Specifically Measure Target Cell Death in a Co-Culture with Immune Cells Through a Non-Radioactive, Dye-Free Cytotoxicity Assay

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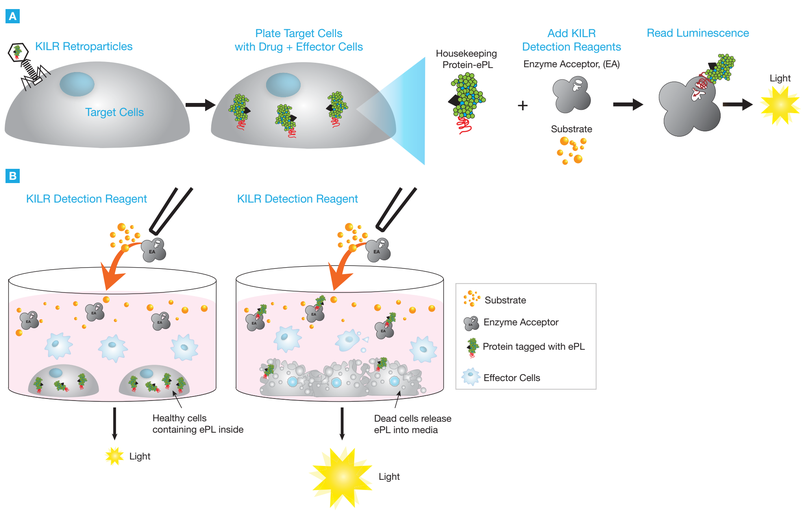
Abstract

Following the recent regulatory approvals of a number of checkpoint inhibitor therapeutics, development of a wide array of immunomodulatory therapeutic antibodies targeting novel antigens and with distinct mechanisms of action (MOA) have emerged. These include classical monoclonal antibodies, but also increasingly bi- and multi-specific antibodies designed to redirect T-cells to tumors and modulate anti-tumor T-cell responses. In addition to the classical antibody (Fc) effector functions that developers must monitor, such as Antibody-dependent cell-mediated cytotoxicity (ADCC) and Complement-dependent cytotoxicity (CDC), there is increasing interest in following another type of effector mediated killing, known as Antibody-dependent cellular phagocytosis (ADCP). Here, we present a novel cytotoxicity assay using our proprietary enzyme fragment complementation (EFC) technology that specifically measures killing of target cancer cells in a co-culture with immune cells, in an easy to use, dye-free & radioactivity-free assay. Importantly, the same technology is applicable to multiple applications such as ADCC, CDC, ADCP and T-cell redirection. We discuss examples where the same engineered target cell line is used to determine ADCC, CDC and ADCP capabilities of an antibody drug. These assays have very low background, thus producing robust assay windows, with excellent precision, and are applicable to multiple stages of drug development, ranging from screening to use in QC lot release of these complex biologic drugs. This assay has been tested using multiple primary effector cells (PBMCs, Macrophages and NKs) or engineered cells lines (NK92). Further, this assay is applicable to other immunotherapy drugs such as T-cell redirecting bi-specific antibodies and chimeric antigen receptor T-cells (CAR-T).

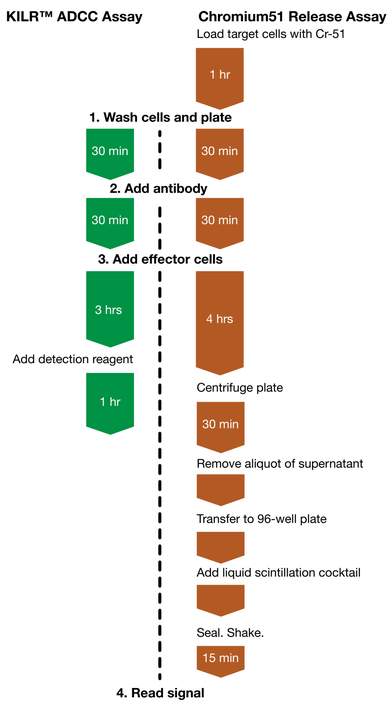
Unparalleled Specificity of the KILR® Cytotoxicity Assays



A. Target cells can be engineered to stably express a housekeeping protein 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.

B. 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 to the well, we cannot detect chemiluminescence as the KILR reporter protein does not leak out through an intact cell membrane into the media. Alternatively, in the well on the right, the target cancer 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.

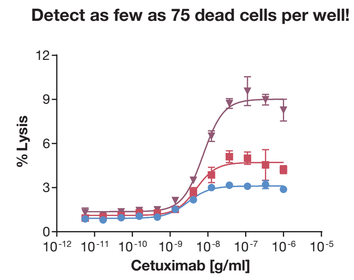
Increase Lab Efficiency with an Easy-to-Use Assay Protocol



- Simple, add and read protocol
- No centrifugation or transfer steps
- Results obtained in nearly half the time needed to run a Chromium 51 release assay
  - KILR= 5hr
  - Chromium51= 8hr
- No radioactivity

Comparison of workflow for KILR ADCC assay to Chromium51 release. Figure highlights the short overall assay time of 5 hr for the homogeneous KILR ADCC assay (green) vs 8hr for Chromium51 release (orange). Using the KILR assays, you can eliminate the need to load target cells prior to every experiment, the use of radioactivity, and reduce the number of steps, increasing the efficiency of the lab.

Unparalleled Sensitivity of KILR Assays



	Hill Slope	EC_{50}	S/B
2.5K (E:T=25:1)	1.509	2.936e-009	4.0
5K (E:T=25:1)	1.653	4.86e-009	5.4
10K (E:T=25:1)	1.602	7.391e-009	8.4

KILR H322 cells were plated at various densities with primary human PBMCs and Cetuximab, an anti-EGFR drug, to measure ADCC response. ADCC is a known mechanism of action for Cetuximab, where the antibody activates immune cells through its Fc effector function to kill target cancer cells. Target cell death is reported as % Lysis, a ratio of experimental signal to total signal generated when the cells are lysed with detergent. The data on the left shows we can robustly detect 3% lysis in the KILR H322 cells when a total of 2500 KILR cells are plated in the well, indicating that we have detected the death of 75 cells inside the well. This exquisite sensitivity of the assay demonstrates its value in applications such as screening and lead optimization, where sensitivity is critical to identify and optimize lead drug candidates.

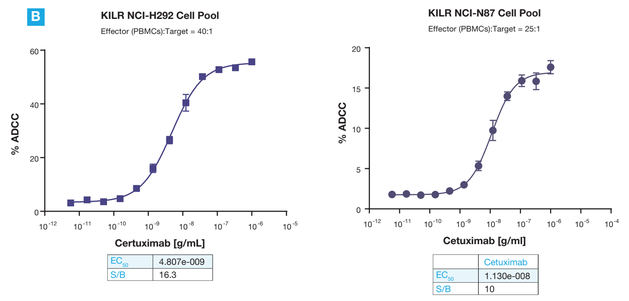
ADCC Response to Cetuximab in EGFR+ KILR Cell Lines

A

Receptor Density Data

Cell Model	EGFR Status	No. of Receptors/Cell
ARH77	Negative	333
NCI-N87	Positive (low)	1,245
A549	Positive (med)	1,636
H322	Positive (med)	1,875
SK-MEL-5	Positive (med)	2,198
SK-MEL-28	Positive (med)	2,149
NCI-H292	Positive (high)	3,022
U-2 OS +EGFR	Positive (high)	3,970

B

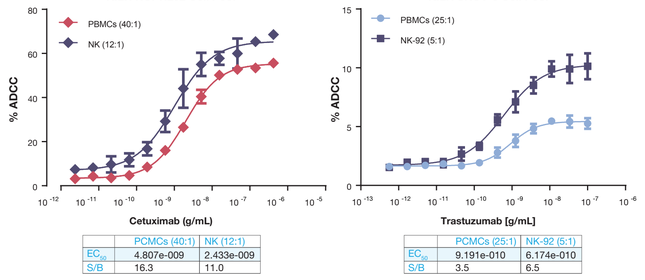


EC_{50}	4.807e-009
S/B	16.3

	Cetuximab
EC_{50}	1.130e-008
S/B	10

A. Quantitative receptor density data are shown indicating that we have cell lines with low, medium and high levels of receptor expression. B. ADCC response to cetuximab in NCI-H292 (high EGFR expression) and NCI-N87 (low expression) KILR cell pools is shown (displayed as % ADCC). Both cell models generate robust assay windows (>10-fold) with an effector:target ratio of 25:1 or more using primary PBMCs.

KILR Assays are Compatible with Multiple Types Of Effector Cells

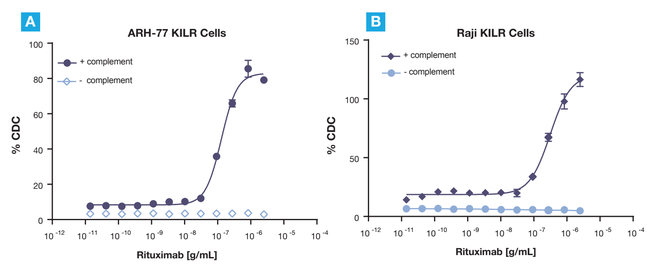


	PCMCs (40:1)	NK (12:1)
EC_{50}	4.807e-009	2.433e-009
S/B	16.3	11.0

	PCMCs (25:1)	NK-92 (5:1)
EC_{50}	9.191e-010	6.174e-010
S/B	3.5	6.5

KILR ADCC assay format is compatible with multiple effector cell types. A. Cetuximab–mediated ADCC in NCI-H292 cells with primary PBMCs and NK cells. NCI-H292 cells, which endogenously express high levels of EGFR, were stably transduced with the KILR reporter protein and treated with Cetuximab in the presence of effector cells. 10,000 cells/well were incubated with cetuximab and purified NKs (E:T=12:1) or PBMCs (E:T = 40:1) for 3hr prior to detection of target cell death with the KILR detection kit. B. Trastuzumab–mediated ADCC in SKOV-3 cells was tested with primary PBMCs or the NK92 cell line (ConkWest). NK-92 cells (E:T= 5:1) or primary PBMCs in 25-fold excess (E:T= 25:1) were incubated with SKOV-3 cells in the presence of trastuzumab and ADCC was detected by the addition of KILR detection reagents.

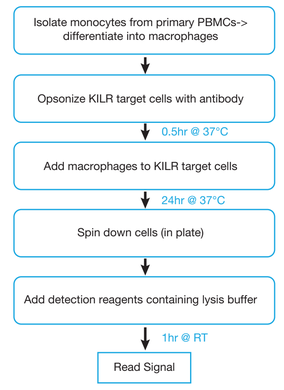
Robust CDC Response with Rituximab in KILR Cells



A simple protocol was developed to test for CDC, mediated by Rituximab and complement in CD20-positive cells. In brief, target cells are opsonized with Rituximab for 15 minutes prior to treatment with 5% baby rabbit complement for 45 minutes, followed by addition of KILR detection reagents. A. Results with ARH-77 cells, plotted as % CDC (or a percentage of total lysis). Note no lysis is observed in the absence of complement. B. Results with Raji cells, plotted as % CDC (or a percentage of total lysis). Note no lysis is observed in the absence of complement.

A Plate-Based Antibody Dependent Cellular Phagocytosis Assay Measuring Cell Death

A



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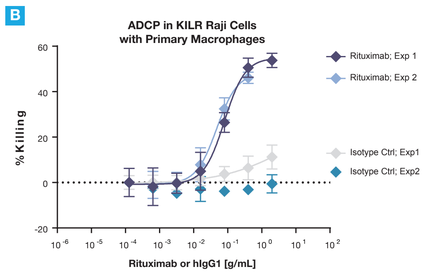
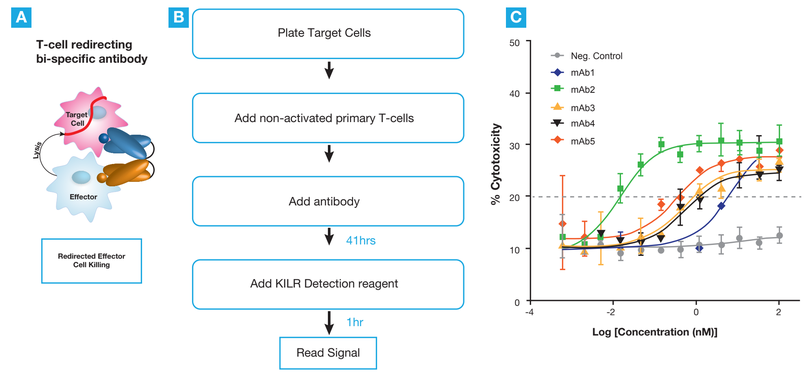


A. Protocol for the use of KILR Raji cells in an ADCP assay. Monocytes from primary PBMCs are isolated by standard techniques, then differentiated to M1 macrophages for 7 days (cultured for 7 days in presence of M-CSF, and differentiated with IFNg for last 24hr). KILR cells are then opsonized with antibody of interest for 30 minutes, then co-incubated with macrophages (E:T=4:1) for 24hr. Plate is spun down to concentrate cells and then the cells are lysed in the presence of detection reagents, and signal is read on a chemiluminescent plate reader. B. ADCP results with Rituximab, an anti-CD20 antibody, and isotype control in KILR Raji cells using M1 macrophages derived from frozen PBMCs relative to an isotype control. In this case, the same frozen PBMCs were used in two independent experiments (dark blue vs light blue), and results are highly reproducible. Data presented as % killing: (100 x {RLU (cells with antibody) – RLU (cells without antibody)} / RLU (cells without antibody)).

Simple Detection of Bispecific Antibody Mediated T-Cell Redirection



A. The mechanism of action (MOA) of certain bi-specific antibodies is to bind to T-cells with one arm and to the target antigen on the cancer cells with the other arm. B. An assay was developed to confirm that activation of T-cells was occurring with the bi-specific antibody. Target cells expressing the antigen of interest and the KILR reporter protein were seeded into a 384-well plate, with non-activated T-cells and bi-specific antibodies (5 with one arm targeting a cancer-specific antigen and one negative control). The assay was incubated for 41hr at 37C. KILR detection reagent was added for 1hr at R/T prior to reading signal on a chemiluminescent plate reader. C. The 5 antigen-specific antibodies produced a dose-dependent increase in killing with high reproducibility. One molecule displayed a significantly potent response, while the negative control shows no dose-dependent increase in killing. Rank order of the antibodies matched results obtained with a more labor intensive flow-based killing assay (data not shown).

Summary

Benefits of the KILR Cytotoxicity Assays

- Broad applications for cancer immunotherapy drug development
- Unparalleled Specificity – Signal only from dead target cells
- Easy to use – Simple add and read protocol with chemiluminescent readout
- Exquisite Sensitivity – Detect as few as 75 dead cells with high reproducibility
- Ultimate Flexibility – Ability to run cytotoxicity assay from 30 minutes to 72 hours
- Use available KILR cell lines or make your own stable KILR cell lines with retroviral particles!!

