Plating

The following is a protocol for harvesting cells from a T75 flask (with cell density of 1 x 10^6 cells/mL) and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that cells have been cultured in their specified cell culture medium. The cell culture medium comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the AssayComplete Cell Culture Kit and supplemented with appropriate selection antibiotics.

Culturing the target cells in cell culture medium without selection antibiotics in a 37°C and 5% CO_2 humidified incubator for 48 hours before running the KILR assay may increase assay performance. A shorter incubation time maybe acceptable, but needs to be determined empirically. Refer to the KILR Cell Line-specific datasheet for cell culture and plating requirements.

1. On the day of the assay, pre-warm AssayComplete Cell Plating 39 Reagent in a clean 37°C water bath for at least 20 minutes. Add fresh L-glutamine to the Cell Plating 39 Reagent to a final concentration of 2 mM. See table below for suggested volumes. Set aside the L-glutamine supplemented Cell Plating 39 Reagent (cell plating 39 medium) until it is needed at Step 9. Use only cell plating 39 medium when executing the following protocol.

The cell plating 39 medium comprises of the AssayComplete Cell Plating 39 Reagent supplemented with L-glutamine provided in the AssayComplete Cell Plating 39 Reagent Pack. The cell plating 39 medium is light sensitive, thus protect from light while working with it in the culture hood.

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

2. Gently mix the cells in the flask to ensure an equal distribution of cells.

3. Immediately transfer 10 mL of the cell suspension from the flask to a 15 mL conical tube.

4. 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 cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL conical tube.
5. Determine the number of cells that will be needed for the experiment. For example, an entire 96-well plate with 5,000 cells/well in 50 µL would require 0.6 x 10^6 cells. Refer to the cell line-specific datasheet to determine the optimal assay cell number.
6. Calculate the volume of cell suspension that contains this desired number of cells, based on the cell concentration determined at Step 4.c. Transfer this volume of cell suspension from the 15 mL conical tube to a second clean 15 mL conical tube.
7. Centrifuge these collected cells at 300 X g for 4 minutes.
8. Wash the cells by adding 10 mL of the cell plating 39 medium. Gently invert the tube several times.
9. Centrifuge the cells at 300 X g for 4 minutes.
10. Carefully decant supernatant or remove it with a 10 mL pipette without disturbing the cell pellet.
11. Gently resuspend the cell pellet in the cell plating 39 medium. Based on the cell number obtained in Step 5 above, dilute the resuspended cells to the desired concentration (e.g. if 0.6 x 10^6 cells obtained then add 6 mL to obtain 100,000 cells/mL or 5,000 cells/50 µL).
12. Transfer 50 µL/well of the cell suspension to a 96-well assay plate. Load enough 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 Antibody Addition section (Section III).

Section II: 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.
    a. For each antibody, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
    b. 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 per dose.
    c. 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 a 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.

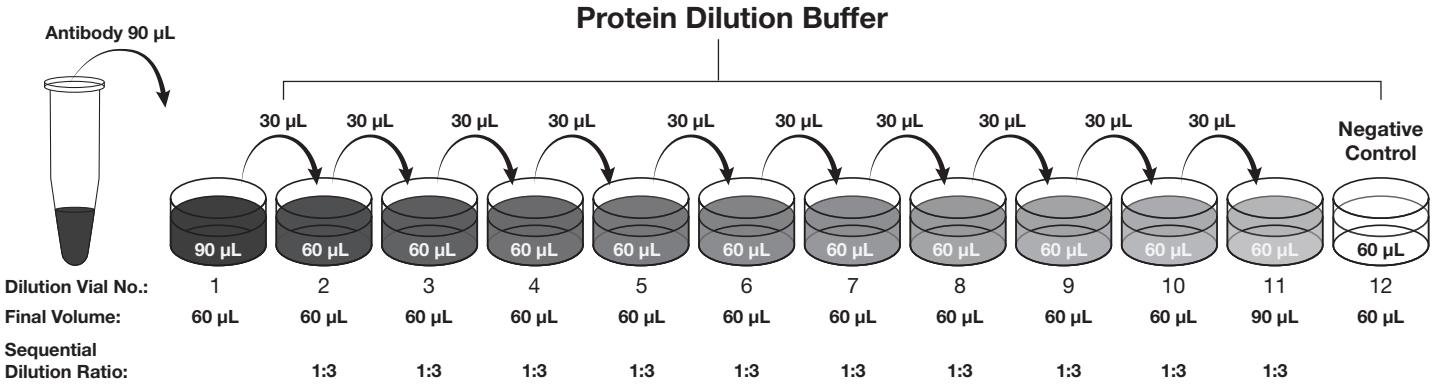
d. Add 90 µL of the highest concentration of antibody to well No. 1 (see figure: Antibody Serial Dilutions).

e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

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.

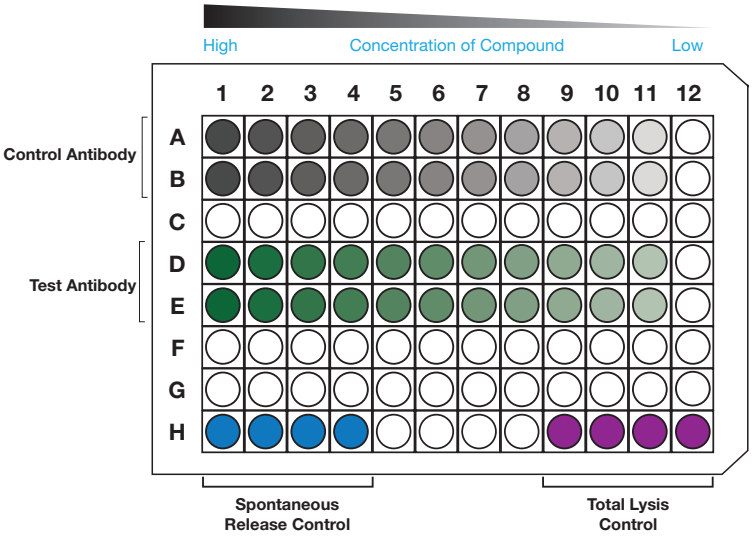
2. Set up serial dilutions for any additional antibodies in a similar manner.



Antibody Serial Dilutions: Make eleven 3-fold serial dilutions of antibody in a separate dilution plate.

Section III: Antibody Addition

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



Assay Plate Map: Plate layout with 11-point dilution curves, for Test and Control antibodies run in duplicates, and including Spontaneous Release and Total Lysis Controls.

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 or to the Total Lysis Control.

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 many of the KILR cell lines, no reference control antibody was available. Datasheets for these cell lines indicate an incubation time and temperature for a non-antibody control reagent (e.g. Triton X-100 or Digitoxin) that provide a reference for potential assay signal. For many of our cell lines validated with an antibody, we opsonize for 30 minutes at 37°C. For the best results, the optimal incubation time and temperature should be empirically determined.

Section IV: Effector Cell Preparation

The following is a procedure for preparing the effector cells for the ADCC assay. If using the single donor-derived KILR CD16 Effector Cells (Cat. No. 97-0007 Series) with the assay, refer to their specific user manual (Document No. 70-388) for instructions for effector cell preparation.

1. 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 a ratio ranging from 12.5:1 to 25:1 for optimal results (Note: for difficult cell types, a higher E:T ratio, e.g. 40:1, may be required). For isolated natural killer (NK) cells, we recommend a ratio ranging from 5:1 to 10:1 for optimal results.

Refer to the KILR Assay Cell Line or Pool-specific datasheet for optimal E:T ratios.

Section V: 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.

1. Using a multichannel pipettor and a reagent reservoir, add 50 µL of effector cells (prepared as described in the Supplemental Information section) into each well of the 96-well assay plate. Do not add effector cells to Spontaneous Release Control wells H1 through H4 and Total Lysis Control wells H9 through H12 as shown in the described assay plate map.

2. Incubate assay plate for 3 hours (or optimal incubation time for the relevant KILR cell model as indicated on the datasheet) in a 37°C and 5% CO_2 humidified tissue culture incubator.

Section VI: 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 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.

1. For Spontaneous Release Controls (wells H1 through H4), add 50 µL of cell plating 39 medium.

2. For Total Lysis Control Wells (wells H9 through H12), add 48 µL of cell plating 39 medium and 2 µL of KILR Total Lysis Control Reagent (included in the KILR Detection kit; Cat. No. 97-0001 Series).

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).

Section VII: 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 more 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. Refer to the user manual for the KILR Detection Kit 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**

Working KILR detection solution is light sensitive, thus incubation in the dark is necessary.

2. Add 100 µL of working KILR detection solution to wells containing cells in the assay plate. It is not recommended to mix solutions in the wells by pipetting up and down or by vortexing the assay plate.
3. Place lid back on plate and incubate assay plate for at least 1 hour at room temperature in the dark.
4. 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. The plate could be incubated overnight and the signal should be measured within 24 hours. 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 manufacturer may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.
5. 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.).

Section VIII: Calculations

Calculate % ADCC

The following are instructions for calculating % **ADCC** values. The resulting calculated data 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:

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

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 Calculate % ADCC section for each antibody concentration.

The following are hypothetical values (RLU) created for this demonstration.

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

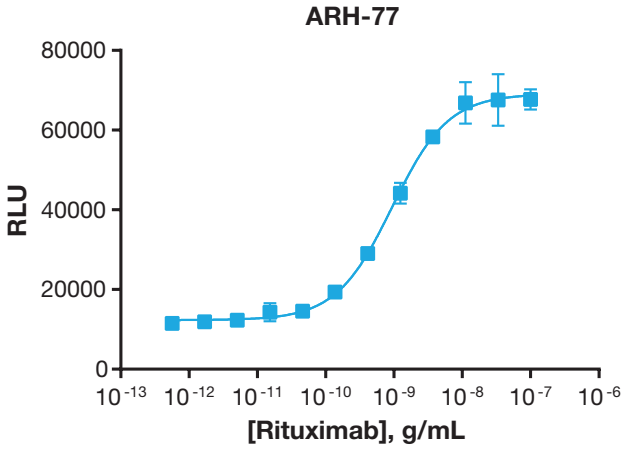
Antibody Values (1 data point): 65,000

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	

Typical Results

Typical results are shown for Rituximab-mediated killing in the CD20-positiive KILR ARH77 cell model. ARH77 cells stably expressing the ePL-labeled housekeeping protein were washed, opsonized with a titration of Rituximab (anti-CD20 antibody), then incubated with primary PBMCs (25:1 ratio of effector: target). After 3 hours, KILR Detection Reagents were added to the medium and incubated for one hour prior to detection of luminescent signal on a luminometer (Envision, PE). The EC_{50} of 0.94 ng/mL is consistent with EC_{50} observed for Rituximab with other ADCC assay formats (e.g. chromium-51 release; europium).



EC_{50}	S/B	Hill Slope
0.9 ng/mL	5.9	1.058

Supplemental Information

Recommended Cell Densities for KILR Suspension Cell Lines

Cell Line	Recommended Starting Density (Viable Cells/mL)	Range for Cell Maintenance (Viable Cells/mL)	Maximum Cell Density (Viable Cells/mL)
ARH77	2×10^5	$1 \times 10^5 – 1 \times 10^6$	1×10^6
Ramos	2×10^5	$2 \times 10^5 – 1 \times 10^6$	1×10^6
Raji	2×10^5	$4 \times 10^5 – 2 \times 10^6$	$<3 \times 10^6$
Daudi	3×10^5	$3 \times 10^5 – 2 \times 10^6$	3×10^6
WIL2-S	2×10^5	$1 \times 10^5 – 1 \times 10^6$	1×10^6
THP-1	2×10^5	$1 \times 10^5 – 0.8 \times 10^6$	$<1 \times 10^6$
MOLT-4	4×10^5	$4 \times 10^5 – 1 \times 10^6$	$<2 \times 10^6$
EL4	2×10^5	$2 \times 10^5 – 1 \times 10^6$	1×10^6
Jurkat	2×10^5	$1 \times 10^5 – 1 \times 10^6$	1×10^6
CCRF-CEM	2×10^5	$2 \times 10^5 – 2 \times 10^6$	2×10^6
HuT78	2×10^5	$5 \times 10^4 – 8 \times 10^5$	$<1 \times 10^6$
T2	3×10^5	$3 \times 10^5 – 1 \times 10^6$	1×10^6
RPMI 8226	5×10^5	$5 \times 10^5 – 2 \times 10^6$	$<3 \times 10^6$
K562	2×10^5	$1 \times 10^5 – 1 \times 10^6$	1×10^6

Note: If the cell line in use is not listed, the optimal density range for cell maintenance should be determined empirically. Refer to the cell line datasheet for its specific recommendations. If developing a cell line using Retroparticles, the technical information provided by the cell vendor may include appropriate recommendations.

Effector Cell Preparation: Isolated PBMCs

The following sections contain two separate protocols, for preparing effector cells: one protocol for PBMCs (peripheral blood mononuclear cells) and one for primary NK (Natural Killer) cells.

Preparation of PBMCs Day 1: Thawing PBMCs

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

Components	2 Plates	10 Plates	25 Plates
AssayComplete™ Cell Plating 39 Reagent (mL)	100	500	1,250
L-glutamine (100X) (mL)	1	5	12.5
Total Volume (mL)	**101**	**505**	**1,262.5**

1. Pre-warm AssayComplete Cell Plating 39 Reagent in a clean 37°C water bath for at least 20 minutes. Add fresh L-glutamine to the Cell Plating 39 Reagent to a final concentration of 2 mM, see table above for suggested volumes. Use only L-glutamine supplemented Cell Plating 39 Reagent (cell plating 39 medium) when executing the following protocol.

The cell plating 39 medium comprises of the AssayComplete Cell Plating 39 Reagent supplemented with L-glutamine provided in the AssayComplete Cell Plating 39 Reagent Pack. The cell plating 39 medium is light sensitive, thus protect from light while working with it in the culture hood.

2. Remove the cell cryovials 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. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

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.

Prolonged thawing at 37°C may result in cell death.

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 cell plating 39 medium.

7. Add an additional 2 mL of cell plating 39 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 cells and calculate the concentration of cells in the suspension.

   d. Calculate the total number of cells remaining in the 15 mL conical tube.

9. Centrifuge at 300 X g for 15 minutes at room temperature to pellet cells.

10. Decant supernatant or carefully remove media with a 10 mL pipette without disturbing the cell pellet.

11. PBMC's must be suspended in cell plating 39 medium to a density of 1 x 10^6 cells/mL

    a. Calculate the total volume of cell plating 39 medium required to generate a density of 1 x 10^6 cells/mL .

    b. Add the required volume of cell plating 39 medium to a T25 or T75 flask. The table below provides recommendations for flask size:

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

    c. Transfer 10 mL of the cell plating 39 medium from the flask to the 15 mL tube containing the pellet of PBMCs.

    d. Using the pipette, break up the pellet of PBMCs by gently pipetting up and down.

    e. Transfer the entire volume of the PBMC suspension back to the tissue culture flask.

12. Incubate overnight at 37°C and 5% CO_2 with the flask standing on end. This rest 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).

Optional: PBMCs may be stimulated during the overnight rest to promote activation. Add a combination of 20 ng/mL recombinant human IL-2 and 10 pg/mL recombinant human IL-15 to media. However, generally a larger assay window is obtained from KILR ADCC assays in the absence of stimulating cytokines.

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 performance. Monocytes in PBMCs will attach to the plastic in about 2-3 hours when incubated at 37°C. Longer incubations will result in firm attachment. Lymphocytes are not adherent, they will mostly be in suspension, and can be removed by mildly flushing the wells with medium.

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.

   Trypan blue stains dead cells only. Count only the unstained cells to determine viable cell density.

   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:

   Refer to the KILR Assay Cell Line or Pool-specific datasheet for optimal E:T ratios. PBMCs are the effector cells.

   a. The E:T ratio.

   b. The total number of desired assay wells with 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 PBMCs in culture flask.

   e. The table below includes an example calculation, and space to use for calculating the volume of PBMC suspension required for 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 PBMCs = 1.5 x 10^7 PBMCs	
Volume of PBMC Suspension (including overage)	(1.5 x 10^7 PBMCs) / (1 x 10^6 /mL) = 1.2 mL	

* Refer to the KILR cell line-specific datasheet for the recommended E:T ratio and target cells/well.

** 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 cell plating 39 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 cell plating 39 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 x 10^7 PBMCs	
PBMCs Needed per Well	25 x 5,000 target cells/well* = 125,000 PBMCs/well	
Required PBMC Density	125,000 PBMCs / 0.05 mL = 2.5 x 10^6 PBMCs/mL	
Volume Added to PBMC Pellet, including overage*	(1.5 x 10^7 PBMCs) / (2.5 x 10^6 PBMCs/mL) = 6 mL	

* Cell plating 39 medium is added to the suspension to reach the required final volume.

8. Leave cells in a 37°C incubator until needed for the Effector Cell Addition section (Section V).

Effector Cell Preparation: Isolated Primary NK Cells

Preparation of Frozen Primary NK Cells Day 1: Thawing NK Cells

The following is a procedure for preparing primary NK cells from frozen stocks. This procedure should be initiated the day before the opsonization step of the ADCC assay.

1. Pre-warm AssayComplete™ Cell Plating 39 Reagent in a clean 37°C water bath for at least 20 minutes. Add fresh L-glutamine to the Cell Plating 39 Reagent to a final concentration of 2 mM, see table above for suggested volumes. Use only L-glutamine supplemented Cell Plating 39 Reagent (cell plating 39 medium) when executing the following protocol.

Components	2 Plates	10 Plates	25 Plates
AssayComplete Cell Plating 39 Reagent (mL)	100	500	1,250
L-glutamine (100X) (mL)	1	5	12.5
Total Volume (mL)	**101**	**505**	**1,262.5**

The cell plating 39 medium comprises of the AssayComplete Cell Plating 39 Reagent supplemented with L-glutamine provided in the AssayComplete Cell Plating 39 Reagent Pack. The cell plating 39 medium is light sensitive, thus protect from light while working with it in the culture hood.

2. Remove the cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them on dry ice prior to thawing.
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.

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. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

4. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.
5. With a 2 mL wide-bore pipette, carefully transfer the thawed cells to a sterile 15 mL conical tube.

Prolonged thawing at 37°C may result in cell death.

6. Slowly add 10 mL pre-warmed cell plating 39 medium.
7. Add an additional 2 mL of cell plating 39 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 cells and calculate the concentration of cells in the suspension.
   d. Calculate the total number of cells remaining in the 15 mL conical tube.

9. Centrifuge at 300 X g for 15 minutes at room temperature to pellet cells.
10. Decant supernatant or carefully remove media with a 10 mL pipette without disturbing the cell pellet.
11. NK cells must be suspended in cell plating 39 medium to a density of 1 x 10^6 cells/mL.
    a. Calculate the total volume of cell plating 39 medium required to generate a density of 1 x 10^6 cells/mL.
    b. Add the required volume of cell plating 39 medium to a T25 or T75 flask. The table below provides recommendations for flask size:

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

    c. Transfer 10 mL of the cell plating 39 medium from the flask to the 15 mL tube containing the pellet of NK cells.
    d. Using the pipette, break up the pellet of NK cells by gently pipetting up and down.
    e. Transfer the entire volume of the NK cell suspension back to the tissue culture flask.
12. Incubate overnight at 37°C and 5% CO_2 with the flask standing on end. This rest allows the NK cells to recover functionality, and 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).

Optional: NK cells may be stimulated during the overnight rest to promote activation. Add a combination of 20 ng/mL recombinant human IL-2 and 10 pg/mL recombinant human IL-15 to media. However, generally a larger assay window is obtained from KILR ADCC assays in the absence of stimulating cytokines.

Preparation of NK Cells Day 2: Final Preparation of NK Cells

The following is a procedure for final preparation of the effector NK cells 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.

Trypan blue stains dead cells only. Count only the unstained cells to determine viable cell density.

    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 15 mL conical tube.
2. Determine the number of NK cells required for the assay. This number is dependent on the following factors:
    a. The E:T ratio.
    b. The total number of desired assay wells with 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 NK cells in 15 mL conical tube.

e. The table below includes an example calculation, and space to use for calculating the volume of NK suspension required for the assay:

The optimal E:T ratio when using NK cells must be determined empirically.

Determination of Number of NK Cells		
Variable	**Example**	**Experiment**
E:T ratio*	5:1	
Number of Assay Wells	96 wells	
Number of Target Cells per Well*	5,000 cells	
Density of NK in 15 mL Tube	1 x 10^6 /mL	
NK Cells Needed per Well	5 x 5,000 cells/well = 25,000 cells/well	
Overage Factor**	0.25	
Total NK Cells Needed per Assay (accounting for overage factor)	[96 wells + (96 wells x 0.25)] x 25,000 NK cells = 3 x 10^6 NK cells	
Volume of NK Cells Suspension (including overage)	(3 x 10^6 NK cells) / (1 x 10^6 /mL) = 3 mL	

* Refer to the KILR cell line-specific datasheet for the recommended target cells per well. The optimal E:T ratio must be determined empirically.

** It is advisable to factor in at least 25% more NK cells (and therefore a greater volume of NK cell 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 NK cell suspension (as calculated in the table above) from the 15 mL conical tube into a separate sterile 15 mL conical tube.

4. Centrifuge at 300 X g for 15 minutes at room temperature to pellet cells.

5. Decant supernatant or carefully remove media with a 10 mL pipette without disturbing cell pellet.

6. Resuspend cell pellet in pre-warmed cell plating 39 medium. Suspend the NK cells in a volume that produces the desired NK cell 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 NK suspension required for the assay.

Preparation of NK Cells Suspension for Assay		
Variable	**Example**	**Experiment**
Number of Assay Wells	96 wells (plus overage)	
Volume of NK Cells per Assay Well	50 µL (0.05 mL)	50 µL (0.05 mL)
Number of NKs in Pellet, including overage	3 x 10^6 NK cells	
NK Cells Needed per Well	5 x 5,000 Target cells/well* = 25,000 NK cells/well	
Required NK Cell Density	25,000 NK cells / 0.05 mL = 5 x 10^5 NK cells/mL	
Volume Added to NK Cell Pellet, including overage*	(3 x 10^6 NK cells) / (5 x 10^5 NK cells/mL) = 6 mL	

* Cell plating 39 medium is added to the suspension to reach the required final volume.

7. Leave cells in a 37°C incubator until needed for the Effector Cell Addition section (Section V).

Frequently Asked Questions

What types of applications can the KILR cell assay be used for?

- The KILR stable cell line/pool can be used for a variety of cytotoxicity applications that measure target cell death, including ADCC and ADCP assays. The following link is to webpages that contain links for downloading an application note with protocols for KILR ADCP Assays: discoverx.com/ADCP.
- Protocols for all other cytotoxicity applications (e.g. CDC, redirected T cell mediated cytoxicity, and Adoptive T cell therapies) must be developed and optimized by the user.
- The mechanism of cell death will dictate which detection reagent will be needed for developing an assay other than ADCC or ADCP. Cytotoxic sample treatments that compromise plasma membrane integrity and result in cell lysis (similar to ADCC mediated apoptosis) and releasing of the KILR Reporter Protein into the assay media require the use of KILR Detection Kit (Cat. No. 97-0001 Series). However, cytotoxicity assays that kill cells without compromising the integrity of the plasma membrane (e.g. ADCP assays) will require the PathHunter® PL/PK Detection Kit (Cat. No. 93-0812 Series) which contains a cell lysis buffer to chemically lyse the cells.

Can overexpression of the KILR Reporter Protein interfere with my assay in any way?

- The KILR Reporter Protein is a housekeeping gene that is endogenously expressed at high levels in many cell lines. Introduction of the labeled Reporter Protein has been evaluated in many cell lines without quantifiable detrimental effects.

Why do we offer these KILR assays as cell pools?

- The stable target cell pools are more representative of the heterogeneity of the original cancer line than a stable clone. Since we do not know a priori which antigen is of primary interest, we have elected to produce a stable pool that best represents the starting state of the cell line.

Are these cell pools stable? Do we have data to support any questions on stability of these cell pools?

- Each KILR target cell pool is assessed for the stability of the KILR Reporter Protein expression as well as comparable % lysis achieved either with an antibody or a chemical agent that will induce killing, over at least 15 passages. Some tested cell pools have over 30 passages of stability recorded, indicating that these cell pools are very stable in culture.

Under what circumstances would you generate clonal target cells?

- We recommend making clonal target cells when the antigen of interest is clearly known, and it is desirable to have the receptor under selective pressure (e.g. over-expression of the antigen of interest). Additionally, if the cell line is being used for QC lot release of a biologic drug, making a single cell clone will ensure stability over a longer period of time. However, given the known stability of the cell pools in our hands, we believe that this may not be necessary.

We need these assays in many different cancer cell backgrounds. Can we get all the vectors to make the cell line ourselves?

- We do offer our KILR Retroparticles for Cytotoxicity Assays which are reagents that enable transduction of the KILR reporter into any cell host of your choice. Go to discoverx.com/kilr, click on the Assay List tab and look for links to the KILR Retroparticles assays.
- If clonal target cell or a target cell line with a new target of interest is required, these can be generated as custom assay. to request details, request information at CAD@discoverx.com.

Which effector cells have you tried?

- We have tested KILR CD16 Effector Cells (single donor derived primary human cytotoxic T lymphocytes engineered to stably express CD16), isolated human primary PBMCs and NK cells, NK-92 cell line (engineered to express CD16), CTLs, CAR-T cells and primary human macrophages.

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

- The assay is a simple way to measure target cell death. If the drug molecule causes target cell death, this assay will be able to measure it. If regulatory bodies have accepted the Chromium 51, LDH or other means of measuring target cell death for evaluating your molecule, the KILR assay will also be deemed acceptable. In addition, the KILR assay has the advantage of reading the same assay endpoint (effector cell-mediated target cell death) but with greater simplicity, specificity and sensitivity than the alternatives.

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

- The KILR Cytotoxicity platform delivers 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 type of transfection has been used to create the cell line? Are the cells transient or stable?

- Each cell line is retrovirally-transduced with the KILR reporter construct to produce a stable cell pool. These stable pools maintain the expression of their reporter and of the antigen used for testing for anywhere from 15 to 30 passages. If required, the cells can be tested to demonstrate that they do not produce any additional virus (i.e. the virus used was replication-deficient).

I understand that you generate your cell lines via retroviral infection as it is a very efficient system. Do the final cell lines produce any viable retroviral particles?

- No. We have confirmed the lack of retroviral particles using a Marker Rescue Assay.

What is the sensitivity of your assay? What is the minimal number of target cells you could put in your well?

- We have observed that the absolute % lysis induced by ADCC varies greatly between cell lines, and is likely dependent on the target and cell lines. For most cell lines, we have tested a minimum of 1,500-2,000/well in a 96-well format. The assay can reproducibly detect as low as 5% cell death (% lysis), which would equate to the signal from 75-100 cells. To increase cell lysis, you can optimize a number of parameters such as the E:T ratio 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 increases 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 are able to produce good results. Increasing serum concentration may increase background, thus reducing the assay window, but a robust response is generally still observed.

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.

What is the stability of the tagged housekeeping protein in culture supernatant? Do we have a killing time curve?

- Stability of the reporter protein in the medium can be influenced by multiple factors, including the target cells, the type of effectors used, and medium components (e.g. serum). Typically, once released into the medium, highest signal is observed within 16-24 hours. However, depending on the kinetics of killing, the signal from the released protein can still be detected with longer incubation periods. Cells lysed with serial dilutions of dilute detergent and assayed after 24, 48 and 72 hours showed a robust signal at 24 hours (81% lysis), 48 hours (55% lysis) and 72 hours (~48% lysis). This suggests that it is feasible to use a longer incubation time (> 24 hours), if the molecule being tested does not mediate rapid killing of the cell population.

What is the passage number of the frozen cells in the vials I receive when I purchase the cell line?

- Passage 2-3.

What is the general liquid dispensing speed that you would recommend when using robotic dispensing instruments?

- This will depend on the particular instrument and has to be determined empirically, but in general, we would recommend a speed of 15-20 µL/second.

What if the variability between replicates is high?

- Make sure dispensing equipment is properly calibrated and proper pipetting technique is used.
- Make sure to avoid cell clumps when plating. Uneven number of cells in the walls can cause high variability.
- The normal 1 hour incubation period for the detection step can be extended for an additional 1 to 15 hours. In some case, the additional incubation period can reduce variability of the replicates.

Troubleshooting Guide

Problem	Cause	Solution
No Response	Unoptimized or sub-optimal cell growth conditions	See datasheet for cell culture conditions.
	Incorrect antibody used or improper antibody incubation time for opsinization	See datasheet for recommended assay conditions.
	Improper opsonization time	Optimize opsonization time with control antibody.
	Poor quality or dead effector cells	Use trypan blue or other appropriate method to test cell viability.
Decreased Response	Higher passages over P15 may give reduced performance	KILR cells are stable for at least 15 passages. It is recommended to use low passage cells whenever possible.
Low or No Signal	Improper preparation of detection reagents	Make sure that the cell line-specific detection reagents were used, and were stored and prepared as indicated in the datasheet. Detection reagents should be prepared just prior to use and are sensitive to light.
	Problem with cell growth, cell viability, or cell density	Refer to cell line-specific datasheet for recommended cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 second/well.
Experimental S:B does not match datasheet value	Incorrect incubation temperature	Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
	Improper incubation time	Consult datasheet, and optimize incubation time with control antibody.
	Improper AssayComplete™ Cell Plating reagent used	Use the recommended Cell Plating Reagent.
	Detection incubation step	The normal 1 hour incubation period for the detection step can be extended for an additional 1 to 15 hours. In some cases, it may improve S:B ratio.
EC_{50} is right-shifted	Antibody not stored properly	Determine proper storage conditions for the antibody.
	Use of trypsin for cell plating	Use only AssayComplete Cell Detachment reagent to harvest cells for the assay.
High Background	Inappropriate cell incubation time	Follow the protocol specified in the user manual and cell line datasheet.
	Lysis Control mistakenly added to assay wells	Follow the protocol specified in the user manual for proper usage of the Lysis Control Reagent.
	Quality of effector cell preparation	Follow the protocol specified in the user manual for preparing PBMCs and NK cells.
	Presence of selection antibiotics in medium used for reviving target cells	Culture cells for at least 24 hours in the absence of antibiotics.
	Sub-optimal number of target cells per well	Follow the recommendations provided on the cell line datasheet for target cells per well or determine optimal cell numbers empirically.
	Sub-optimal E:T ratio	Follow the recommendations provided on the cell line datasheet for E:T ratio or determine optimal E:T ratio empirically.
	Incorrect AssayComplete Cell Plating reagent	Refer to cell line-specific datasheet for the recommended AssayComplete Cell Plating Reagent.
	Excess serum in assay	Reduce or eliminate serum from the assay.
	Poor target cell health	Thaw a new vial of cells to start a new culture. Use the recommended Cell Culture kit and Cell Plate Reagents.
	Use of reagents (e.g. serum or antibiotics) with residual β-gal activity	Use DiscoverX assay medium (Cell Plating 39 Reagent Cat. No. 93-0563R39 Series).

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