A human FcγRIIa (H131 variant)-specific reporter assay for evaluating antibody activity in antibody-dependent phagocytosis (ADCP) pathway activation

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1. Introduction

Antibody-dependent cellular phagocytosis (ADCP) is an important mechanism of action of therapeutic antibodies that is effective at killing tumor cells in addition to antibody-dependent cell-mediated cytotoxicity (ADCC). Antibody drug development is beginning to address and realize the potential of this mechanism of action more attentively. Additionally, viral vaccine development is realizing the potential of an ADCP response for vaccine effectiveness.

However, even more so than classic ADCC bioassay, classic ADCP assays are tedious and highly variable. They are FACS-based rather than plate-based, and rely on isolation of primary cells, typically monocytes, that then need to be differentiated into phagocytic macrophages in culture for many days, and the need for multiple labeling reactions of effector and target cells. We sought to develop a plate-based, less tedious, faster and less variable ADCP bioassay. With this, understanding, manipulating and quantifying ADCP in both therapeutic antibody and vaccine development could be improved.

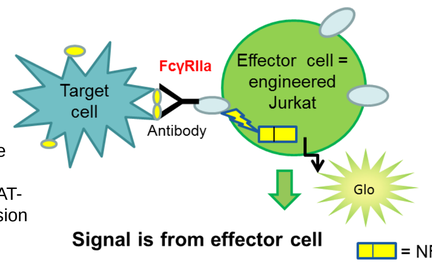
Macrophage effector cells and other myeloid-derived phagocytes mediating ADCP are more dependent on Fcγ activating receptors beyond the NK-cell expressing and ADCC-predominant FcγRIIIa. The FcγRIIa receptor has been reported to have the dominant role in ADCP by macrophages.

We describe here our development and evaluation of a human FcγRIIa (H131 variant)-specific reporter bioassay for quantifying antibody bioactivity in ADCP pathway activation. The H131 variant is able to bind all human IgG isotypes. The assay uses dual-engineered Jurkat effector cells that express the receptor and mediate FcγRIIa–dependent NFAT activation of reporter luciferase expression only when receptors are bound by target cell-bound antibodies.

We confirmed surface expression of the FcγRIIa (H131 variant) in our effector cells via FACS and passage stability of the cell line response for cell bank development. We developed and optimized the ADCP reporter bioassay using the effector cells in thaw-and-use format (immediate use, no culture), for ease of use and assay consistency. Rituximab, a chimeric IgG1 antibody drug with known ADCP activity through FcγRIIa, was used in much of the bioassay development. Using a series of rituximab isotype variants, we demonstrate that all four human IgG isotypes are active in the assay with expected relative activities. The assay is also specific, induction being dependent on the presence of FcγRIIa (H131 variant) and target-cell bound antibody. We further demonstrate that the assay can quantify the biological activity of other antibodies such as anti-EGFR panitumumab (an IgG2), anti-Her2 trastuzumab and an anti-GD2 antibody via this receptor. In a bioassay qualification study quantifying relative potency, the assay was accurate, precise and demonstrated good linearity and range. We also demonstrated the assay was stability-indicating using heat stressed rituximab and that it was robust.

2. Generation of engineered FcγRIIa/ NFAT-RE-luc2 effector cells

Reporter-based FcγRIIa ADCP bioassay



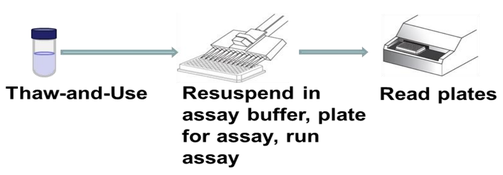
Stable single cell clone selected on:

- Fold of induction
- Biologically relevant EC_{50}
- Passage stability

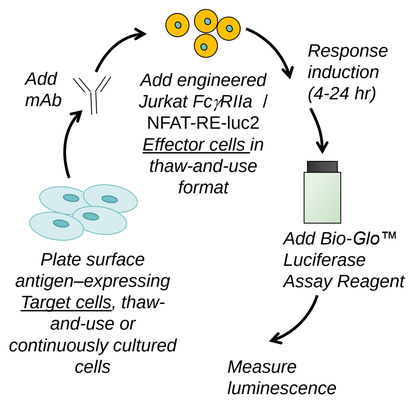
Jurkat cells were transfected with FcγRIIa and NFAT-RE-luc2 expression plasmids.

Jurkat cells have the intracellular signaling pathway elements for ADCP mechanism of action (MOA) that include NFAT activation.

Cells were prepared in thaw-and-use assay-ready format



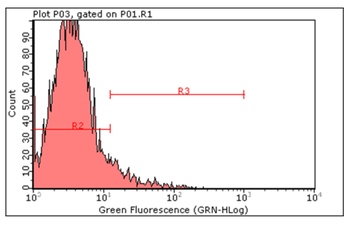
3. Protocol and advantages of the bioassay



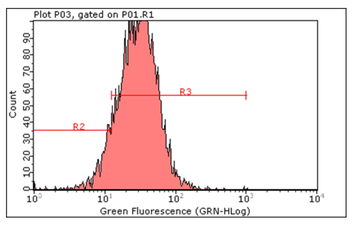
- 96-well plate based assay.
- Eliminates sourcing, purification, multi-day cytokine differentiation of primary monocyte-derived macrophage effector cells and associated inherent variability.
- Eliminates target and effector cell labeling needs and associated inherent variability.
- Than-and-use engineered reporter cells allow same day assay results without culturing or banking concerns.
- Simple, low variability, homogeneous, convenient bioassay.

4. Confirmation of cell surface FcγRIIa expression by FACS analysis

NFAT-RE-luc2 parent line

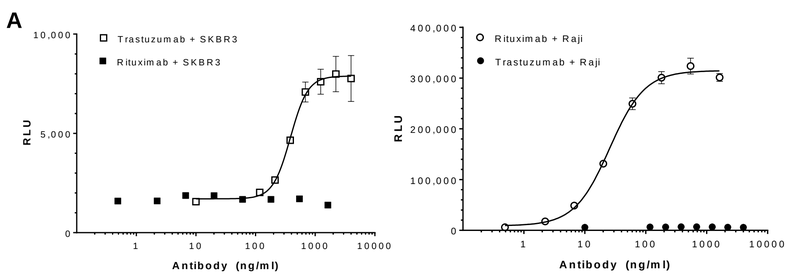


FcγRIIa H131 variant / NFAT-RE-luc2 ADCP reporter bioassay effector cell



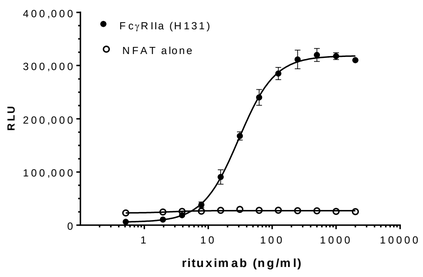
Anti-human FcγRIIa (clone IV.3) staining of parent line (lacking FcγRIIa) and FcγRIIa (H131) effector cell demonstrates cell surface expression of FcγRIIa in effector cells.

5. The bioassay is specific



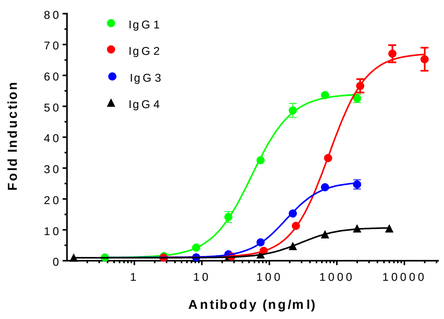
The assay shows high specificity, as demonstrated with SKBR-3 (Her2+) or frozen Raji (CD20+) target cells. Addition of anti-Her2 trastuzumab or anti-CD20 rituximab in combination with the appropriate antigen-expressing target cell gives an assay response, whereas no response is obtained when antibody cannot bind to target cells.

B



Both FcγRIIa receptor and NFAT-RE are necessary for an effector cell response.

6. FcγRIIa (H131) bioassay response shows appropriate IgG isotype bioactivities

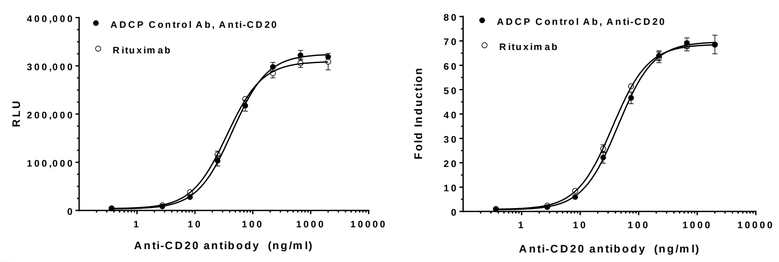


	IgG1	IgG2	IgG3	IgG4
EC_{50} (ng/ml)	54.7	759	176	294

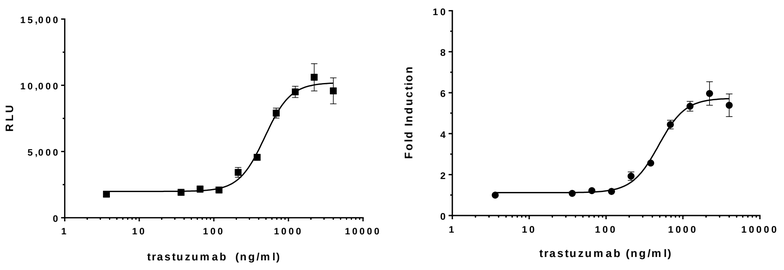
Using an anti-CD20 human IgG isotype series, the assay demonstrates expected EC_{50} ranking based on published SPR binding affinities for FcγRIIa H131 variant.

7. Bioassay can quantify bioactivity in ADCP pathway activation of a variety of drug and research antibodies

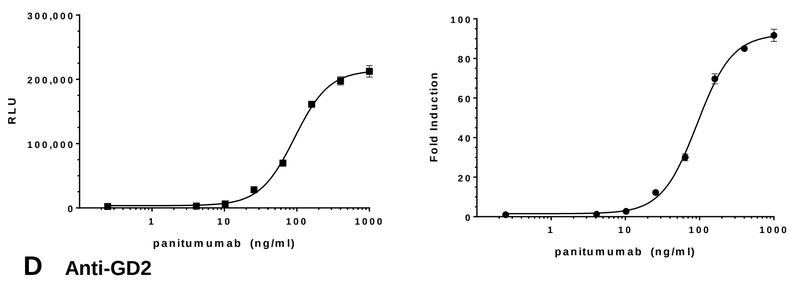
A Rituximab and research-grade 'equivalent' ADCP Control Ab, anti-CD20



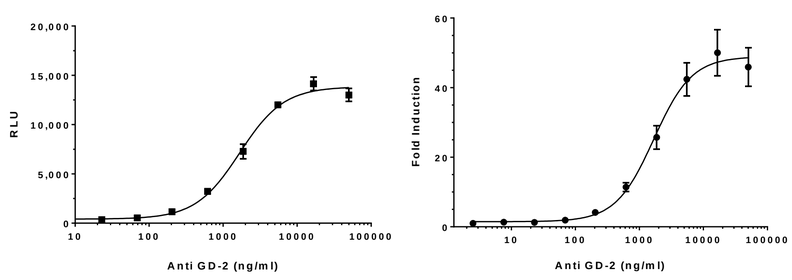
B Trastuzumab



C Panitumumab



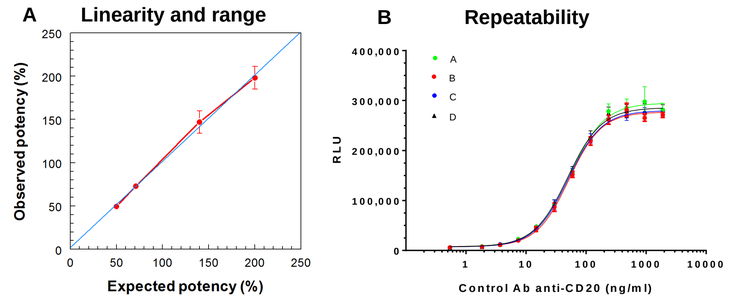
D Anti-GD2



FcγRIIa (H131) bioassay response with a variety of antibodies and target cells using thaw-and-use effector cells. All data obtained following a 6 hour induction. **A**. Rituximab and ADCP Control Ab, Anti-CD20 (IgG1) response using thaw-and-use Raji target cells. **B.** Trastuzumab response with SKBR-3 target cells. **C.** Panitumumab (IgG2) response with A-431 target cells. **D**. Anti-GD2 (clone 14.G2a, mouse IgG2a) response with SK-MEL-1 target cells.

Note: ADCP Control Ab, Anti-CD20 is a research grade hIgG1chimeric antibody with rituximab variable regions that was used as a control antibody during reporter bioassay development.

8. Potency qualification using ADCP Control Ab, Anti-CD20

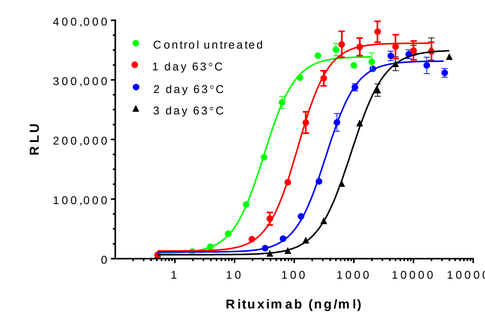


Qualification results:

Parameter		Results
Accuracy	**Expected Relative Potency (%)** 50 71 140 200	**Recovery (%)** 99.1 102.7 105 99.1
Repeatability (%CV)	100% Reference of 3 days	1.58, 1.11, 2.43
Intermediate precision (%CV)		1.71
Linearity (r^2)		0.997
Linearity (y=mx+b)		y= 0.997x+1.95

Potency qualification (3-day) with ADCP Control Ab, anti-CD20 (IgG1) using frozen thaw-and-use FcγRIIa (H131) effector and Raji target cells. Assay was performed on three days using 35K effector cells + 15K Raji target cells, with data analyzed using JMP 11 software.
A. Linearity and range. **B**. Example within-one-day repeatability (100% reference sample EC_{50} values on 4 plates: 43.6, 46.4, 46.7, and 47.1 ng/ml).

9. Bioassay demonstrates stability indicating properties using heat stressed rituximab

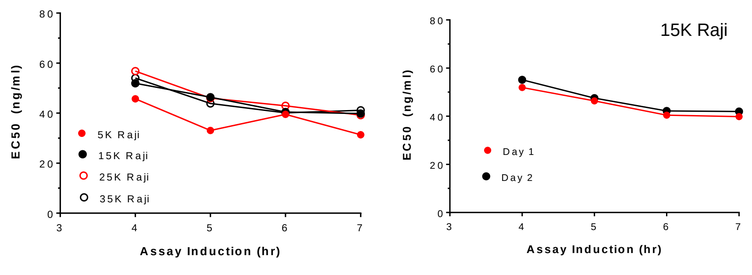


	Untreated	1 day 63C	2 day 63C	3 day 63C
EC_{50} (ng/ml)	31.7	116	339	904

Rituximab was heat stressed at 63°C for 1-3 days and compared to control unheated sample. Results obtained following a 6 hour induction using frozen thaw-and-use FcγRIIa (H131) effector and Raji target cells.

10. ADCP reporter bioassay is robust

Target cells/well and induction time: **Assay day and induction time:**



Bioassay robustness was tested using thaw-and-use FcγRIIa (H131) effector cells, Raji target cells, and ADCP Control Ab, Anti-CD20. The bioassay is robust for EC50 within an induction time of 5 -7 hours and in the range of 15K-35K Raji target cells/well. Effector cells were at 35K/well.

Summary

1. We have developed an FcγRIIa-specific ADCP reporter assay that quantifies ADCP MOA pathway activation for the predominant Fcγ receptor mediating macrophage ADCP.

2. The luminescent 96-well plate assay is simple, homogeneous, convenient, and fast. It should provide a much less variable and tedious method of assessing ADCP pathway MOA than FACS analysis using monocyte-derived macrophages differentiated in culture.

3. It is able to quantify ADCP pathway activation for a variety of research and drug antibodies and demonstrates appropriate human IgG isotype relative activities.

4. The bioassay is specific, robust and stability-indicating. In a qualification study it demonstrated excellent linearity, recovery and intra- and intermediate assay precision, indicating potential application for plate-based lot release testing.