Automated Non-Radioactive Assay Methods For ADCC and CDC Assays

Introduction

It is becoming more evident that monoclonal antibody (mAb) immunotherapeutics will dominate Pharma's next generation of blockbuster drugs. With a fading traditional product pipeline, pharmaceutical companies are increasingly conducting research into biologics, or forming partnerships with smaller biologics research companies. Additionally, many existing biologics blockbusters, including Gentech and Biogen Idec's jointly developed Rituxan® and Amgen's Epogen® and Neupogen® lose patent protection shortly[1], so it makes sense that Pharma companies are exploring biosimilar products, while staving off competitive threats from those seeking the same biosimilar goal.

In response to the increased biosimilars research focus, the European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) have each drafted guidelines[2,3] to assist the industry in biosimilars product development. Both entities recommend performing extensive structural and functional characterization of the proposed product, with the U.S. going one step further, asking for the same characterization studies for the reference product.

The EMA additionally recommends *in vitro* non-clinical studies including binding to the target antigen, Fcγ receptors, FcRn and complement, and also showing Fc-associated functions through antibody-dependent cell-mediated cytotoxicity (ADCC) assays, complement-dependent cytotoxicity (CDC) assays and complement activation, saying, "Together these assays should cover all functional aspects of the mAb."

The U.S. FDA has similar guidelines, including bioassays, biological assays, binding assays, enzyme kinetics and other tests that are cell-based in nature. Additionally, they require companies to "provide additional evidence that the Method of Action (MOA) of the two products is the same to the extent the MOA of the reference product is known."

Here we will discuss the principles of ADCC and CDC assays used in mAb immunotherapeutic development, and how specific new automated, non-radioactive, cell-based assays provide simple methods that are safer than those based on radioactive materials. Primary and immortalized NK cells, and bioengineered cells will be used in the assays. We will show that immortal NK and bioengineered cells provide similar results as primary NK cells, and save time and effort associated with primary cell purification steps. Additionally, when these assays are automated using BioTek's Precision™ Microplate Pipetting System, MultiFlo™ Microplate Dispenser and Synergy™ Multi-Mode Microplate Readers, the process is streamlined with less active labor required, and results are more robust than manual methods.

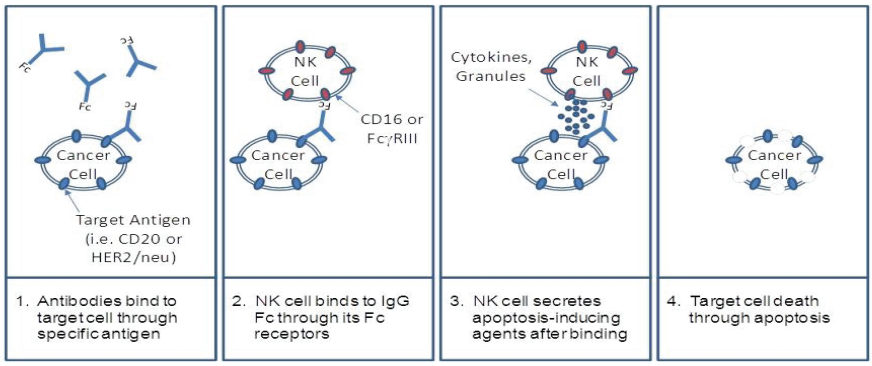

Figure 1. ADCC Mechanism of Action.

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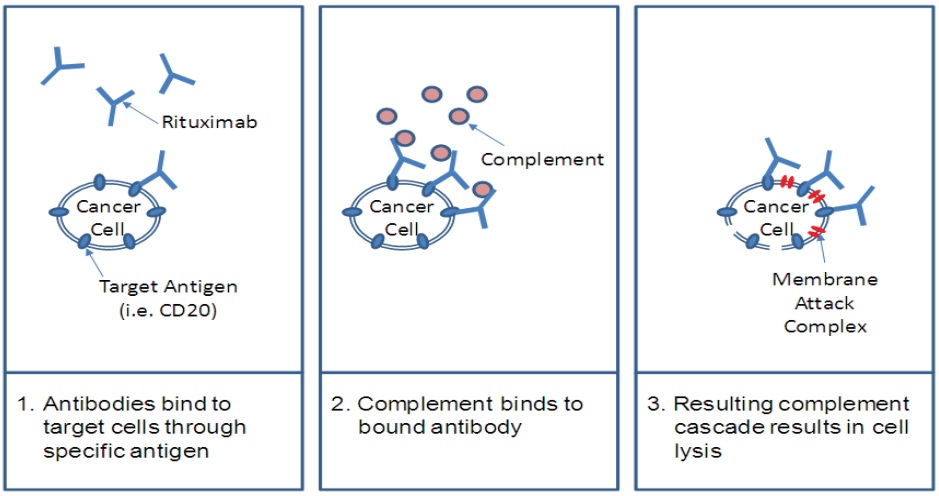

Figure 2. CDC Mechanism of Action.

Assay Theory

The body uses antibody-mediated cytotoxicity to destroy cells that could otherwise replicate and cause disease. This includes the response to virally infected cells, exogenous organisms, cancer cells and others, and may involve other host responses such as inflammation.

Two cytotoxicity mechanisms of action critically important to the current therapeutics market are ADCC and CDC. Although their biologies differ, these lytic systems both act to destroy specific target cell classes. The importance of each system, and any synergistic or antagonistic inter-relation or outside influence is not yet well-defined[4], so it is important for biologics research to include both ADCC and CDC assays to create a true mechanistic profile.

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, thus destroying the target cell.

The CDC mechanism works in a similar manner, whereby antibodies once again bind to a target cell through a specific antigen (Figure 2), and activate a multi-pathway attack on the target cell, called the complement cascade, rather than recruiting NK cells. Binding of complement leads to induction of a membrane attack complex, resulting in cell lysis.

The current gold standard target labeling species for antibody-mediated cytotoxicity assays is the stable isotope Chromium-51 (^{51}Cr). The isotope is pre-loaded into the target cells prior to performing the assay. When lysed, the ^{51}Cr-loaded target cells release ^{51}Cr 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.

Recently developed cell-based methods, such as those discussed here, bypass the need for radioisotope-labeled cells, and rely instead on measurement of molecules released by lysed target cells, or measurement of binding of antibodies to target or FcγRIII receptors. The assays discussed here each have their own strengths, and are chosen depending on the lab's needs and drug development research stage. When automated for medium- and high throughput, these new assays create simple, robust processes that require less active labor, and increased consistency over time and with multiple users.

Instrumentation

BioTek's compact Precision™ Microplate Pipetting System automates pipetting processes for walk-away operation. A user-configurable deck, along with four liquid handling transfer tools, provides flexible experimental design. Its small footprint allows for easy insertion into laminar flow hoods, making it especially suited for use with cell-based assay procedures that require sterility. In the assays detailed here, Precision 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.

The MultiFlo™ Microplate Dispenser from BioTek combines multiple dispensers in one compact unit, thus reducing overall instrument costs, saving processing time, and simplifying the process. Its parallel dispense technology, combines up to two eight-tip non-contact peristaltic dispensers and two variable-tube syringe pump dispense manifolds, to maximize assay flexibility in 96-, 384-, and 1536-well microplates. For use with cell-based assays, the MultiFlo can be placed in a laminar flow hood for sterile operation. In the assays detailed here, MultiFlo was used to dispense cells and media, and also to dispense reagents to assay wells in 384-well format.

A variety of BioTek's Synergy™ Multi-Mode Microplate Readers are suitable for many budgets and applications such as cell-based assays. Each incorporates high precision and performance in fluorescence, luminescence and absorbance modes, and are all driven by integrated Gen5™ Data Analysis Software. Two Synergy readers that are especially useful for cell-based assays such as the ADCC and CDC assays discussed here are the Synergy NEO HTS Multi-Mode Microplate Reader and Synergy H1 Hybrid Multi-Mode Microplate Reader.

Synergy NEO is specifically designed for today's smarter HTS screening applications, with multiple parallel detectors for ultra-fast measurements and dedicated filter-based optics for live cell assays. At the heart of Synergy NEO is patented Hybrid Technology™, which combines monochromator- and filter-based fluorescent optics for endless flexibility and high performance in HTS, research, and drug discovery applications. Filter cubes and minimal optical components provide higher light transmission and sensitivity than readers using fiber optics and reflecting mirrors. Additionally, Synergy NEO can simultaneously read donor and acceptor fluorescence using dual photomultiplier tubes (PMTs), and also features a 100 mW laser-based excitation source for Alpha-based assays.

Synergy H1 also provides high sensitivity and flexibility for cell-based and biochemical assays, with UV-visible absorbance, luminescence and quadruple grating monochromator optics for top and bottom fluorescence. Synergy H1 may be upgraded at any time with an optional filter-based optics module for advanced read modes like fluorescence polarization, time-resolved fluorescence and time-resolved fluorescence resonance energy transfer. As with Synergy NEO, the Synergy H1 optics are devoid of fiber optic cables, reflective mirrors and other components that limit light transmission and reduce sensitivity.

aCella-TOX Assay

Assay Principle

The aCella-TOX™ bioluminescent ADCC and CDC assays from Cell Technology, Inc. (Mountain View, CA), use the same general ADCC and CDC processes (Figure 3) where target cell apoptosis is induced. This lysis leads to the release of endogenous Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), an enzyme in glycolysis and gluconeogenesis pathways, from the cytoplasm. Through the addition of coupling enzymes, adenosine triphosphate (ATP) is generated, and when coupled to a luciferase/luciferin reaction, produces a measureable bioluminescent signal. This signal is proportional to the amount of GAPDH release and target cell lysis. The aCella-Tox assay is non-destructive, homogenous and readily adapted to automated formats. Additionally, as GAPDH is a natural cell component, it is unnecessary to prelabel, transfect, transform or otherwise introduce molecules into the target cells.

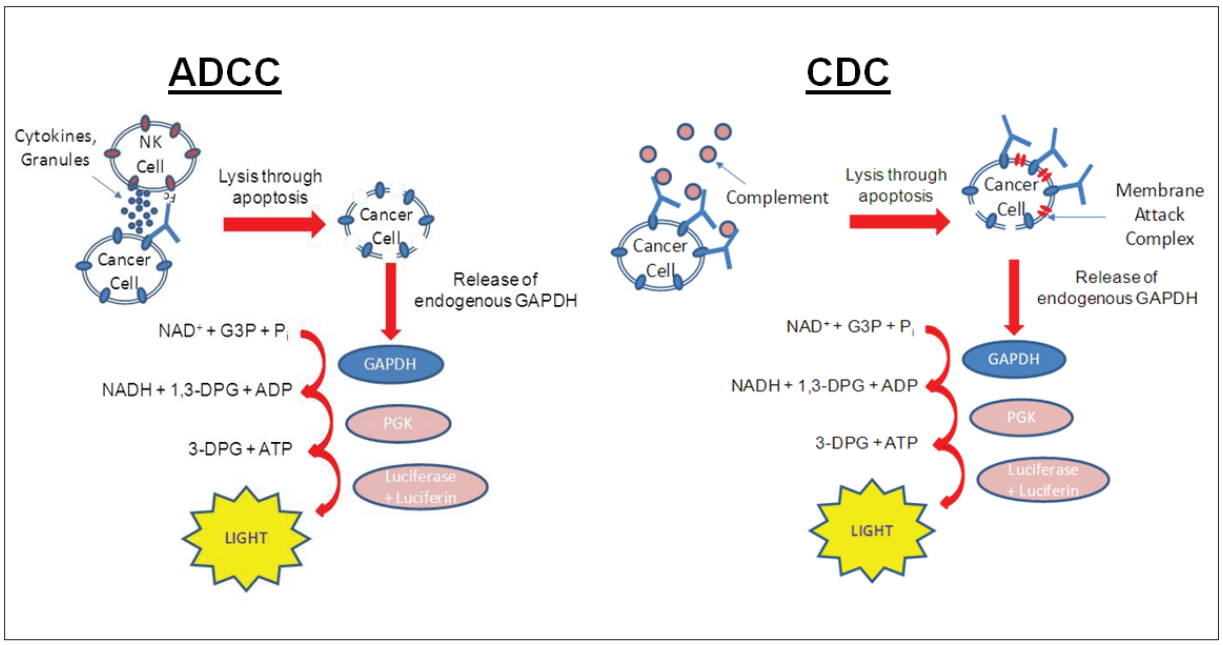

Figure 3. aCella-TOX assay principle showing effector cells (ADCC) or the complement cascade (CDC) causing target cell lysis. GAPDH is released from dying cells, leading to ATP production, which is coupled to a luciferase/luciferin reaction to produce light.

Method Summary

Prior to running the aCella-TOX assay, the kit manufacturer recommends determining the number of target cells to be plated via a cell titration, and adapting those cells in low IgG media for a few passages prior to running the assay. They also recommend determining the optimal effector:target ratio by using varying ratios of effector cells and keeping the target cell number and antibody concentration constant. Per ADCC assays, the antibody should be titrated to determine the dynamic range required to obtain an optimal dose-response.

Daudi target cells were added to a 96-well assay plate. A multi-point titration curve of rituximab antibody was then created and added to the plate. The plate was then incubated at 37°C/5% CO_2 to allow the cells to opsonize. Freshly isolated NK effector cells were then added to each well, the plate was centrifuged, and then incubated again under the same conditions. The plate was then equilibrated to room temperature (RT). The target cells in the maximum lysis control wells were lysed by adding Lysis Buffer, and the plate was incubated at RT. ADCC Medium was then added to each well, and the plates were centrifuged. Enzyme Assay Diluent was then transferred to the appropriate wells of an opaque white luminescence plate. Each reaction supernatant was transferred to wells containing the assay diluent. Then Enzyme Assay Reagent was added to each well followed by Detection Reagent. The plate was immediately read using the Synergy Multi-Mode Microplate Reader's luminescence kinetic mode at five-minute intervals. Endpoint measurements can also be taken.

The assays were repeated using cryopreserved Human Peripheral Blood NK cells from STEMCELL Technologies (Vancouver, BC). Cryopreserved NK cells increase assay reproducibility as multiple vials from single cell lots can be procured at one time. Additionally, purification systems and consumables/reagents associated with freshly isolated NK cells are eliminated, saving overall costs and time for lab personnel.

Experiments were also performed incorporating complement instead of NK cells to create the cytotoxic effect on target cells using the same assay procedure.

Note that for those performing batch processing, enzyme assay reagent is added to the wells to initiate ATP production, and after incubation, the plate may be stored. Following removal from storage, the Detection Reagent is added, and the luminescent signal is read using kinetic or endpoint measurements.

Results

After determining the proper cell concentration, effector:target (E:T) ratio, and antibody titration (data not shown), multiple aCella-TOX runs were completed using freshly isolated NK cells from different donors with a 20:1 E:T cell ratio to demonstrate that similar data could be generated despite the donor. This was demonstrated by the similarity in EC_{50} values for the test antibody, rituximab. Additionally, data generated using manual and automated methods (Figure 4) show equivalent results. The assay was also run using a 10:1 E:T ratio to further demonstrate that lower effector cell numbers may be used; thus saving time and money.

Variability within percent cytotoxicity data was seen using freshly isolated NK cells, with 20:1 E:T cell ratio variation from 60-100% and 10:1 E:T cell ratio variation from 40-100%.

The 20:1 and 10:1 E:T cell ratios were then used to validate the assay using cryopreserved NK cells. Per Figure 5, similar EC_{50} values and dose-response curves were seen with cryopreserved NK cells when compared to that generated with freshly isolated NK cells, demonstrating that cryopreserved cells may be used with aCella-TOX assays.

Finally, CDC assay optimization included an analysis of complement concentration percentage as well as incubation time for complement, antibody, and target cells. Validation data (Figure 6) prove that the automated assay is once again repeatable over multiple runs.

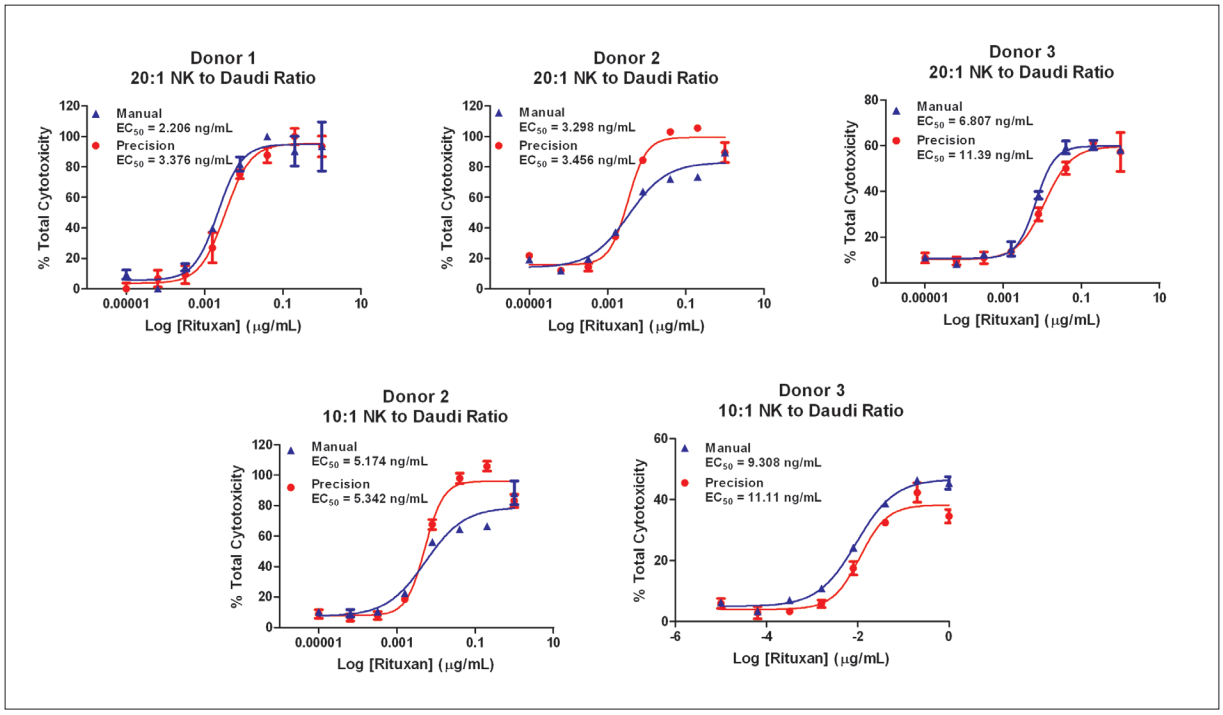

Figure 4. Manual vs. Automated Data Comparison Using Freshly Isolated NK Cells. Equivalent results seen using manual and automated assay methods. Data generated using different donors also shows similarity regardless of the donor.

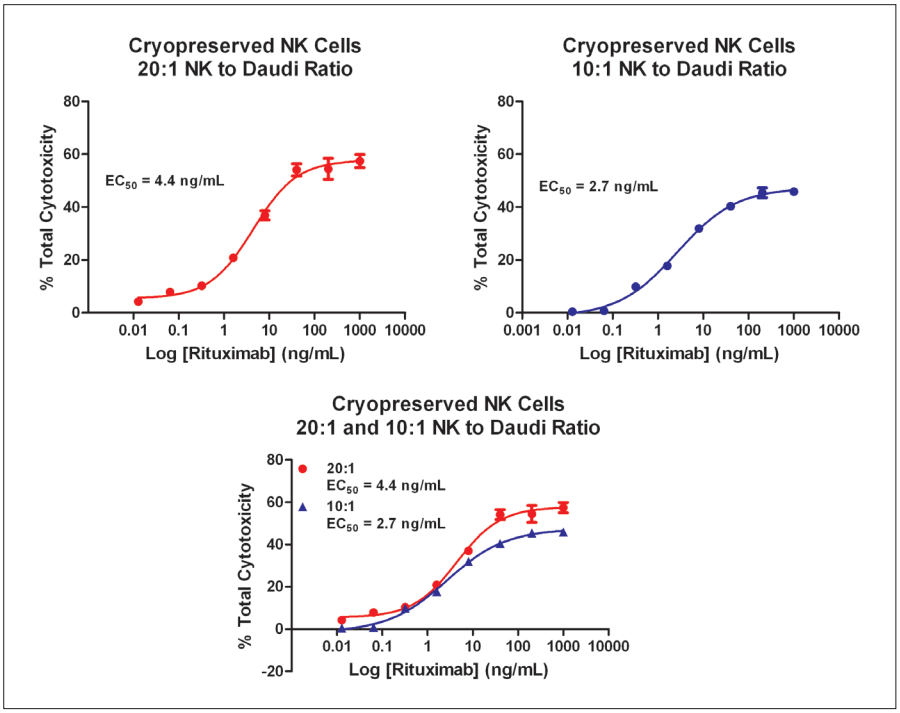

Figure 5. Cryopreserved NK Cell Test Data.

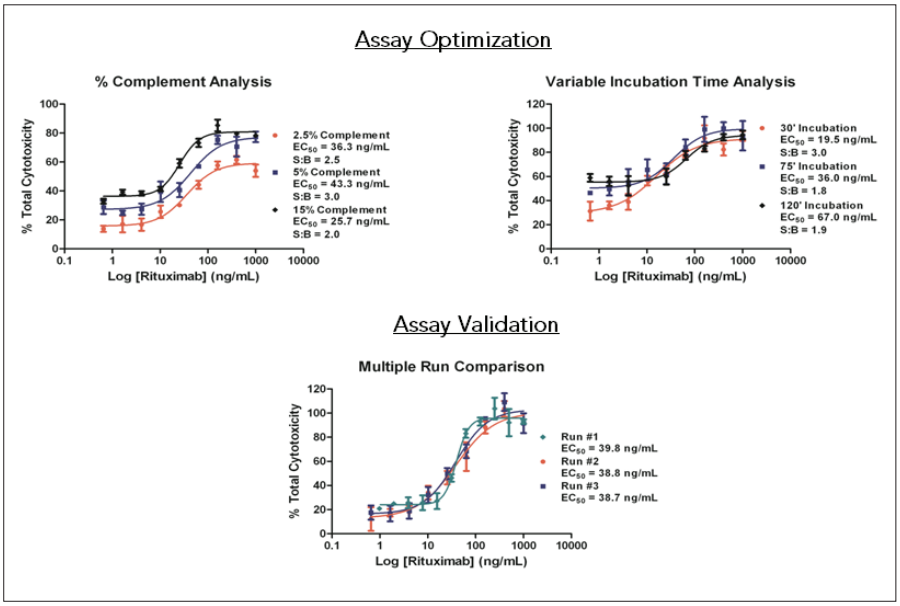

Figure 6. CDC assay optimization and validation data showing that the automated assay using complement is accurate and repeatable across multiple runs.

DELFIA® TRF ADCC Assay

Assay Principle

The Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA) from PerkinElmer, Inc. (Waltham, MA) calls for loading the target cells with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA). Inside the cell membrane, the ester bonds hydrolyse to form a non-cell permeable hydrophilic ligand (TDA). These loaded target cells are added to microplate wells (Figure 7) in addition to titrated antibody and effector cells. Binding of the test antibody to the two cell types again leads to target cell lysis. Once the target cell is lysed, the TDA ligand is released, and the cells are pelleted. A supernatant aliquot is transferred to a separate plate where the TDA binds to europium to form the highly fluorescent and stable chelate, EuTDA. The resulting signal is measured via time-resolved fluorescence (TRF), and correlates directly with the amount of target cell lysis within the microplate well. The DELFIA assay does not require physical or chemical treatment of the cell membrane, and BATDA is quickly accumulated into the target cells, thus facilitating excellent recovery of the labeled cells. Additionally, the process is readily adapted to automated methods.

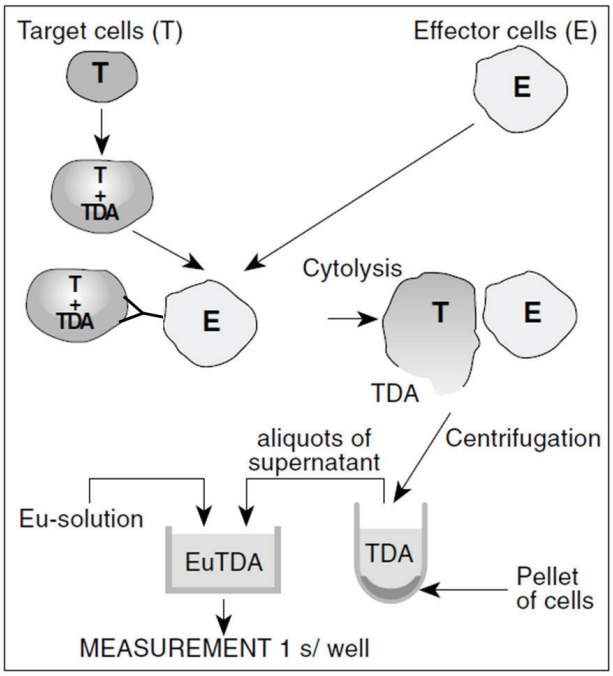

Figure 7. DELFIA assay principle, showing loaded target cells binding to antibody that is also bound to effector cells, target cell lysis, pelleting, and TDA binding with europium to form a fluorescent chelate.

The assay model tested here incorporated ovarian carcinoma SKOV3 as target cells, as well as immortalized NK-92.CD16 cells from Conkwest (Del Mar, CA) as effector cells. These cells are derived from parental NK-92 cells, developed at the Fox Chase Cancer Center (FCCC), and are part of their Neukopanel ADCC Assay System. They are transduced to express either the high affinity variant CD16.176V or the low affinity variant CD16.176F, which are allelic variants common in normal human populations. The advantage of NK-92.CD16 cells is that they have an easier cell preparation process, and therefore lower cost and time required, versus NK cells purified from blood. They also provide greater consistency in assay results versus repeated cell purifications from blood.

Method Summary

Prior to running the DELFIA ADCC assays, the optimal cell loading time (specific release) for the fluorescence enhancing ligand and Cell Mediated Cytotoxicity (spontaneous release) values were determined to maximize assay window and ensure that little to no target cell lysis takes place in the absence of antibody (data not shown). This guarantees a true specific release percentage during ADCC testing.

Multiple runs of the ADCC assay were then performed using a 10:1 NK-92:SKOV3 cell ratio. Pre-loaded SKOV3 target cells were added to each well of a 96-well assay plate. A multi-point titration curve of Herceptin antibody was created, and each antibody concentration was added to the assay plate along with NK-92.CD16 effector cells. The plate was centrifuged then incubated to allow opsonization and cell lysis to take place. After incubation, the plate was centrifuged again to pellet the cells, and supernatant was transferred to a detection plate. Europium Solution was added to each well, and the plate was shaken at room temperature. The fluorescent signal was then measured with the Synergy Multi-Mode Microplate Reader using a time resolved format.

The assay was repeated using a 5:1 effector to target cell ratio, and also comparing microplate types.

Results

Per Figure 8, the NK-92.CD16 176V strain variation was found to have increased cytotoxicity compared to the 176F strain at lower concentrations, consistent with previously generated data[5]. Additionally, calculated EC_{50} values agree with previously generated values of 5.2 ng/mL and 15.4 ng/mL, respectively using the chromium-based method.

When the DELFIA assay was run using a 5:1 NK-92:SKOV3 cell ratio (Figure 9), the cytotoxic effects between the NK-92 176V and 176F strain variations provided a more realistic representative of the actual cytotoxic effects. Therefore, a 5:1 E:T cell ratio is recommended for experiments using NK-92 cells. In addition, similar results were seen with both plate types tested. This confirms that each is suitable for use with the DELFIA ADCC assay.

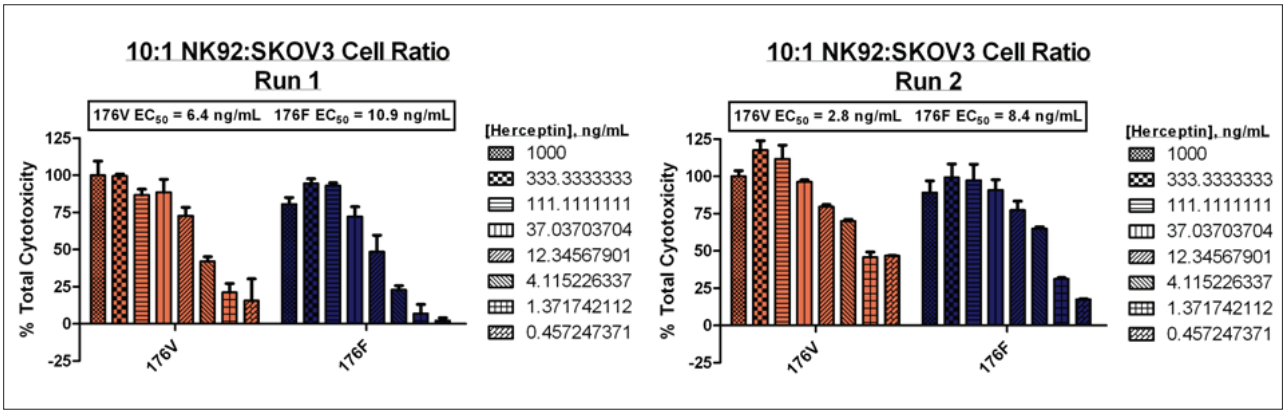

Figure 8. DELFIA automated assay validation data using a 10:1 E:T ratio.

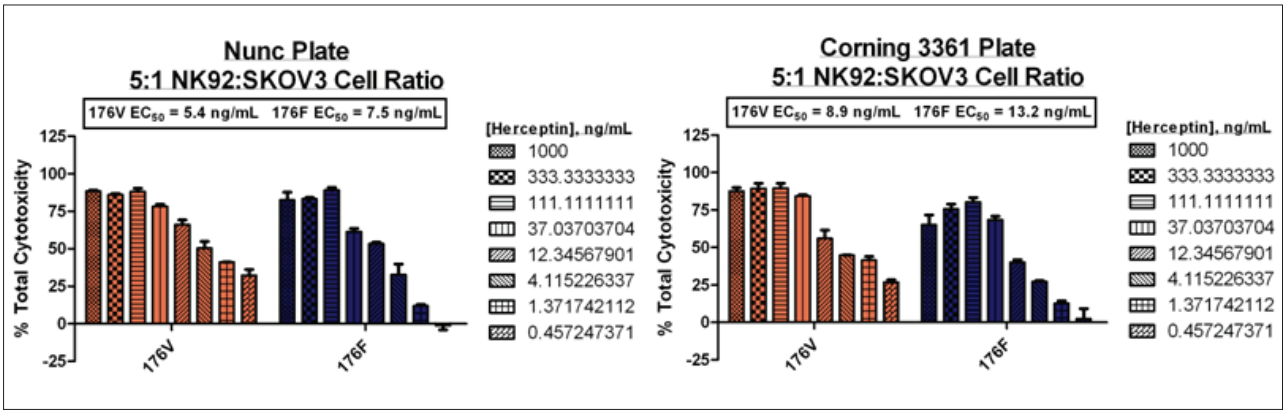

Figure 9. DELFIA automated assay validation data using a 5:1 E:T cell ratio.

ADCC Reporter Bioassay

Assay Principle

The ADCC Reporter Bioassay from Promega Corporation (Madison, WI) uses the same binding process as the aforementioned ADCC assays. Test antibodies bind to the specific target cell antigen. However, in the reporter bioassay, engineered immortalized T lymphocyte cells known as Jurkat cells, which express the FcγRIIIa receptor are used as effector cells instead of NK cells (Figure 10). These Jurkat cells eliminate the need for complex cell preparation, and can provide more consistency than NK cell methods. In this process, antibodies bound to target cell antigens then bind to the FcγRIIIa receptor on the Jurkat cell. Receptor binding stimulates the nuclear factor of activated T-cells (NFAT) pathway and activates an NFAT response element upstream of the luciferase gene; leading to luciferase enzyme production. Once the effector cells are lysed, the luciferase interacts with luciferin and ATP in solution, leading to the generation of a stable luminescent signal. The assay is a mix-and-read format, and does not require centrifugation or transfer steps seen in the previous methods, and also allows for method of action (MOA) determination.

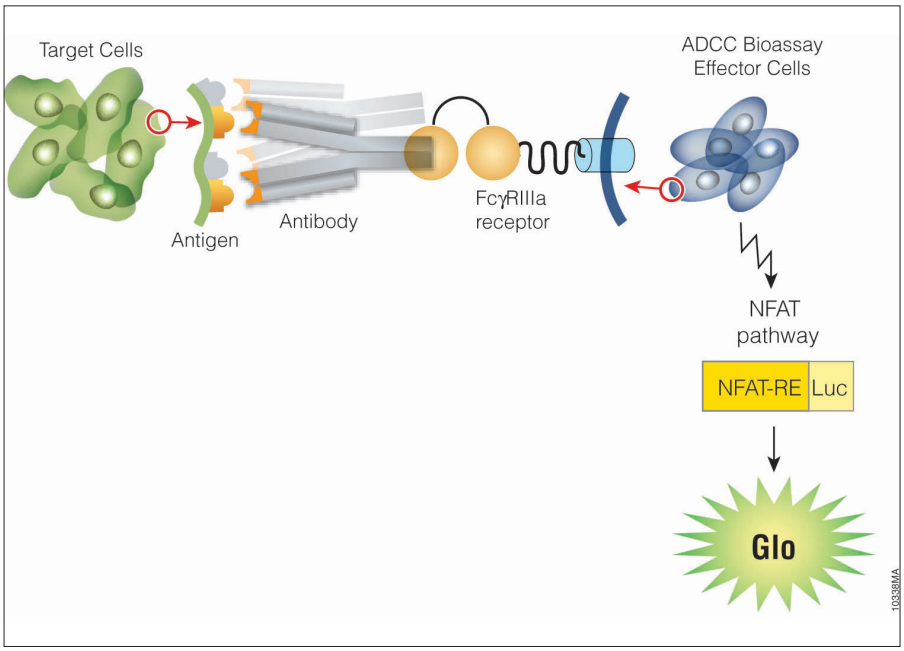

Figure 10. ADCC Reporter Bioassay principle showing antibody binding to target cell antigens and Jurkat cell expressed FcγRIIIa receptors. Following binding and effector cell lysis, the generated luminescent signal is detected.

Method Summary

Target Raji (lymphoblastoid) or WIL2-S (lymphoma B) cells were added to the wells of a 96-well assay plate along with titrated anti-CD20 monoclonal antibody. Engineered Jurkat effector cells were then added to each well, and the plate was incubated. After incubation, the plate was cooled to room temperature, and Bio-Glo™ Reagent was added. The plate was incubated again, then read using the Synergy Multi-Mode Microplate Reader's luminescence mode. An increase in luminescent signal is therefore proportional to the binding level of the test antibody to the expressed FcγRIIIa receptor.

Results

Using Raji cells and anti-CD20 monoclonal antibody, and a 6:1 E:T cell ratio, manual and automated assay methods were compared. Per Figure 11, manual and automated methods generated equivalent values, and EC_{50} results also closely correlate to previously generated data of 0.743e-07 g/mL[6]. It is important to note that since the measurement is of antibody binding to the engineered effector cell, data will not show percent total cytotoxicity. However, positive fold induction is seen with increased binding, and EC_{50} values are equivalent to those generated using more traditional methods.

The Synergy Multi-Mode Microplate Reader's performance was also compared to that of a luminescence-focused microplate reader (Figure 12). Equivalent fold induction and EC_{50} values (1.01e-07 g/mL and 0.88e-07 g/mL, respectively) are seen across the readers, thus demonstrating Synergy's high sensitivity and accuracy, along with its increased functionality.

Finally, the automated ADCC Reporter Bioassay was also validated using WIL2-S cells, and a 6:1 E:T cell ratio. As seen with the Raji validation data, WIL2-S EC_{50} values (Figure 13) are similar to the previously generated and manually-derived EC_{50} value of 2.29e-08[6]. This demonstrates the accuracy and robustness of an automated method using the ADCC Reporter Bioassay technology.

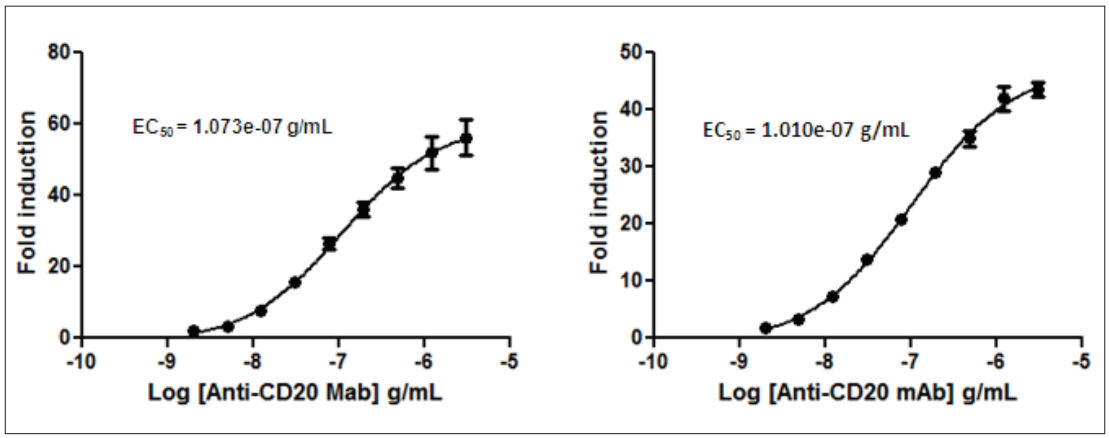

Figure 11. ADCC Reporter-based bioassay comparison of automated versus manual methods.

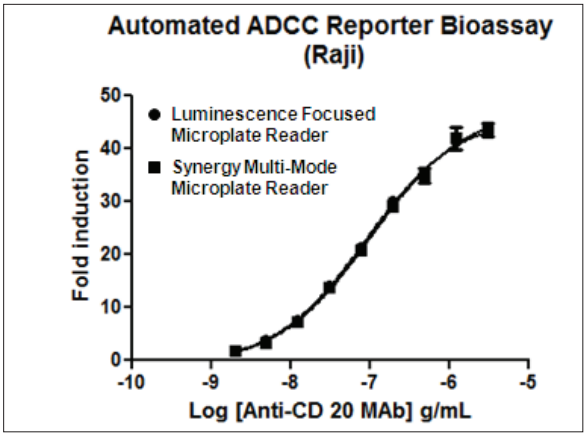

Figure 12. Comparison of results between the Synergy Multi-Mode Microplate Reader and a luminescence-focused microplate reader.

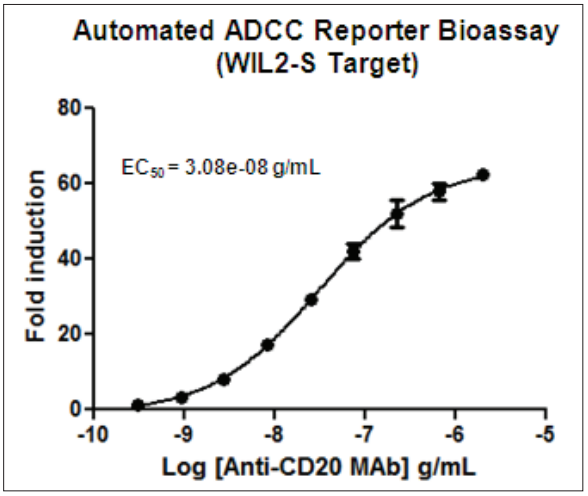

Figure 13. Automated ADCC Reporter Bioassay validation using WIL2-S cells.

TAG-Lite Assay

Assay Principle

Tag-lite® assays (Cisbio Bioassays, Codolet, France) do not use effector:target cell formats as the previously described assays, but rather combine target receptor:antibody and FcγRIIIa receptor:antibody binding assays together to demonstrate ADCC potential. Each incorporates homogeneous time-resolved fluorescence (HTRF®) and SNAP-tag™ technologies as part of the detection step. HTRF detects biomolecular interactions via fluorescence resonance energy transfer (FRET) between Lumi4®-Tb cryptate (donor fluorophore) and a modified allophycocyanin (acceptor fluorophore), whereas SNAP-tag is a small, self-labeling fusion protein that is a precise and highly specific alternative to antibody capture or labeling. Indirect and competitive binding may be measured for increased assay versatility.

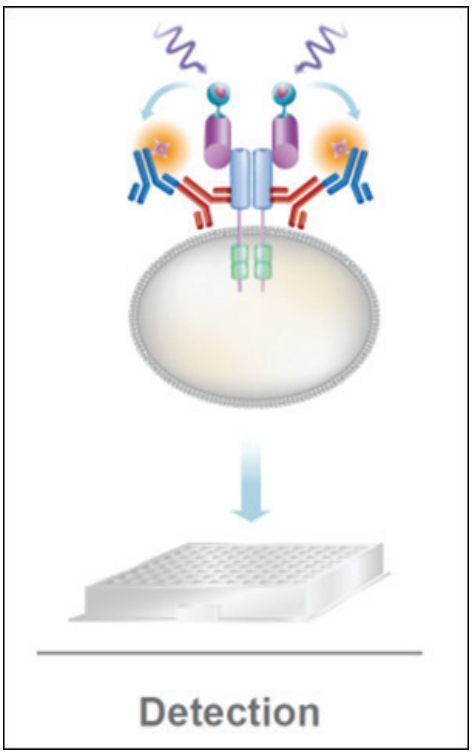

Figure 14. Tag-lite indirect binding assay principle with primary (red) and secondary (blue) antibodies.

In the Tag-lite process, engineered cells containing the test antigen and the SNAP-tag sequence are labeled with the donor terbium molecule. When measuring indirect binding (Figure 14), the primary test antibody binds to the antigen, and a secondary antibody, labeled with the d2 acceptor fluorophore, binds to the Fc portion of the primary antibody. This complex brings the donor and acceptor molecules in close proximity, allowing FRET to take place, and generating an increased HTRF value. Per competitive binding studies (Figure 15) using another known ligand or biosimilar antibody, a d2 acceptor labeled antibody binds to the antigen, allowing FRET to take place. If preferential binding takes place once an unlabeled test antibody is added to the solution, the labeled antibody is displaced, thus interfering with FRET and lowering the HTRF value. This combination of target and Fc receptor binding assays also allows for MOA determination. The assay is also a mix-and-read format, and does not require centrifugation or transfer steps seen in other methods.

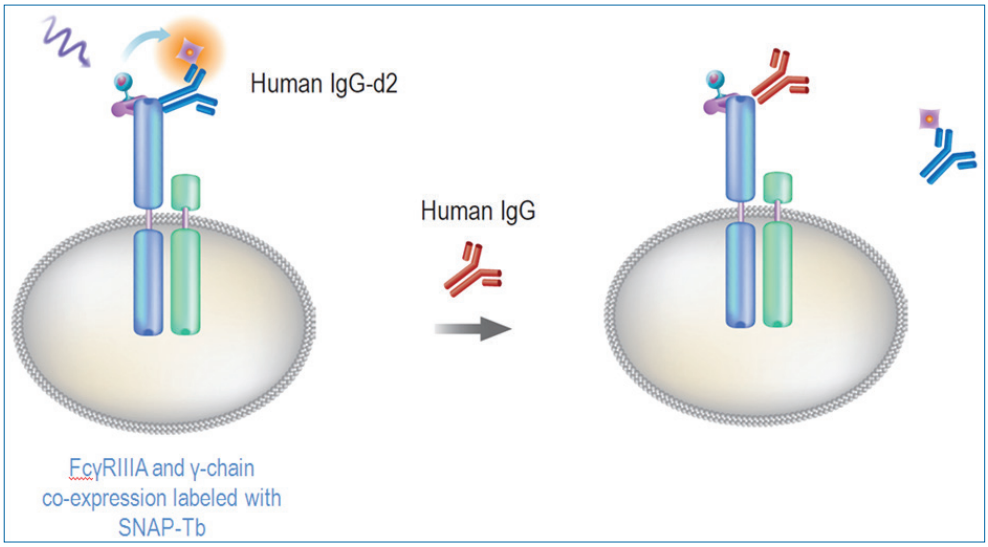

Figure 15. Tag-lite competitive binding assay principle showing labeled primary antibody bound to the target antigen (left), and secondary antibody competitive binding (right).

Method Summary

Using the indirect binding method, titrated antibody, Tag-lite buffer, d2-labeled acceptor antibody and thawed, cryopreserved cells were added to low-volume (LV) 384-well microplates. Per the competitive binding method, competitive binding protein, primary binding antibody, d2-labeled acceptor antibody and thawed, cryopreserved cells were added to LV 384-well microplates. The microplates were incubated, then read in the Synergy Multi-Mode Microplate Reader set to detect the assay's dual 620 nm and 665 nm fluorescent emissions from the donor and acceptor molecules when the excitation was set to 330 nm.

Results

K_d values were generated to assess test antibody binding to the target receptors, including a G-protein coupled receptor (GPCR) and receptor tyrosine kinase (RTK). EC_{50} and EC_{80} values were also calculated to determine binding to target receptors, and also for use in competitive binding experiments (data not shown).

The K_i value for known receptor ligands and potential biosimilar antibodies could then be calculated from results of a separate competitive binding experiment using the test antibody EC_{80} value (Figure 16).

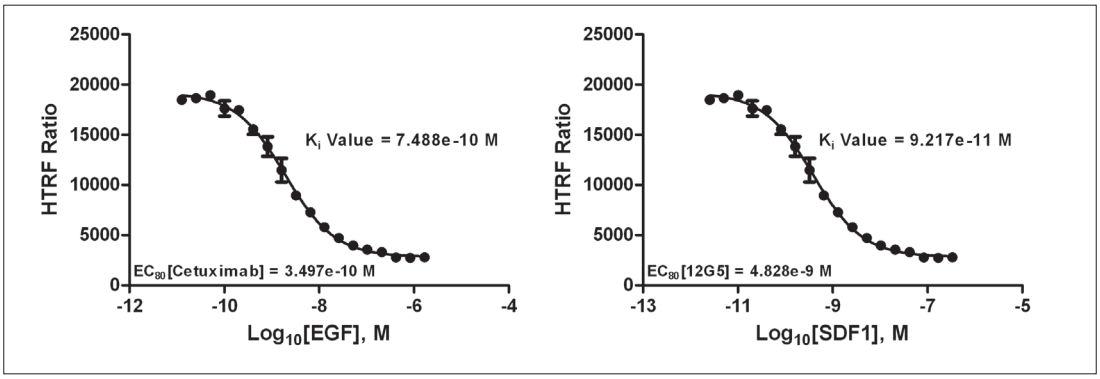

Figure 16. Competitive binding assessment of known receptor ligands.

In order to assess CD16a binding, four IgG antibodies with differing receptor binding qualities were tested (Figure 17), in addition to the therapeutic antibody, cetuximab, and 12G5. IgG1 and cetuximab both bind to CD16a (Figure 18), while 12G5 does not bind. Since the assay uses a humanized CD16a receptor, and 12G5 is an anti-mouse antibody, binding should not occur. Therefore, the antibody served as a negative control. Antibody binding to both target and FcγRIIIa receptors demonstrates ADCC activity and validates the instrumentation's ability to generate accurate data.

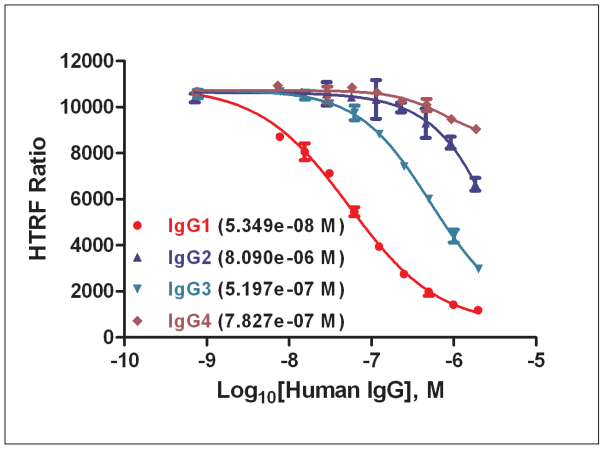

Figure 17. Characterization of IgG antibodies.

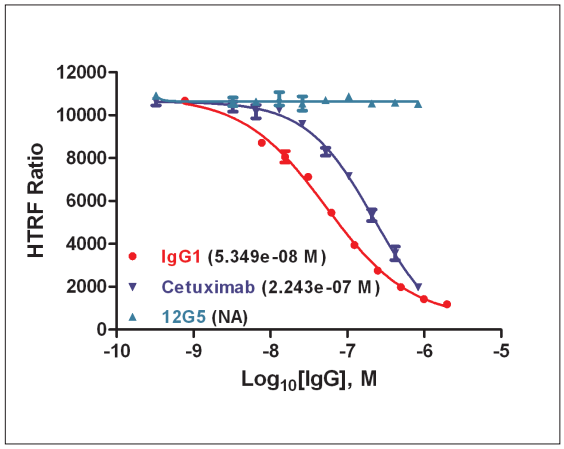

Figure 18. Comparison of IgG1, cetuximab and negative control 12G5 antibody.

Conclusions

We have demonstrated several easy-to-use, non-radioactive assay platforms for ADCC studies using luminescent, TRF or TR-FRET detection and primary, immortalized or genetically engineered cells. Each of the assay workflows can be easily automated with instruments like BioTek's Precision and MultiFlo to increase throughput and repeatability. The various read modes in Synergy Multi-Mode Microplate Readers add even more flexibility to the processes, and increase efficiency to the overall laboratory workflow. The combination of these new assay chemistries, appropriate cell models, and automated instrumentation can increase productivity and throughput, simplify processes, and generate high-quality, reproducible results for the discovery of new antibody therapeutics.

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