

Assessment of New Developments in ADCC Assays

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Introduction

The success of biologic therapeutics has begun to reshape today's pharmaceutical market. The first and most successful of these antibody therapies, Rituximab (Rituxan®; Roche/Genentech), showed worldwide sales in 2009 of \$5.6 billion (GEN News Highlights, 2011). This, among others including Trastuzumab (Herceptin®; F Hoffman-La Roche), have shown great promise for treatment of patients with leukemia, lymphomas, breast, and other cancer types due to their specificity and reduced side effects (Zhou, 2007). One of the mechanisms which play a central role in the response to clinical antibody therapy is antibody-dependent cell-mediated cytotoxicity (ADCC) (Wang, 2008). This involves the response of natural killer (NK) cells to bind to specific antibody-coated target cells, such as CD20 and HER2 expressing cells, to promote the death of the target cell.

With many of the existing patents covering these treatments set to expire in the next few years, the development of biologic therapeutics similar to the original drug (biosimilars) has become increasingly important. This is highlighted by the report that Spectrum Pharmaceuticals and Viropro are set to work together to develop a biosimilar to Rituximab (GEN News Highlights, 2011). As a direct result, assays that can assess the ability of a biosimilar to act in a manner similar to the original biologic have also seen increased interest. The current "gold standard" ADCC assay incorporates ⁵¹Cr. The procedure involves labeling and incubating target cells with the radioligand, assessment of the labeling procedure, and finally performance of the actual assay. Not only is this time consuming, but involves the use and eventual costly disposal of radioactive material. Newer ADCC assays, however, have recently been developed. They have incorporated luminescent, time-resolved fluorescence (TRF), or homogeneous time-resolved fluorescence (HTRF) technologies in substitution for the previously used radioligand. These assay chemistries have proven to be easier to use and more amenable to automation, while still delivering accurate results.

As no technology is completely foolproof, it is important to test lead antibodies using a number of different assays. Therefore, an overview of these technologies will be presented here in a toolbox approach. An emphasis will be placed on how each procedure can be automated in 96- or 384-well format to allow for hands-free, higher throughput processing. Experimental data using freshly isolated or cryopreserved NK cells, NK cell lines, or other relevant genetically modified cell lines will be shown to prove the validity of each method.

BioTek Instrumentation

Precision™ Microplate Pipetting System. The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to titrate test antibodies in 96-well format and transfer them into the 96- and 384-well assay plates. Additionally, it was used to transfer cells, media and reagents to 96-well cell and assay plates.

MultiFlo™ Microplate Dispenser. The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 0.5-3000 µL. The instrument was used to dispense cells and media, and also to dispense reagents to assay wells in 384-well format.

Synergy™ Multi-Mode Microplate Readers. While a number of Synergy readers can be adopted for use with the ADCC assay technologies discussed here, two are especially useful; the Synergy H1 Hybrid Multi-Mode Microplate Reader and Synergy NEO HTS Multi-Mode Microplate Reader. Synergy H1 combines monochromator and filter-based detection systems in one unit, along with temperature and gas control features for use with live-cell assays. Synergy NEO combines the capabilities of other Hybrid readers with new features such as a high performance luminescence detection system, dedicated filter-based detection for live-cell assays, and dual PMT technology to make it ideal for use with high throughput cell-based assays.

ADCC Assay Principle

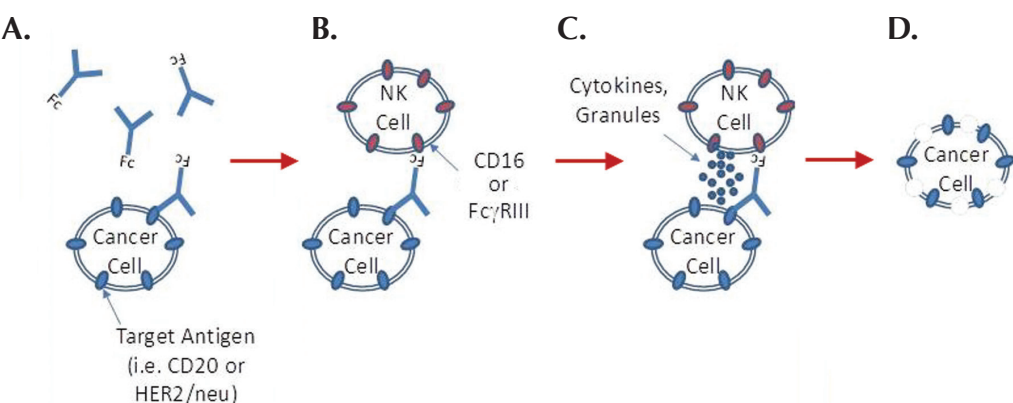


Figure 1 – (A.) Fab region of antibody binds to specific target cell antigen. **(B.)** Fc region of antibody binds to FcγRIII (CD16) receptor on NK cell. **(C.)** Following binding, NK cell secretes apoptosis-inducing agents. **(D.)** Target cell death through apoptotic pathway.

ADCC is a prominent mechanism in the host immune defense where the Fab region of an antibody binds to a specific antigen on a target cell (Figure 1), commonly an infected cell or pathogen. The Fc region of the same antibody then binds to a FcγRIII or CD16 receptor on an effector cell, commonly a natural killer (NK) cell. The bound NK cell then secretes apoptosis-inducing agents, destroying the target cell.

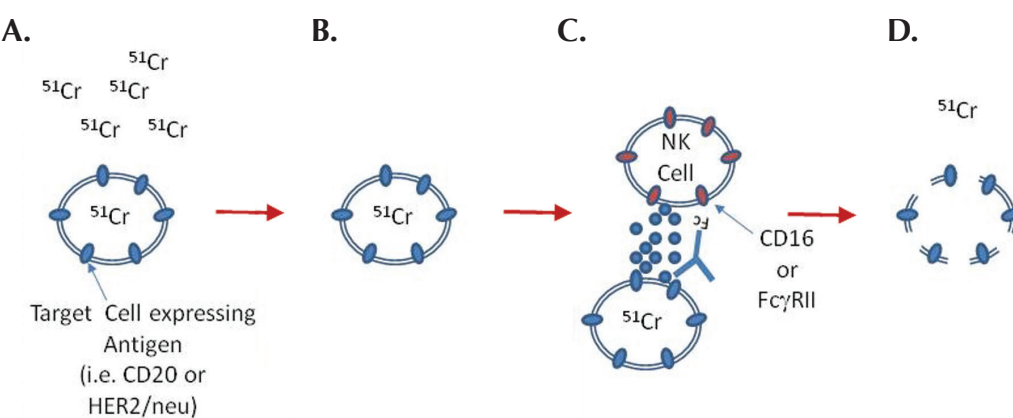


Figure 2 – (A.) Target cells are pre-loaded with ⁵¹Cr isotope. **(B.)** ⁵¹Cr not taken up into cells is washed away. **(C.)** ADCC assay performed with pre-loaded target cells and effector cells. **(D.)** ⁵¹Cr released into supernatant upon cell lysis.

The current gold standard target labeling species for antibody-mediated cytotoxicity assays is the stable isotope Chromium-51 (⁵¹Cr). Per Figure 2, the isotope is pre-loaded into the target cells prior to performing the assay. When lysed, the ⁵¹Cr-loaded target cells release ⁵¹Cr into the supernatant; the measured radioactivity in the supernatant indicates the extent of cell lysis. Radioactive assays such as these pose obvious safety threats and waste disposal concerns. The labeling procedure can also be arduous and time consuming. Additionally, artifacts from the labeling process and chromate ion toxicity are possible.

aCella-TOX™ Bioluminescent ADCC Assay

The aCella-TOX™ bioluminescent ADCC assay from Cell Technology, Inc. (Mountain View, CA), uses the same general ADCC process as explained in Figure 1. Target cell lysis leads to the release of endogenous Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and finally to a measurable bioluminescent signal. Additionally, as GAPDH is a natural cell component, it is unnecessary to pre-label, transfect, transform or otherwise introduce molecules into the target cells.

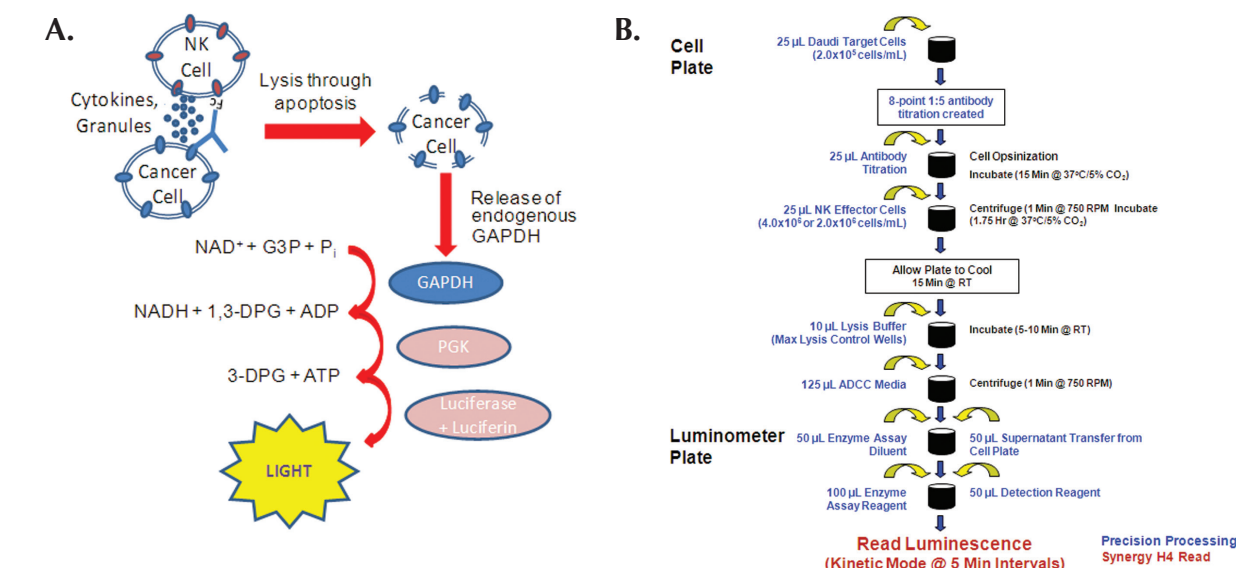


Figure 3 – aCella-TOX Assay Principle and Automated Procedure. (A.) ADCC process leading to target cell lysis and release of endogenous GAPDH. Through the addition of coupling enzymes and finally luciferase and luciferin, a luminescent signal is created that is proportional to cell lysis. **(B.)** All liquid handling steps automated using Precision. Luminescent signal detected by Synergy H4 using kinetic read mode.

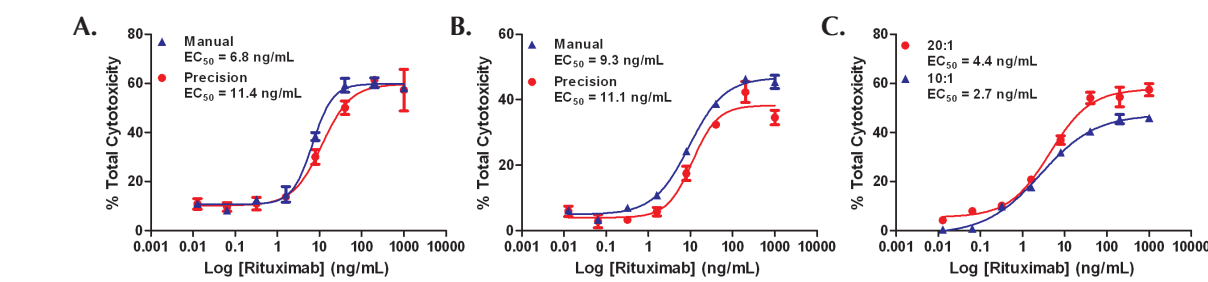


Figure 4 – aCella-TOX Robotic and Manual ADCC Assay Results. Data generated with freshly isolated Natural Killer (NK) cells from blood using manual or automated assay procedure and a 20:1 (A.) or 10:1 (B.) NK to Daudi target cell ratio. Cryopreserved cells from Stemcell Technologies (Vancouver, BC) also tested with automated method (C.) using 20:1 and 10:1 NK:Daudi cell ratios.

Data generated using the automated procedure compares well to that generated manually. Results shown here are also similar to EC₅₀ values generated previously for Rituxan using Daudi target cells, freshly isolated NK cells, and the ⁵¹Cr assay, ranging from 1.7-11.7 ng/mL.

Conclusions: The aCella-TOX assay represents an easy-to-use method that generates accurate data from test antibodies. Due to the fact that no pre-loading of target cells is required, the assay typifies an excellent substitute for the ⁵¹Cr method when the use of actual natural killer cells is either necessary or desired.

DELFIA® TRF ADCC Assay

The DELFIA® TRF ADCC assay from PerkinElmer, Inc. (Waltham, MA) incorporates a pre-loading step where the fluorescent enhancing ligand, BATDA, is added to the target cells. Upon hydrolysis inside the cell, the final TDA molecule is created. During the detection step, an Europium solution is added, which binds to the TDA creating a highly fluorescent chelate. A NK-92.CD16 cell line, available from Conkwest (Del Mar, CA), was incorporated into the assay procedure. The cells are derived from a parent NK-92 cell line and express one of two CD16 allelic variants commonly found in human populations.

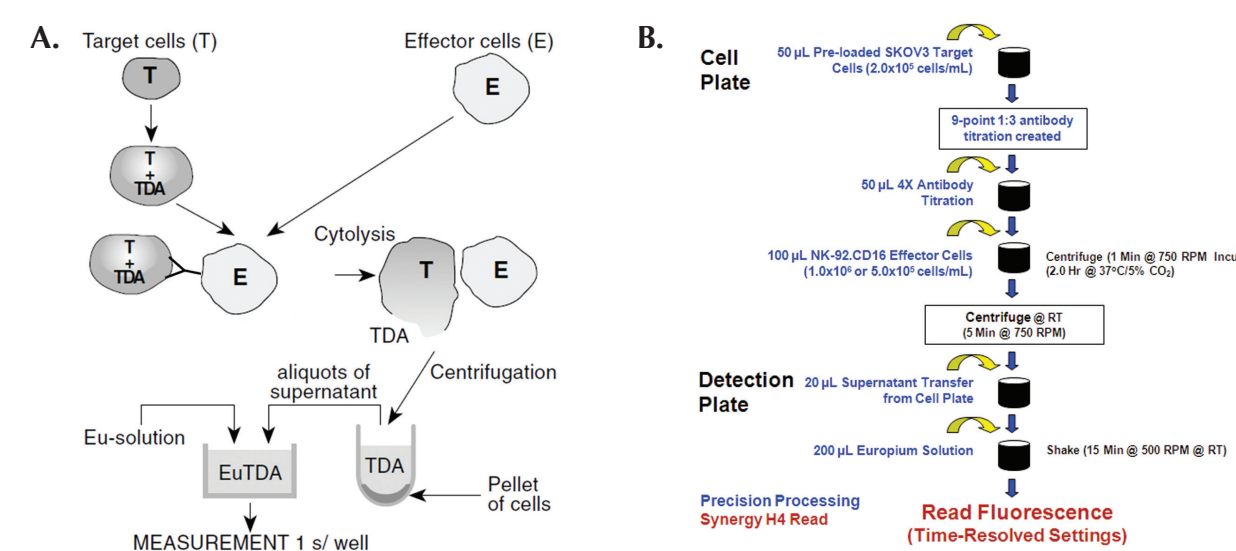


Figure 5 – DELFIA Assay Principle and Automated Procedure. (A.) ADCC process leading to release of fluorescence enhancing ligand (TDA) upon target cell lysis. Transfer of a supernatant aliquot to a separate plate containing Eu-solution allows formation of the highly fluorescent EuTDA molecule. **(B.)** All liquid handling steps of the ADCC assay procedure automated using Precision. Fluorescent signal from the EuTDA molecule captured by the Synergy H4 using a time-resolved read mode.

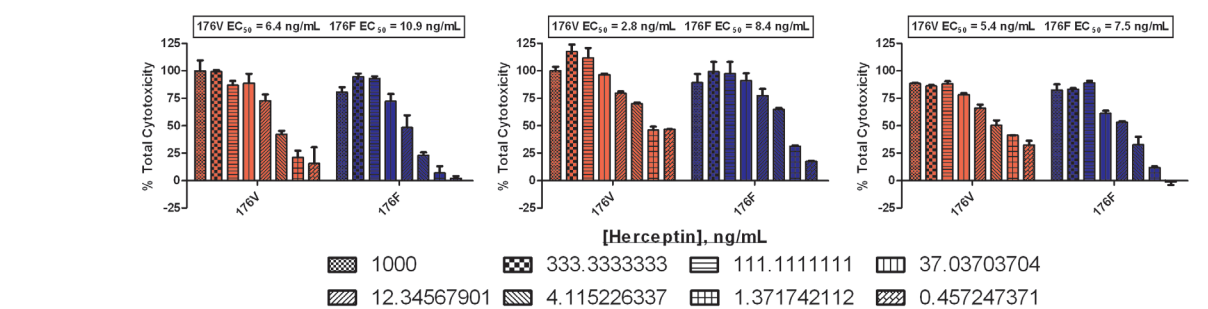


Figure 6. DELFIA Robotic ADCC Assay Results using NK-92.CD16 Cell line. (A. and B.) Multi-run data with CD16.176V or CD16.176F variant effector cells, SKOV3 target cells, and Herceptin control antibody titration using 10:1 effector to target cell ratio. **(C.)** Repeat test using 5:1 effector to target cell ratio.

EC₅₀ values calculated from 176V and 176F variant dose response curves correlate with previously generated data using same cells and antibody and the ⁵¹Cr assay, of 5.2 and 15.4 ng/mL, respectively. 5:1 effector to target cell ratio recommended for tests using NK-92.CD16 cell lines.

Conclusions: The DELFIA ADCC assay represents a suitable surrogate to the ⁵¹Cr method. Despite the fact that pre-loading of target cells is necessary, no radioactivity is used in the procedure, creating a simpler, more automatable assay process. Data generated using the NK-92.CD16 cell line also demonstrates that these cells can be used in substitution for freshly purified cells.

ADCC Reporter Bioassay

In the ADCC Reporter Bioassay from Promega Corporation (Madison, WI), engineered Jurkat cells, expressing the FcγRIIIa receptor, are used in place of purified NK cells or NK cell lines. Upon antibody binding to the target cell antigen and the FcγRIIIa receptor, a luminescent signal is generated that is proportional to receptor binding. This eliminates the need for attaining NK cells in any format, and provides a more repeatable result. The assay can also be used for Method of Action (MOA) determination.

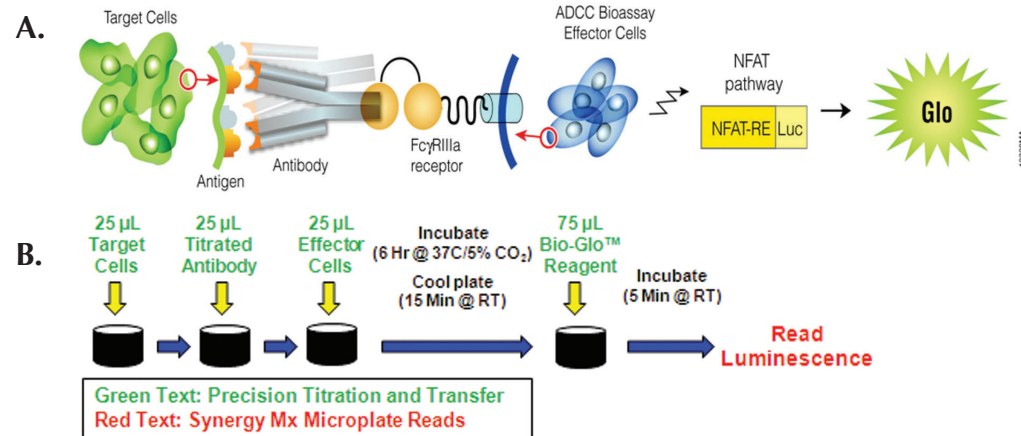


Figure 7 – ADCC Reporter Bioassay Principle and Automated Procedure. (A.) Engineered Jurkat cells, expressing the FcγRIIIa receptor, are used in place of NK cells. Upon binding of the test antibody to the target cell antigen and effector Jurkat cell FcγRIIIa receptor, the NFAT pathway is activated leading to luciferase enzyme production. Following effector cell lysis and detection reagent addition, a luminescent signal is generated that is proportional to antibody:FcγRIIIa receptor binding. **(B.)** All liquid handling steps automated using Precision. Luminescent signal detected by Synergy Mx.

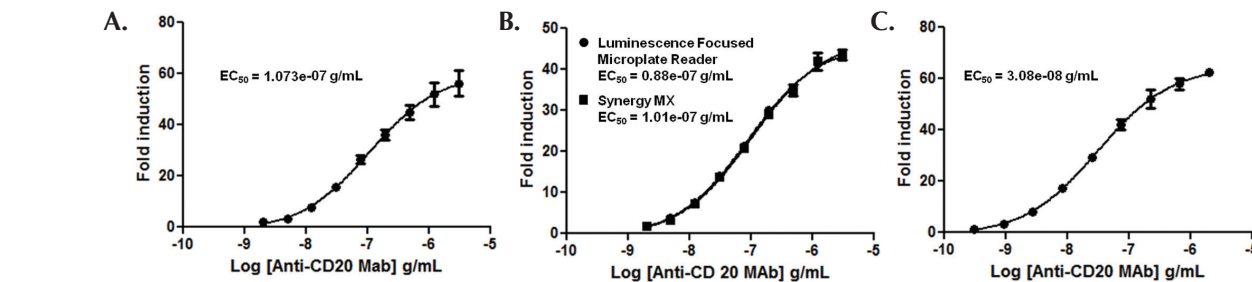


Figure 8 – aCella-TOX Robotic and Manual ADCC Assay Results. Data generated with Raji target cells and a 6:1 effector to target cell ratio using the manual (A.) or automated (B.) procedure. Luminescent signal detected using Synergy Mx for robotically prepared plate, and luminescence focused microplate reader for manual and robotically prepared plates. **(C.)** Results generated using WIL2-S cell model and automated assay procedure.

Results from tests incorporating the automated procedure show similar EC₅₀ values to those previously generated with the manual method using either Raji (see Figure 8A) or WIL2-S cells (2.29e-08 g/mL). Reader comparison data using Raji cells also demonstrates that the luminescent detection system on the Synergy reader is as sensitive, with a similar dynamic range as a luminescence dedicated microplate reader.

Conclusions: The ADCC Reporter Bioassay represents an easy-to-use, homogeneous method for analysis of antibodies that requires no centrifugation or plate transfer steps, and is easily automatable in 96- or 384-well format. The use of FcγRIIIa receptor expressing Jurkat effector cells also creates a more repeatable process which allows for method of action determination.

Tag-lite® Target Receptor and CD16a Assays

The Tag-lite® assays from Cisbio Bioassays (Codolet, FR) combine homogeneous target receptor:antibody and FcγRIIIa (CD16a) receptor:antibody binding assays to demonstrate ADCC potential. Each assay incorporates engineered cell lines expressing either target or CD16a receptors, SNAP-tag™ technologies and homogeneous time-resolved fluorescence (HTRF®). Together the two assay chemistries can be used to make method of action determinations with test antibodies in a high throughput format.

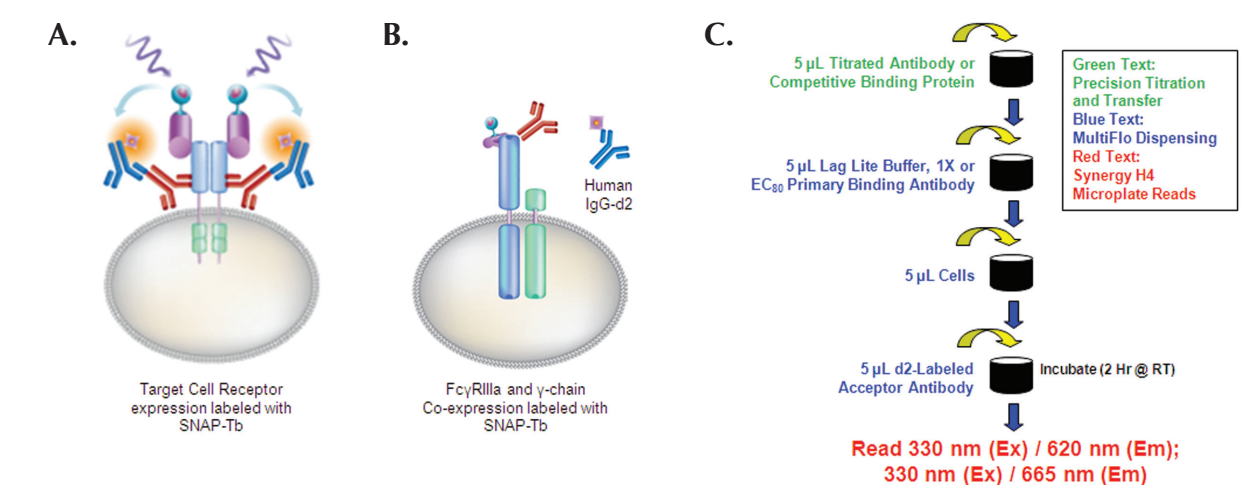


Figure 9 – Tag-lite Assay Principles and Automated Procedure. (A.) Target receptor expressed with SNAP-Tag and labeled with Tb donor molecule. Primary antibody (Ab) binds to target receptor, and acceptor labeled secondary Ab binds to Fc portion of primary Ab, allowing FRET between donor and acceptor molecules. **(B.)** FcγRIIIa receptor expressed with SNAP-Tag and labeled with Tb donor molecule. d2 labeled human IgG Ab binds to receptor, allowing FRET between donor and acceptor molecules. Other human IgG Ab binding to receptor displace d2 labeled Ab, decreasing FRET. **(C.)** All liquid handling steps automated using Precision and MultiFlo. Donor and acceptor fluorophore signals detected by Synergy H4.

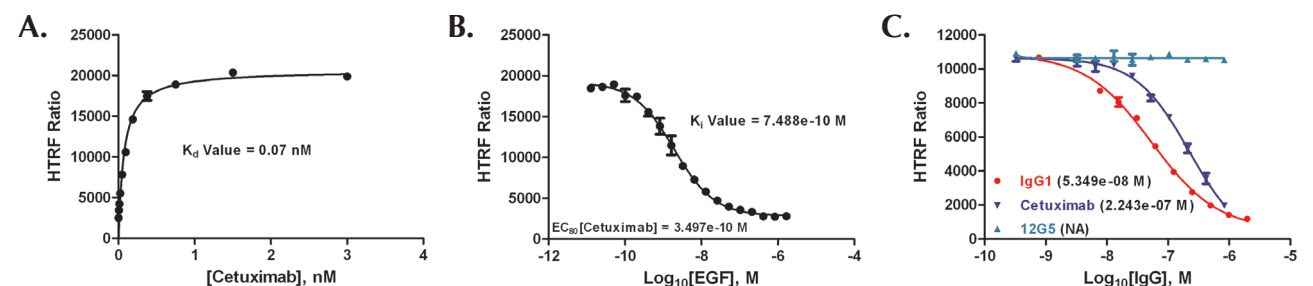


Figure 10 – Target and CD16a Receptor Assay Test Results. (A.) Cetuximab K_d determination for Ab binding to target EGFR receptor. **(B.)** Competitive binding data generated using EGF receptor ligand and test antibody. **(C.)** Binding characteristics of human IgG antibody, cetuximab, and anti-mouse 12G5 antibody to FcγRIIIa receptor.

A K_d value was generated to assess test antibody binding to the target EGFR receptor. EC₅₀ and EC₈₀ values were also calculated for use in competitive binding experiments (data not shown). The K_d value for known receptor ligands and potential biosimilar antibodies can then be calculated from results of a separate competitive binding experiment using the test antibody EC₈₀ value (Figure 10B). Finally, binding to CD16a was assessed. Human IgG1 and cetuximab demonstrate receptor binding, while the mouse 12G5 antibody does not dissociate the IgG-d2 antibody from the CD16a receptor.

Conclusions: The Tag-lite target and CD16a receptor assays represent straight forward, rapid, sensitive methods for antibody analysis. The two assay method, and incorporation of cell lines expressing the receptors of interest, allow for accurate and repeatable binding determinations to be made for both target and FcγRIIIa receptors. The homogeneous low volume 384-well assay format is easily automatable and amenable to higher throughput experimentation.