A novel dye-free cytotoxicity assay to selectively measure target cell death in ADCC, CDC CAR-T or T-cell activation assays

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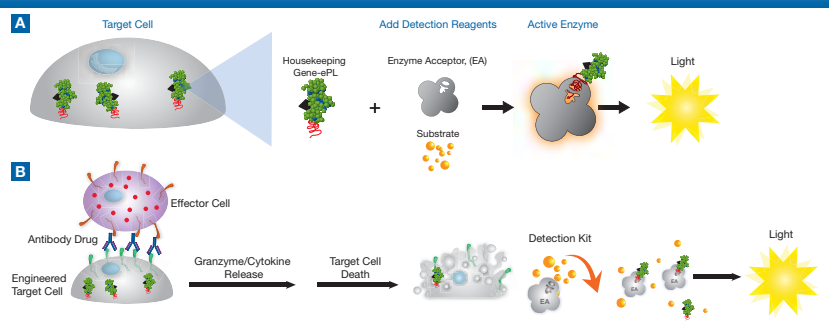
Abstract

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the mechanism by which many Class I therapeutic antibodies, including rituximab and trastuzumab, achieve clinical efficacy. In particular, molecules based on IgG1 isotype may be expected to elicit ADCC and it is recommended that developers assess an antibody's potential to elicit ADCC throughout the development process. Current ADCC assay techniques, such as chromium or calcein release assays, suffer from drawbacks like cellular "leakiness" leading to high background and variability, or lack of specificity for target cell killing (such as LDH release). We present a robust assay format using our proprietary enzyme fragment complementation (EFC) technology that specifically measures killing of target cells in a dye-free & radioactivity-free assay. These assays have very low background, thus producing robust assay windows, with excellent precision. Importantly, the same assay can be used for both ADCC and CDC, as well as other cell death mechanisms. Examples of rituximab-, cetuximab- and trastuzumab-mediated ADCC in multiple cancer cell models, with the use of primary NK cells and PBMCs, will be presented.

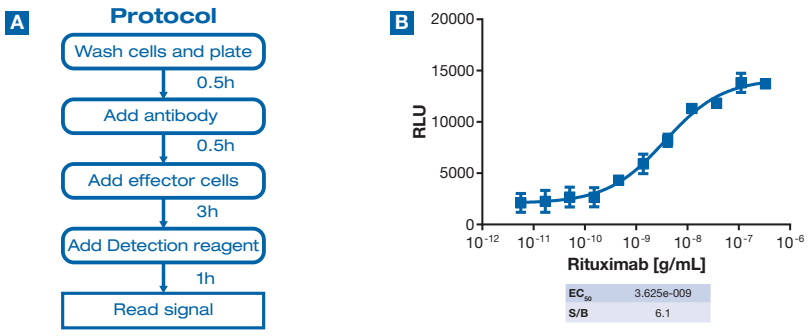
KILR™ ADCC Assay Principle



A. Target cells expressing the antigen of interest are engineered to stably express a housekeeping protein that is tagged with the ProLabel (ePL), a β-gal reporter fragment. When the target cell lyses and releases the tagged protein into the media, we can detect the cell lysis by the addition of detection reagents that contains 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. The engineered target cells are incubated with antibody drug and effector cells, leading to activation of the effector cells. This causes target cell death through the release of various granzymes and cytokines. The PL-tagged protein is released into the media and can be detected by addition of detection reagent (as noted in figure A). Conversely, very little signal is observed from healthy intact cells, as the tagged protein is retained inside the cells. Signal is highly specific to target cell death as only target cells express the β-gal fragment.

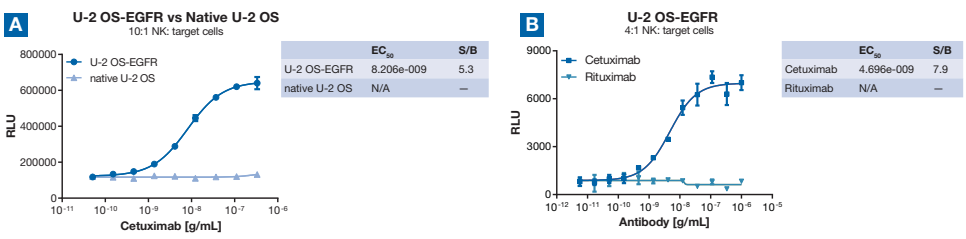
Rituximab-mediated ADCC Assay



EC_{50}	3.625e-009
S/B	6.1

ADCC with Rituximab in ARH-77 cells. **A.** Protocol: CD20+ ARH77 cells stably expressing the housekeeping protein were washed, opsonized with a dose response of rituximab (anti-CD20), then incubated with NK cells (4:1 ratio of effector: target). After 3hr, detection reagent containing exogenous EA and enzyme substrate was added to the medium and incubated for one hour prior to detection of luminescent signal on a luminometer (Envision, PE). **B.** Dose-dependent increase in rituximab-mediated ADCC in ARH77 cells. EC_{50} of 3.6ng/ml consistent with EC_{50} observed with other ADCC assay formats (e.g. chromium-51 release; europium).

High Specificity of ADCC Assay Responses

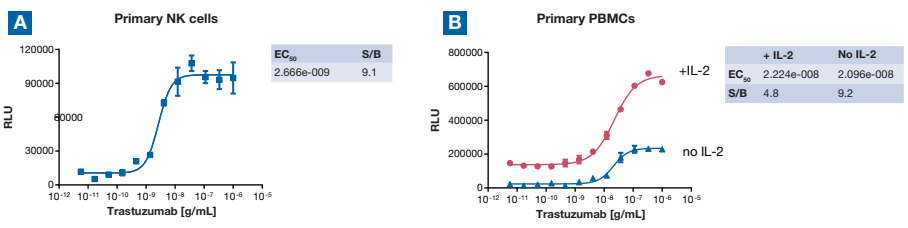


	EC_{50}	S/B
U-2 OS-EGFR	8.206e-009	5.3
native U-2 OS	N/A	–

	EC_{50}	S/B
Cetuximab	4.696e-009	7.9
Rituximab	N/A	–

KILR ADCC assay response is highly specific. A) Cetuximab mediates an ADCC response in U-2 OS cells over-expressing EGFR (dark blue) but not in native U-2 OS cells (light blue) which have very low levels of endogenous EGFR; 5,000 cells/well were incubated with cetuximab (30 minutes) and 50,000 purified NK cells for 3hr prior to detection of ADCC with KILR detection reagent. B) Cetuximab elicits an ADCC response in U-2 OS-EGFR cells, while rituximab (negative control antibody) does not. 10,000 target cells/well were incubated with cetuximab or rituximab (60 minutes) and 40,000 purified NK cells for 6hr prior to detection of ADCC with KILR detection reagent.

Assay Format Compatible with Multiple Effector Cell Types

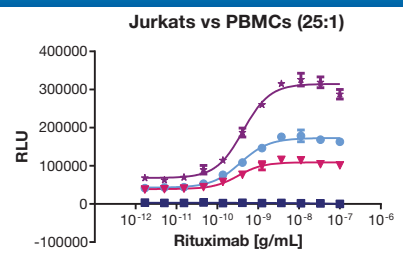


EC_{50}	S/B
2.666e-009	9.1

	+ IL-2	No IL-2
EC_{50}	2.224e-008	2.096e-008
S/B	4.8	9.2

KILR ADCC assay format is compatible with multiple effector cell types. **A.**Trastuzumab–mediated ADCC in SKBR3 cells with primary NK cells. SKBR3 cells (which endogenously over-express ErbB2) stably transduced with the KILR reporter protein were treated with Trastuzumab; 10,000 cells/well were incubated with Trastuzumab (60 minutes) and 40,000 purified NK cells for 6hr prior to detection of ADCC with KILR detection reagent. **B.** Trastuzumab–mediated ADCC in SKBR3 cells with primary PBMCs. Frozen primary PBMCs were cultured overnight with or without IL-2, prior to addition in a 10:1 ratio to SKBR3 cells previously opsonized with trastuzumab for 60 minutes. Cells were incubated for 3.5hr prior to addition of KILR detection reagent. Note that the assay window is improved when PBMCs are cultures in the absence of IL-2, with little effect on the pharmacology of the response.

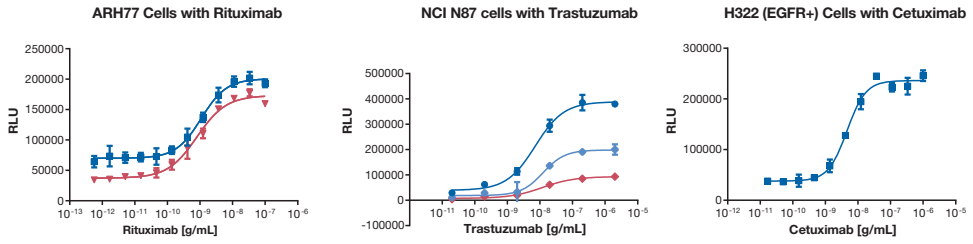
Robust Response with PBMCs From Multiple Donors



	EC_{50}
Jurkats (25:1)	N/A
Donor 1 (25:1)	3.247e-10
Donor 2 (25:1)	4.244e-10
Donor 3 (25:1)	3.674e-10

PBMCs from multiple donors elicit robust ADCC response with Rituximab in ARH77 cells. Frozen PBMCs from three unrelated donors or Jurkat cells were incubated with ARH77 cells (opsonized with Rituximab in dose response for 30') at a effector to target ratio of 25:1. All 3 donors elicited at least a 2.5-fold assay window, with comparable EC_{50}'s, while Jurkat cells produced no ADCC effect, as expected.

Assay Validation with Marketed Drugs



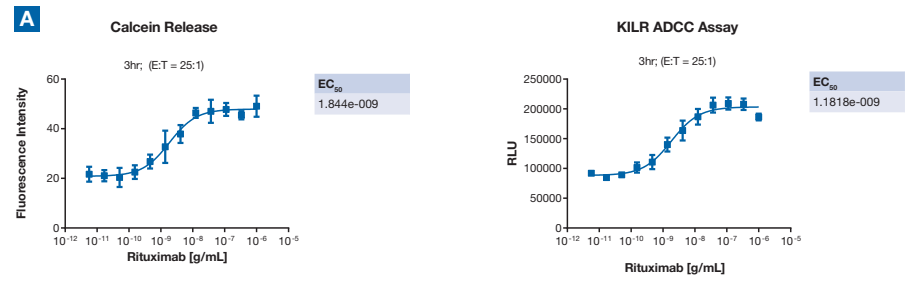
	EC_{50}	S/B
Stable pool (25:1 E:T)	1.089e-009	3.1
Stable clone (25:1 E:T)	8.087e-010	5.1

E:T Ratio	EC_{50}	S/B
40:1	7.187e-009	9.9
20:1	1.243e-008	10.6
10:1	1.207e-008	12.4

EC_{50}	4.611e-009
S/B	7.7

Assay validation with marketed drugs rituximab, cetuximab and trastuzumab. **A.** Example of Rituximab-mediated ADCC in the B lymphoblastoid cells, ARH77. Comparison of performance of stable pool vs stable clone. **B.** Trastuzumab-mediated ADCC in ErbB2-positive NCI N87 cells with various ratios of PBMCs: target cells. **C.** Cetuximab treatment induces ADCC in the EGFR- positive NSCLC cell line H322 with NK cells.

KILR™ ADCC Assay Performance vs Calcein Release Assay



EC_{50}
1.844e-009

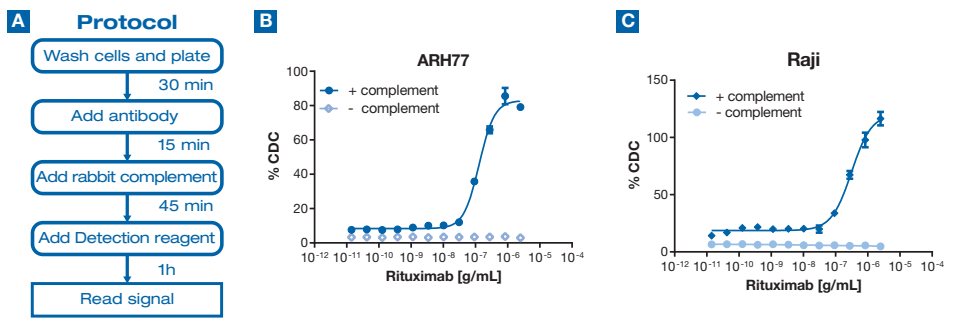
EC_{50}
1.1818e-009

B

Antibody	Chromium assay ED_{50} Range	aCELLA-Tox assay ED_{50} Range	KIL ADCC Assay ED_{50} Range
Rituximab	1-10 ng/ml	2-11 ng/ml	0.5-6 ng/ml

KILR ADCC assay performance comparable to other technologies for ADCC. **A.** Direct comparison to Caclein release. ARH77 cells were loaded with calcein for 30 minutes prior to treatment with antibody and primary PBMCs. In parallel, KILR assay format was performed with same lot of PBMCs. Both assay formats produced similar EC_{50}s. **B.** Comparison of KILR assay performance to other commercial ADCC assay formats.

Robust KILR™ CDC Response with Rituximab



KILR CDC Assay. **A.** Simple protocol for CDC. 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 substrate and detection reagent. **B.** Results with ARH77 cells, plotted as % CDC (or a percentage of total lysis). Note no lysis is observed in the absence of complement. **C.** Results with Raji cells, plotted as % CDC (or a percentage of total lysis).

Summary & Conclusions

- Universal technology
  - Apply to any cell type, suspension or adherent
  - Applicable for various cytotoxicity assays
    - ADCC, CDC, CAR-T, CTL, T-cell activation assay, Apoptosis or necrosis
- Enzymatic chemiluminescent output
  - No radioactivity
- Highly specific
  - Monitor only target cell death & lysis – no signal from effector cells
  - Protein label so no leakiness from target cells
- Simple assay protocol
  - Simple one-step add & read detection protocol
  - Homogenous protocol
- Scalable for screening or high throughput analysis of samples

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