DiscoverX

User Manual

KILR®

Retroparticles for Cytotoxicity Assay

Retroviral Particles to Generate KILR Cell Lines

Table of Contents

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

w discoverx.com usa 1.866.448.4864 e SupportUS@discoverx.com eu +44.121.260.6142 e SupportEurope@discoverx.com

Overview

The KILR Retroparticles provide a potent vehicle for the stable delivery of the KILR reporter construct into almost any dividing cell line, enabling the creation of KILR cytotoxicity assays in the cell line expressing the target of your choice. The KILR Cytotoxicity cell line you generate 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 Cancer Immunotherapy (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.

Technology Principle

The KILR retroviral particle is a replication incompetent Moloney Murine Leukemia Virus (MMLV) engineered to drive expression of the KILR Reporter Protein, a housekeeping protein tagged with Enhanced ProLabel® (ePL), a β-galactosidase (β-gal) enzyme fragment. The KILR Retroparticles for Suspension cells contain a hybrid LTR promoter for expression of the fusion protein in difficult-to-transduce suspension cell lines, whereas the KILR Retroparticles for Adherent cells use a CMV promoter for expression of the KILR construct in most adherent cell lines. When transduced with the KILR Retroparticles, the target cells stably express the KILR Reporter Protein inside the cells. Death of the cells leads to rupture of the cell membrane and the KILR Reporter Protein is released into the media. 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.

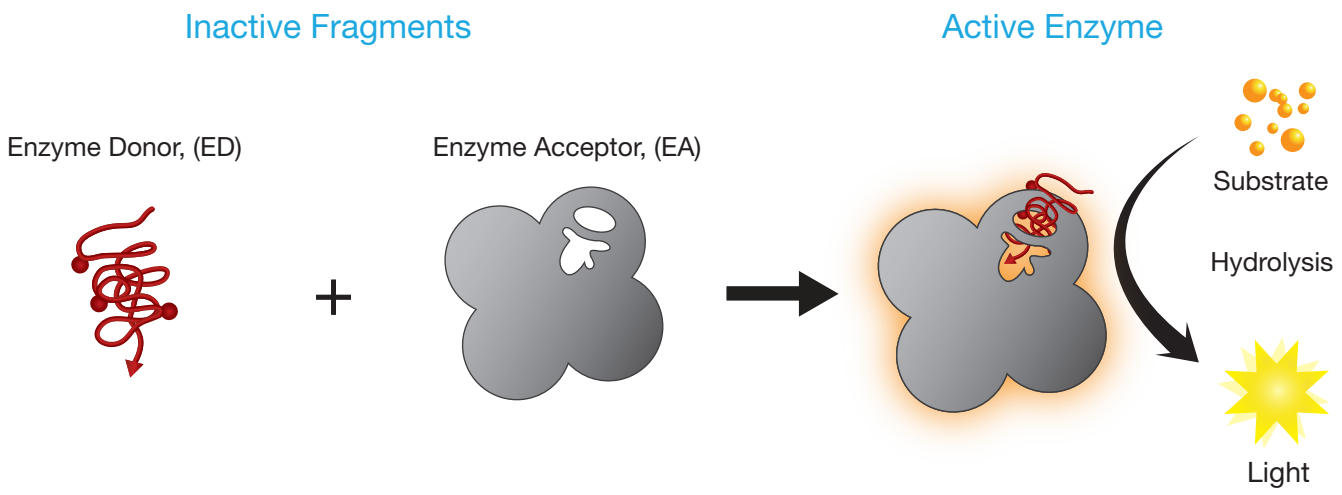


Figure 1. DiscoverX's proprietary PathHunter enzyme fragment complementation (EFC) technology consists of the β-galactosidase (β-gal) enzyme split into two inactive components, the enzyme donor peptide (ED also known as ePL) and an enzyme acceptor (EA). When brought together ED complements with EA to form active β-gal. The active enzyme then catalyzes the chemiluminescent substrate to generate light, providing an amplified signal to make for a high-sensitivity assay.

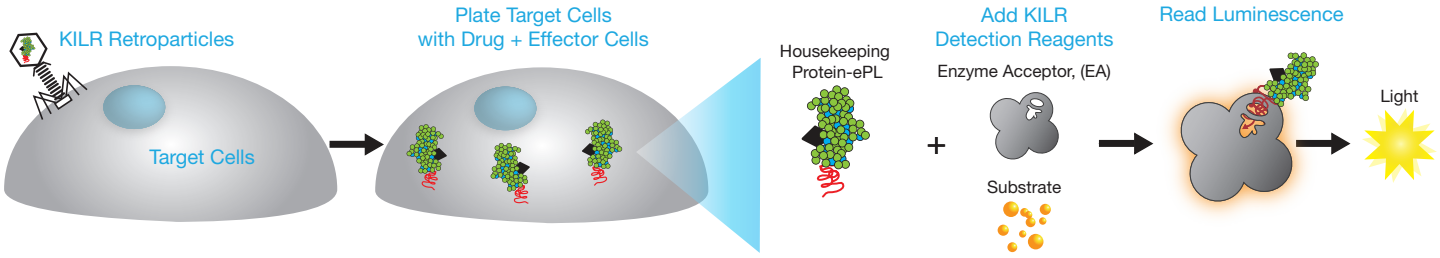


Figure 2. Target cells expressing the receptor antigen can be engineered to stably express a housekeeping protein that is tagged with enhanced ProLabel® (ePL), a β-gal reporter fragment using the KILR Retroparticles. When the stable target cell line is used in a cytotoxicity assay, and its membrane is 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.

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 cell-surface antigens have been coated with antigen-specific antibodies, harboring 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 for T cell redirection 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

List of Components	Configuration
KILR Retroparticles for Suspension Cells	4 x 1.0 mL media containing retroviral particles; 97-0002, 97-0006
KILR Retroparticles for Adherent Cells	4 x 0.5 mL media containing retroviral particles; 97-0003, 97-0005
KILR Retroparticles for Adherent & Suspension Cells	2 x 0.5 mL viral media (adherent cells) + 2 x 1.0 mL viral media (suspension cells) ; 97-0004, 97-0008
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar

Storage Conditions

Store at -80˚C. For one-time use only. Do not freeze-thaw.

Additional Materials Required

Materials	Ordering Information and Order Information
KILR Detection Kit	97-0001 Series*
6-well Cell Culture Plate (clear, non-pyrogenic, sterile treated, polystyrene)	Corning Cat. No. 3506
Tissue Culture Disposables Pipettes (1 mL – 25 mL) and Tissue Culture Flasks (T25 and T75 flasks, etc.)	
15 or 50 mL Polypropylene Tubes	
Cell Culture Media	Refer to cell line-specific datasheet
0.25% Trypsin-EDTA	

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

Safety and Handling Conditions

When working with live retroviral particles, follow the recommended federal and institutional guidelines for working with BSL-2 organisms.

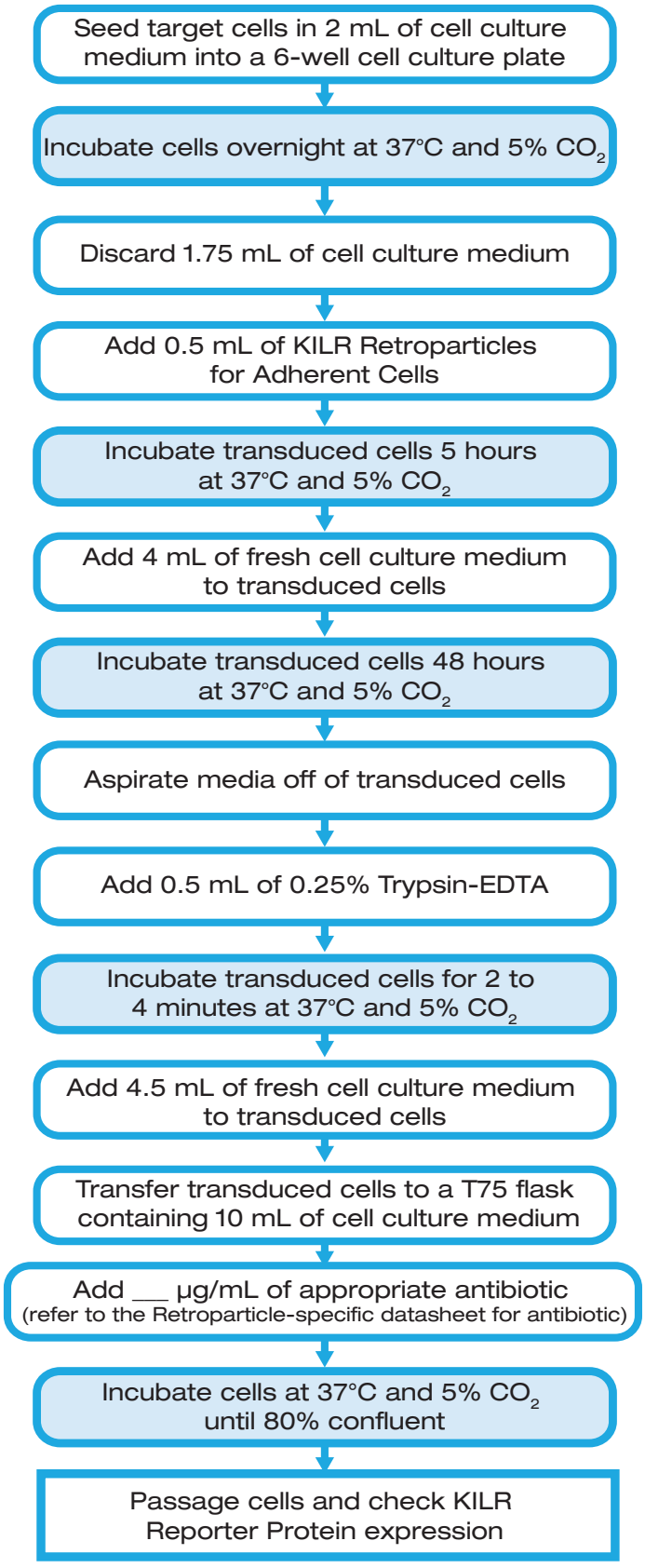
- Perform all manipulations within a certified biosafety cabinet.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.
- Dispose of used pipettes, pipette tips, and other tissue culture supplies as biohazardous waste in accordance with your federal and institutional guidelines.

Strictly follow all published Biosafety Level 2 (BSL-2) safety guidelines when working with retroviral particles with proper waste decontamination. For more information about the BSL-2 guidelines and retrovirus handling, refer to the document, “Biosafety in Microbiological and Biomedical Laboratories,” 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at US National Institutes of Health website at http://bmbl.od.nih.gov.

Handle all retroviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of retroviral particles may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the KILR Retroparticles.

Adherent Cells Protocol Schematic

Quick-Start Procedure: Each experiment requires a 6-well cell culture plate for each cell type and a T75 cell culture flask.



Detailed Assay Protocol for Adherent Cells

The following is a detailed protocol for infecting an adherent cell host with KILR Retroparticles.

Section I: Target Cell Preparation

The following is a protocol for harvesting target cells and preparing them for plating in a 6-well tissue culture plate, prior to their infection with the Retroparticles. The cells are seeded the day before the Retroparticle transduction. The cell culture medium will depend on the cell line being used. Consult the technical information from the vendor of the cell host being used to determine appropriate cell culture conditions. The protocol assumes that the target cell host is being cultured in a T75 flask.

1. Pre-warm cell culture medium chosen for culturing the target cell host in a 37°C water bath for 15 minutes.
2. Remove the T75 flask of target cells from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate medium from the T75 flask.
4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire face of the flask to rinse the cells.
5. Gently aspirate PBS from flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
7. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.
8. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.
10. Add 9 mL of cell culture medium to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added medium.
12. Remove the entire volume of cell suspension from the flask and transfer to a 15 mL conical tube.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

Keep cells on ice to maintain cell viability until ready for transfer to the 6-well plate.

Section II: Target Cell Plating

An appropriate number of cells must be added to a well of a 6-well plate. The required cell density on the plate will depend on the cell host being used. At the point the infection is initiated, a target confluency of 40-60% is desired so that the cells will be in log phase growth during the infection incubation period. To reach this target confluency, an appropriate number of cells per well (6-well plate) for a given cell line must be determined empirically. The table below provides examples of suggested cell numbers per well for a variety of common cell lines.

Target Cell Line	Cell Density (per Well)
U2OS	100,000
A549	100,000
SKOV3	400,000
NCI H292	200,000
SKBR3	400,000
HT-29	100,000

The following is a protocol for adjusting the density of the cell suspension and adding 2 mL of cell suspension into wells of a 6-well tissue culture plate.

1. 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.
2. Centrifuge the collected cells at 300 X g for 4 minutes.
3. After centrifugation, discard the supernatant.
4. Re-suspend the cell pellet in pre-warmed (37°C) cell culture medium. Based on the cell number obtained in Step 1 above, add enough cell culture medium so that the cells are suspended at the desired concentration (e.g. 100,000 cells/mL).
5. Gently mix the cells with a pipette so that the cell pellet is completely resuspended evenly.
6. Transfer 2 mL of the cell suspension to one well of the 6-well plate. There should be one well plated with cells for each infection that will be performed.
7. Incubate the 6-well plate overnight at 37°C and 5% CO_2.
8. Proceed to the Retroparticle Infection section.

Section III: Retroparticle Infection

The following is a protocol for transferring Retroparticles to target cells that were plated in a 6-well plate the previous day. One vial of KILR Retroparticles will be used per well of target cells on the 6-well plate.

1. Remove one vial of KILR Retroparticles for Adherent Cells from the -80°C freezer and allow it to thaw at room temperature for 10 minutes or until completely thawed.

Do not thaw Retroparticles in a 37°C water bath, and do not re-freeze them.

After infection, treat spent media containing viral particles with bleach to destroy any live virus.

2. Retrieve the 6-well cell culture plate containing the target cells from the tissue culture incubator.
3. Remove and discard 1.75 mL of medium from the well. Be careful not to disturb the cells.
4. With a pipette, carefully transfer the entire 0.5 mL of the thawed KILR Retroparticles from the vial directly to a single well of target cells. Do not distribute the contents of a single vial of Retroparticles across multiple wells with target cells. This will adversely impact the infection efficiency.
5. Return the 6-well plate to the tissue culture incubator and incubate at 37°C and 5% CO_2 for 5 hours.

Section IV: Cell Recovery

The following is a protocol for initiating target cell recovery.

1. Remove the 6-well cell culture plate containing infected cells from the incubator.
2. Add 4 mL of fresh cell culture medium to the wells containing the infected cells.
3. Incubate plate at 37°C and 5% CO_2 for 48 hours.

Section V: Antibiotic Selection

The KILR Retroparticles contain an antibiotic resistance marker to enable positive selection. The antibiotic concentration required to generate a stable pool is dependent on the cell host being used, and must be determined empirically. It is recommended that a kill curve be generated to determine the antibiotic concentration required to kill non-transduced cells. The appropriate concentration of antibiotic must be determined before the infection experiment is begun. Refer to the Retroparticle-specific datasheet to determine the appropriate selection antibiotic required to select a stable pool.

Take care to use the correct selection antibiotic specified on the Retroparticle datasheet. Using the incorrect antibiotic will result in death of all the cells.

Recommended Antibiotic Concentrations		
Target Cell Line	**G418 (µg/mL)**	**Hygromycin B (µg/mL)**
U2OS	500	250
A549	500	300
SKOV3	500	50
NCI H292	300	Not Determined
SKBR3	250	100
HT-29	400	Not Determined

The table to the left contains recommended concentrations of G418 and Hygromycin B for several commonly used cell lines.

At this point, the infection is complete. The following is a protocol for initiating antibiotic selection of the infected cells to generate a stable pool.

1. Pre-warm cell culture medium in a 37°C water bath for 15 minutes.
2. Remove the 6-well plate from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate medium from the infected cells in the 6-well plate.
4. Add 2 mL PBS into the well, very gently tip the 6-well plate from side-to-side to rinse cells.
5. Gently aspirate PBS from the well.
6. Add 0.5 mL of 0.25% Trypsin-EDTA to the well.
7. Gently rock the 6-well plate back and forth to ensure the surface of the well is thoroughly covered with Trypsin-EDTA
8. Incubate the plate at 37°C and 5% CO_2 for 2 to 4 minutes or until the cells have detached.
9. Remove the plate from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the plate to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.
10. Add 4.5 mL of cell culture medium to the well.
11. Using a pipette, gently rinse the cells from the surface of the well with the added medium.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

12. Transfer 5 mL of cells to a clean T75 tissue culture flask.
13. Add 10 mL of cell culture medium to the T75 flask. The total volume in the flask should be 15 mL.
14. Add an appropriate volume of selection antibiotic to the flask to achieve the predetermined (e.g determined from a kill curve) concentration.
15. To generate a stable pool overexpressing the KILR Reporter Protein, incubate plate at 37°C and 5% CO_2 in a humidified tissue culture incubator until cells reach 70-80% confluency (typically 5 to 10 days).
16. To ensure the complete antibiotic selection has been achieved, passage cells one time using a conservative (i.e 1:2 or 1:3) cell split ratio.
    a. Pre-warm cell culture medium supplemented with the pre-determined concentration of selection antibiotic in a 37°C water bath for 15 minutes.
    b. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
    c. Gently aspirate medium from the T75 flask.
    d. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire face of the flask to rinse the cells.
    e. Gently aspirate PBS from flask.
    f. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.

g. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.

h. Incubate the flask at 37°C and 5% CO_2 for 2 to 4 minutes or until the cells have detached.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

i. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

j. Add 4 mL of the cell culture medium to the T75 flask.

k. Using a pipette, gently rinse the cells from the surface of the flask with the added medium.

l. Add 12 mL of cell culture medium to a separate T75 flask.

m. Transfer one half to one third (depending on whether or not a 1:2 or 1:3 split ratio is used) of the cell suspension from the first flask to the 12 mL of medium in the new T75 flask. Transfer flask to a tissue culture incubator and incubate cells until they reach 70-80% confluency at 37°C and 5% CO_2.

17. Incubate T75 flask at 37°C and 5% CO_2 in a humidified tissue culture incubator until cells reach 70-80% confluency.

Section VI: Detection of Expression Level

By this point, the cell pool should be stably expressing the KILR Reporter Protein. The following is a protocol for evaluating the expression level of the KILR Reporter Protein in this stable pool using the KILR Detection Kit (Cat. No. 97-0001). Cells will be treated with solutions containing cell lysis buffer and either Reagent 2 (the EA reagent) or buffer. The differences of signal generated by the two treatments [with vs. without Reagent 2] will reveal expression of the KILR Reporter Protein. It can also be informative to run this experiment on the native parental cells used to generate the KILR cell pool, to compare the basal activities of the transduced cells versus the non-transduced cells.

1. Remove the T75 flask containing the KILR stable pool from the tissue culture incubator.
2. Aspirate the medium from the flask.
3. Add 5 mL PBS into the T75 flask and very gently tip the flask side to side to allow the PBS to cover the entire face of the flask to rinse the cells.
4. Gently aspirate the PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
6. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.
7. Incubate the flask at 37°C and 5% CO_2 for 2 to 4 minutes or until the cells have detached. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

8. Add 9 mL of cell culture medium to the T75 flask. If also testing non-transduced native cells for their basal activity, follow the same steps for harvesting the cells.

9. Using a pipette, gently rinse the cells from the surface of the flask with the added medium.
10. Remove the entire amount of cells from the flask and transfer to a 15 mL conical tube.
11. 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.

Keep suspended cells on ice to maintain cell viability until ready for transfer to the assay plate.

    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.
12. Based on the cell number obtained in Step 11 above, dilute the resuspended cells to 200,000 cells/mL (i.e. 20,000 cells/100 µL).
13. Transfer a portion of the cells to a sterile reservoir.
14. Transfer 100 µL (20,000 cells) of the cell suspension to each of 6 wells of a 96-well assay plate. If also testing non-transduced cells, be sure to plate them in wells other than the 6 wells occupied by the KILR cell pool.

Be sure to save a portion of the cell suspension for propagating the KILR pool in a separate tissue culture flask.

15. Transfer the assay plate to a tissue culture incubator to incubate the assay plate at 37°C and 5% CO_2 until the KILR Detection Reagents are prepared and ready to be added to the cells.
16. Prepare two working KILR detection solutions in separate tubes: one solution with KILR Detection Reagent 2 (for positive signal detection); the second solution with PBS (for negative control). The two working solutions consist of 4-parts of KILR Detection Reagent 1, 1-part of KILR Detection Reagent 2 (or PBS for the negative control), and 1-part of KILR Detection Reagent 3. Refer to the user manual for the KILR Detection Kit (70-337 KILR Detection Kit) for more complete information on the detection reagents. If testing non-transduced native cells, double the reagent volumes indicated in the table below.

Assay Reagents	With Reagent 2	Without Reagent 2
KILR Detection Reagent 1 (µL)	240	240
KILR Detection Reagent 2 (µL)	60	0
KILR Detection Reagent 3 (µL)	60	60
PBS (µL)	0	60
Total Volume (µL)	**360**	**360**

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

17. Mix reagents by gently inverting the tubes a few times. Briefly centrifuge the tubes to make sure the entire reagent volume is at the bottom of the tube.
18. Remove the 96-well assay plate from the tissue culture incubator.
19. Add 2 µL of the KILR Total Lysis Control reagent to each of the 6 test wells (or 12 wells if also testing non-transduced cells).
20. To each of 3 wells, add 100 µL of working KILR detection solution containing KILR Detection Reagent 2. To each of the other 3 wells, add 100 µL of working KILR detection solution containing PBS. It is not recommended that the solution in the wells be mixed by pipetting up and down or by vortexing the plate.

21. Place lid back on plate and incubate assay plate for at least 30 minutes at room temperature in the dark.

22. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. In general, the signal continues to increase over time. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturer may include a cut-off filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

23. The signal/background ratio must be calculated to evaluate the relative expression of the KILR Reporter Protein.

    a. Calculate the mean of the RLU values generated by the 3 replicate wells treated with the working detection solution containing KILR Detection Reagent 2. The result is the **Mean Signal**.

    b. Calculate the mean of the RLU values generated by the other 3 replicate wells treated with the working solution containing PBS. The result is the **Mean Background**.

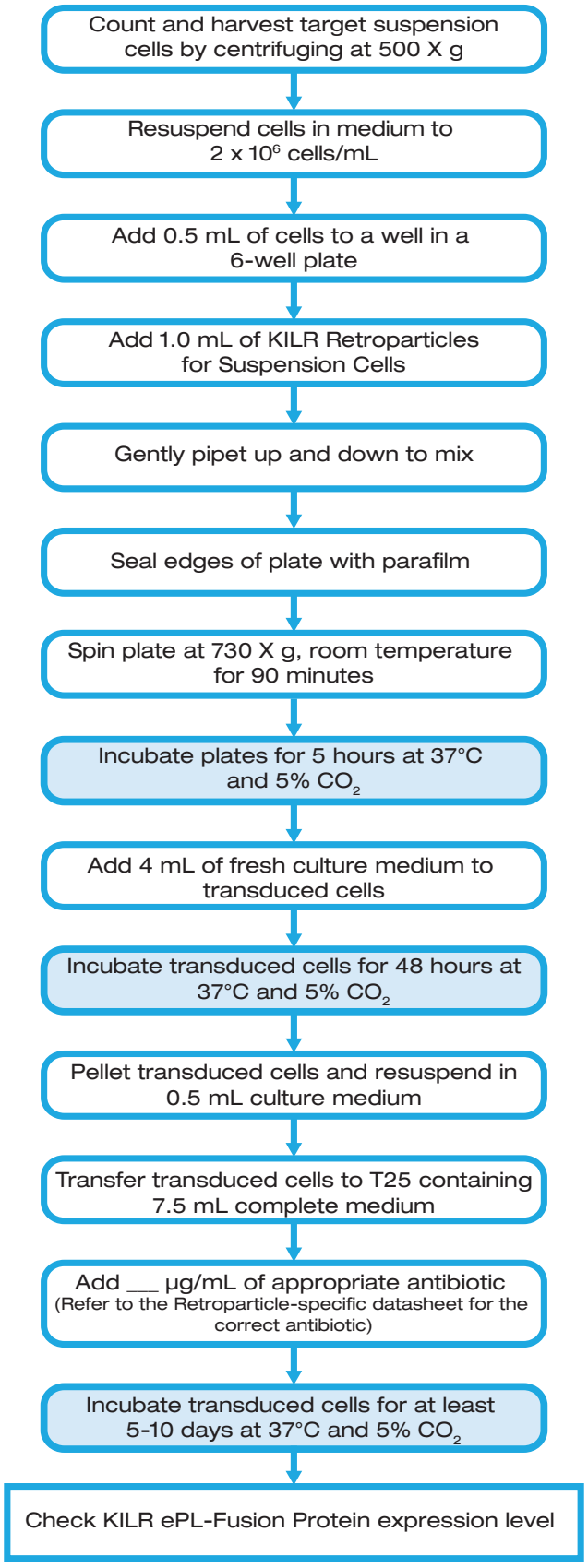
    c. Divide the **Mean Signal** by the **Mean Background**. The result is the Signal/Background Ratio (**S/B ratio**).

       S/B ratio = Mean Signal / Mean Background

24. Refer to Guidelines for Interpreting Results in the Supplemental Information Section.

Suspension Cells Protocol Schematic

Quick-Start Procedure: Each experiment requires a 6-well cell culture plate for each cell type and a T75 cell culture flask.



Detailed Assay Protocol for Suspension Cells

The following is a detailed protocol for infecting a suspension cell host with KILR Retroparticles.

Section I: Target Cell Preparation

The following is a protocol for harvesting target cells and preparing them for plating in a 6-well tissue culture plate, prior to their infection with the Retroparticles. The cells are seeded the same day as the Retroparticle transduction. The cell culture medium will depend on the particular cell line being used. Consult technical information from the vendor of the cell host being used to determine appropriate cell culture conditions. The protocol assumes that the target cell host is being cultured in a T75 flask and the density of the suspension cells are at least 1 x 10^6 cells/mL before starting this protocol. It is recommended that the cells be fed with fresh medium the day before the Retroparticle infection will be initiated. This will ensure the cells are healthy and in log phase growth to maximize the infection efficiency.

1. Pre-warm cell culture medium chosen for culturing the target cell host in a 37°C water bath for 15 minutes.
2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
3. Feed cells with fresh culture medium. It is best to complete this step when the cell density is at least 1 x 10^6 cells/mL to ensure that the cells are in log phase growth. Count cells and calculate their density in the suspension. If cell density is adequate, feed cells by adding fresh cell culture medium at a volume equivalent to 50% of the volume of the cell suspension already present in the flask. For example, if there is 20 mL in the flask, add 10 mL fresh cell culture medium.
4. Return to 37°C and 5% CO_2 incubator overnight.
5. On the day of infection, remove the flask from the tissue culture incubator and place in the sterile tissue culture hood.
6. Gently swirl the flask to mix the cells, transfer at least 10 mL of the cell suspension to a 15 mL conical tube.

Section II: Target Cell Plating

An appropriate number of cells must be added to a well of a 6-well plate. The number of cells can vary depending on cell type. The following is a list of cell types typically require 1 x 10^6 cells per well, provided the cells are in log phase growth: Jurkat, Raji, Daudi, Ramos, and THP-1.

The following is a protocol for adjusting the density of the cell suspension and adding 2 mL of cell suspension into wells of a 6-well tissue culture plate.

1. 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.
2. Centrifuge the collected cells at 300 X g for 4 minutes.

3. After centrifugation, discard the supernatant, and re-suspend the cell pellet in pre-warmed (37°C) cell culture medium. Based on the cell number obtained in Step 1 above, add enough cell culture medium so that the cells are suspended at a concentration of 2 x 10^6 cells/mL.
4. Gently mix the cells with a pipette so that the cell pellet is completely broken up and evenly suspended.
5. Transfer 0.5 mL of the resuspended cells to one well of a 6-well tissue culture plate.
6. Incubate the 6-well plate at 37°C and 5% CO_2 until the Retroparticles are ready to be added to the target cells (on the same day).
7. Proceed to the Retroparticle Infection Section.

Section III: Retroparticle Spin Infection

The following is a protocol for transferring Retroparticles to target cells that were plated in a 6-well plate. These steps should be completed the same day as the cells are plated. One vial of KILR Retroparticles will be used per well of target cells on the 6-well plate.

1. Remove 1 vial of KILR Retroparticles for Suspension Cells from the -80°C freezer and allow it to thaw at room temperature for 10 minutes or until completely thawed.

After infection, treat spent media containing viral particles with bleach to destroy any live virus.

Do not thaw Retroparticles in a 37°C water bath, and do not re-freeze them.

2. Retrieve the 6-well cell culture plate containing the target cells from the tissue culture incubator.
3. With a P1000 pipette, carefully transfer the entire 1.0 mL of the thawed KILR Retroparticles from the vial directly to the target cells on the plate. Then use the pipette to gently mix the virus supernatant and cells together by pipetting up and down. The volume should now be 1.5 mL. Do not distribute the contents of a single vial of Retroparticles across multiple wells of the target cells. This will adversely impact the infection efficiency.

It is important to avoid creating bubbles when mixing.

4. Perform the following spin infection protocol:
   a. Wrap the edges of the 6-well plate with parafilm to contain any leakage. Take care to avoid spilling the medium.
   b. Weigh the 6-well plate. Generate a balance plate with an equivalent weight by adding medium (e.g. 1.5 mL multiplied by number of filled wells) to a second 6-well plate. Wrap parafilm around the edges of the balance plate to contain any leakage.

It is important to ensure both plates have the same weight to prevent damage to the centrifuge.

   c. Carefully put the plates in opposing plate holders of a swinging bucket rotor in a table top centrifuge (e.g. Beckman Coulter Allegra, or similar instrument); spin plates at 730 X g for 90 minutes at room temperature.

Make sure the plates are secured in plate holders before commencing with spinning.

   d. After the 90 minute spin, remove the 6-well plate from the centrifuge. Carefully remove the parafilm from its edges.

e. Return the 6-well plate to the tissue culture incubator and incubate at 37°C and 5% CO_2 for 5 hours.

Section IV: Cell Recovery

The following is a protocol for initiating target cell recovery.

1. Remove the 6-well cell culture plate containing infected cells from the incubator.
2. Add 4 mL of fresh cell culture medium to the wells containing the infected cells.
3. Incubate plate at 37°C and 5% CO_2 for 48 hours.

Section V: Antibiotic Selection

The KILR Retroparticles contain an antibiotic resistance marker to enable positive selection. The antibiotic concentration required to generate a stable pool is dependent on the cell host being used, and must be determined empirically. It is recommended that a kill curve be generated to determine the antibiotic concentration required to kill non-transduced cells. The appropriate concentration of antibiotic must be determined before the infection experiment is begun. Refer to the Retroparticle-specific datasheet to determine the appropriate selection antibiotic required to select a stable pool.

Take care to use the correct selection antibiotic specified on the Retroparticle datasheet. Using the incorrect antibiotic will result in death of all the cells.

Recommended Antibiotic Concentrations

Target Cell Line	G418 (µg/mL)	Hygromycin B (µg/mL)
Jurkat	500	250
Raji	1000	100
Daudi	800	200
Ramos	500	Not Determined
THP-1	500	200

The table to the left contains recommended concentrations of G418 for several commonly used cell lines. Appropriate concentrations of Hygromycin B must be determined empirically.

By this point, the infection is complete. The following is a protocol for initiating antibiotic selection of the infected cells to generate a stable pool.

1. Pre-warm cell culture medium in a 37°C water bath for 15 minutes.
2. Remove the 6-well plate from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently transfer the infected cells from the 6-well plate to a 15 mL conical tube.
4. Centrifuge the collected cells at 300 X g for 4 minutes.
5. Gently aspirate the medium from the infected cells.
6. Add 7.5 mL of cell culture medium to a T25 tissue culture flask.
7. Add 0.5 mL of cell culture medium to the pelleted cells. Gently resuspend the cells using a pipette.
8. Transfer all of the cells to the T25 tissue culture flask containing the cell culture medium.
9. Add an appropriate volume of selection antibiotic to the flask to achieve the predetermined (e.g determined from a kill curve) concentration.

10. To generate a stable pool overexpressing the KILR Reporter Protein, incubate plate at 37°C and 5% CO_2 in a humidified tissue culture incubator until the cells reach a density of 1 x 10^6 cells/mL (typically 5 to 10 days).
11. To ensure the complete antibiotic selection has been achieved, dilute the cells one time using a conservative (i.e. ~1:3) dilution ratio.
    a. Pre-warm cell culture medium supplemented with the pre-determined concentration of selection antibiotic in a 37°C water bath for 15 minutes.
    b. Remove the T25 flask from the tissue culture incubator and place in a sterile tissue culture hood.
    c. Add 17 mL of cell culture medium to a separate T75 flask.
    d. Transfer the entire volume of cell suspension from the T25 flask to the T75 flask.
12. Incubate T75 flask at 37°C and 5% CO_2 in a humidified tissue culture incubator until cells reach a density of 1 x 10^6 cells/mL.

Section VI: Detection of Expression Level

By this point, the cell pool should be stably expressing the KILR Reporter Protein. The following is a protocol for evaluating the expression level of the KILR Reporter Protein in this stable pool using the KILR Detection Kit (Cat. No. 97-0001). Cells will be treated with solutions containing cell lysis buffer and either Reagent 2 (the EA reagent; the compliment to the ePL-tagged KILR Reporter Protein) or buffer. The differences of signal generated by the two treatments [with vs. without Reagent 2] will reveal expression of the KILR Reporter Protein. It can also be informative to run this experiment on the native parental cells used to generate the KILR cell pool, to compare the basal activities of the transduced cells versus the non-transduced cells.

1. Remove the T75 flask containing the KILR stable pool from the tissue culture incubator.
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. Based on the cell number obtained in Step 4 above, dilute the resuspended cells to 200,000 cells/mL (i.e. 20,000 cells/100 µL). If also testing non-transduced native cells for their basal activity, follow the same steps for harvesting the cells and resuspending.
6. Transfer a portion of the cells to a sterile reservoir.
7. Transfer 100 µL (20,000 cells) of the cell suspension to each of 6 wells of a 96-well assay plate. If also testing non-transduced cells, be sure to plate them in wells other than the 6 wells occupied by the KILR cell pool.

Be sure to save a portion of the cell suspension for propagating the KILR pool in a separate tissue culture flask.

8. Transfer the assay plate to a tissue culture incubator to incubate the assay plate at 37°C and 5% CO_2 until the KILR Detection Reagents are prepared and ready to be added to the cells.

9. Prepare two working KILR detection solutions in separate tubes: one solution mixed with KILR Detection Reagent 2 (for positive signal detection); the second solution with PBS (for negative control). The two working detection solutions consist of 4-parts of KILR Detection Reagent 1, 1-part of KILR Detection Reagent 2 (or PBS for the negative control), and 1-part of KILR Detection Reagent 3. Refer to the user manual for the KILR Detection Kit for more complete information on the detection reagents. If also testing non-transduced native cells, double the reagent volumes indicated in the table below.

10. Mix reagents by gently inverting the tubes a few times. Briefly centrifuge the tubes to make sure the entire reagent volume is at the bottom of the tube.

Assay Reagents	With Reagent 2	Without Reagent 2
KILR Detection Reagent 1 (µL)	240	240
KILR Detection Reagent 2 (µL)	60	0
KILR Detection Reagent 3 (µL)	60	60
PBS (µL)	0	60
Total Volume (µL)	**360**	**360**

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

11. Remove the 96-well assay plate from the tissue culture incubator.

12. Add 2 µL of the KILR Total Lysis Control reagent to each of the 6 test wells (or 12 wells if also testing non-transduced cells).

13. To each of 3 wells, add 100 µL of working KILR detection solution containing KILR Detection Reagent 2. To each of the other 3 wells, add 100 µL of working KILR detection solution containing PBS. It is not recommended that the wells be mixed by pipetting up and down or by vortexing the plate.

14. Place lid back on plate and incubate assay plate for at least 30 minutes at room temperature in the dark.

15. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. In general, the signal continues to increase over time. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturer may include a cut-off filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

16. The signal/background ratio must be calculated to evaluate the relative expression of the KILR Reporter Protein.

    a. Calculate the mean of the RLU values generated by the 3 replicate wells treated with the working detection solution containing KILR Detection Reagent 2. The result is the **Mean Signal**.

    b. Calculate the mean of the RLU values generated by the other 3 replicate wells treated with the working detection solution containing PBS. The result is the **Mean Background**.

    c. Divide the **Mean Signal** by the **Mean Background**. The result is the Signal/Background Ratio (**S/B ratio**).
    S/B ratio = Mean Signal / Mean Background

17. Refer to Guidelines for Interpreting Results in the Supplemental Information Section.

Supplemental Information

Guidelines for Interpreting Expression Results

Typical relative expression levels of the KILR Reporter Protein in various cell backgrounds (both adherent and suspension) are shown in the table below. Relative expression is determined with the aid of the KILR Detection Kit (Cat. No. 97-0001). Signal/Background ratios (S/B) >20 suggest expression levels of the KILR Reporter Protein are sufficient for using the assay for studying cytotoxicity.

Cell Line Pool*	Cell Type	S/B Ratio
U2OS	Adherent	180
SKBR3	Adherent	28
SKOV3	Adherent	54
Daudi	Suspension	504
Raji	Suspension	37
A549	Adherent	76

* These cell lines are available for sale. Visit discoverx.com/kilr for more details.

Protocols for Running Cytotoxicity Assays

The newly established KILR stable cell pool can be used for a variety of cytotoxicity applications, including ADCC and ADCP assays. Refer to the following user manuals for KILR ADCC assays using Adherent or Suspension target cells.

KILR ADCC Assays for Suspension Cell Lines discoverx.com/KILRSuspensionUM

KILR ADCC Assays for Adherent Cell Lines discoverx.com/KILRAdherentUM

Refer to the KILR CD16 Effector Cells user manual (document No. 70-388) for using single donor-derived effector cells in any ADCC assay. The following link is to a webpage that contains a link for downloading an application note with protocols for KILR ADCP Assays:

KILR ADCP Assay Application Note discoverx.com/ADCP

Protocols for all other cytotoxicity applications (e.g. CDC, T cell redirection, 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 artificially lyse the cells.

Frequently Asked Questions

Why is my KILR fusion protein expression level low?

Reasons and Solutions for Low KILR ePL-Fusion Protein Expression	
Reasons	**Solutions**
Too much or too little antibiotic used for selection	Determine the antibiotic sensitivity of the target cell line by performing a kill curve experiment.
Viral stocks stored incorrectly	Viral stocks should be stored at -80°C. Do not freeze-thaw. Stock should only be used once.
Cell type difficult to transduce	Some suspension lines are refractory to infection. Perform two consecutive rounds of infection (2 x 1 mL of virus) before putting cells under selection to boost multiplicity of infection (MOI) if insufficient expression was achieved with a single infection. If using Retroparticles for Adherent Cell Lines, try using the Retroparticles for Suspension Cells, which uses a different promoter for expression of the ePL-labeled reporter protein.
Detection reagent degraded	KILR detection kit contains protein components that need to be stored at -20°C. Ensure that the detection kits are stored properly and the detection reagents are not past their expiration date.

What factors affect cell infection efficiency?

Factors that Affect Infection Efficiency	
Factors	**Details**
Characteristics of target cell line	The target cell lines must be dividing, ideally with a doubling time between 16 and 20 hours. Adherent cell lines are generally easier to infect than suspension lines.
Media content of viral supernatant	Viral supernatants are generated by harvesting spent media containing virus from the packaging cells. Spent media lacks nutrients and may contain some toxic metabolic waste products. This may affect growth of target cells.
Silencing of integration site of gene	Repeat infection.

Why do my cells look unhappy after infection?

Factors that Affect Cell Recovery from Infection	
Factors	**Solutions**
Too many/too few target cells plated for infection; Cells not growing/dividing optimally	Make sure target cells are plated at correct cell density.
Target cells should be healthy and in log phase growth	Be sure cells are healthy and in log phase growth before starting infection.
Low transduction efficiency	Reduce antibiotic concentration; use conditioned media; repeat transduction and increase target cell density (e.g. reduce the well size/media volume so cells can condition their media).

Why are all the cells dead after addition of selection antibiotic?

Factor that Affects Cell Viability	
Factor	**Solution**
Incorrect selection antibiotic used	If incorrect selection antibiotic was used, repeat the Retroparticle infection with a fresh culture of target cells. Select stable pool with the correct antibiotic. Refer to Retroparticle-specific datasheet to determine the correct selection antibiotic.

Limited Use License Agreement

These products may be covered by issued US and/or foreign patents, patent application and subject to Limited Use Label License.

Please visit discoverx.com/license for limited use label license and trademark information for this product.