# Improving Cell-Mediated Cytotoxicity Assessment through the Use of an Automated Luminescent ADCC Assay

Brad Larson[1], Sumant Dhawan[2], Shalini Wadwani[2], and Peter Banks[1]

[1]BioTek Instruments, Inc., Winooski, Vermont, USA • [2]Cell Technology, Inc., Mountain View, California, USA

## 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/neu 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 $^{51}$Cr. 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. Here we describe the use of a non-radioactive luminescent chemistry to simplify the assay process and provide improved data quality. Recruitment of NK cells by the proper antibody leads to lysis of the target cell. This causes release of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) from the cell. ATP production ensues, which is coupled to a luciferase/luciferin reaction producing a luminescent signal. No target cell preparation time is necessary, and shortened incubation times mean the procedure can be completed in half the time of the original procedure, without any complicated and expensive regulatory permissions. The assay is easily automated in 96-well format to further simplify the procedure. Experimental data generated with fresh as well as cryopreserved NK cells, using multiple effector:target cell ratios, will be shown to prove the validity and flexibility of the method.

## Materials

**Cells –** Target Cells: Human Daudi cell line derived from African-American Burkitt's Lymphoma (ATCC, Catalog No. CCL-213); Effector Cells: Freshly isolated NK cells from whole blood, or cryopreserved NK cells (Stemcell Technologies, Catalog No. PB012F)

**Media –** Daudi Cell Propagation Media: RPMI 1640 (Life Technologies, Catalog No. 11875), FBS, 10% (Life Tech., Catalog No. 10437); ADCC Assay Media: RPMI 1640 (Life Technologies, Catalog No. 11875), Ultra-Low IgG FBS, 10% (Life Tech., Catalog No. 16250), NEAA, 1X (Life Technologies, Catalog No. 11140), Pen-Strep-Glutamine, 1X (Life Tech., Catalog No. 10378)

**Test Antibody –** Rituximab (Genentech, South San Francisco, CA)

**Assay Chemistry –** aCella-TOX™ Bioluminescence Cytotoxicity Assay (Cell Technology, Mountain View, CA)

## aCella-TOX™ ADCC Assay

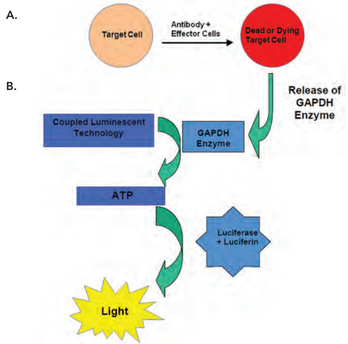


***Figure 1** – A. Effector cells cause lysis of target cells through the antibody-dependent cell-mediated cytotoxicity process. B. GAPDH is released from dying cells, leading to ATP production. The ATP produced is then coupled to a luciferase/luciferin reaction producing light.*

## BioTek Instrumentation

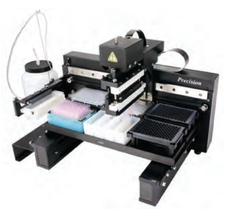

***Figure 2** – The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dispense all assay components, including target and effector cells, serially titrate antibody across a 96-well PP plate, transfer samples from plate to plate, as well as dispense the aCellaTOX assay components.*

***Figure 3** – The Synergy™ H4 Hybrid Multi-Mode Microplate Reader combines a filter-based and monochromator-based detection system in the same unit. A dedicated luminescence detection system was used to quantify the luminescent signal from each assay well. The plates were read in kinetic mode to capture the luminescent signal every 5 minutes.*

## Automated ADCC Assay Procedure

Daudi target cells, at a concentration of 2x10$^5$ cells/mL in 25 µL were added to the 96-well assay plate. An 8-point titration curve of the test antibody was then created using serial 1:5 dilutions beginning at 1 µg/mL. 25 µL of each antibody dilution was added to the plate to start the reaction. The cells were allowed to opsonize for 15 minutes at 37°C. NK effector cells, at a concentration of 4x10$^6$ or 2x10$^6$ cells/mL, were then added (in 25 µL) to give an E:T ratio of 20:1 or 10:1, respectively. The plate was centrifuged for one minute, and incubated at 37°C for 1.75 hours. The plate was then removed from the 37°C incubator and allowed to cool to room temperature for 5-10 minutes. The target cells in the maximum lysis control wells were then lysed by adding 10 µL of the Lysis buffer, and the plate was incubated for an additional 5 minutes. 125 µL of ADCC media was then added to each well to bring the volume to 200 µL. The plates were centrifuged for one minute. 50 µL of Enzyme Assay Diluent was then transferred to the appropriate wells of an opaque white luminescence plate. 50 µL of each reaction supernatant was transferred to wells containing the assay diluent. 100 µL of 2X Enzyme Assay Reagent (containing G3P), followed by 50 µL of 1X Detection Reagent was added to each diluted supernatant. The plates were immediately read using the Synergy H4 at 5-minute intervals. The RLUs were graphed and the data reduced by four-parameter fits for analysis.

## % Total Cytotoxicity Calculation

The luminescent signal from the wells containing media and other assay components was subtracted from all other wells. Average non-lysed effector cell only and target cell only control well signal was then subtracted from all sample wells, while average non-lysed target cell only signal was subtracted from the average maximum lysis signal. % Total Cytotoxicity was then calculated by dividing adjusted sample well signal by the adjusted average maximum lysis signal, and multiplying the result by 100.

## aCella-TOX Assay Optimization

Due to the extreme sensitivity of aCella-TOX, especially if serum-free or heat-killed media are used, it is frequently possible to shorten the incubation time for the lytic process. It is also many times possible, and desirable, to use smaller numbers of target cells than are needed for the $^{51}$Cr-release assay, due to this and the fact that excessive numbers of effector cells can increase the background signal. Two tests were performed to validate the assay's ability to use shorter incubation times, as well as incorporate lower cell numbers.

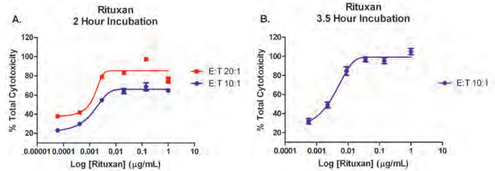


***Figure 4** – A. Rituxan cytotoxicity curves using 5000 Ramos target cells, a 20:1 or 10:1 ratio of effector:target cells, and 2 hour incubation time following effector cell addition. B. Rituxan cytotoxicity curve using a similar target cell number, 10:1 target:effector cell ratio, and 3.5 hour incubation period.*

The % Total Cytotoxicity values seen in part A of figure 4, ranging from 65% to nearly 100% for 10:1 and 20:1 effector:target (E:T) cell ratios, respectively, demonstrate that efficient cell lysis is seen with a two hour lysis incubation period. The curve in part B illustrates that more complete lysis can be achieved by using a longer post-effector cell addition incubation time, should a lower E:T ratio be desired in the assay, in order to lower background signal.

## aCella-TOX/$^{51}$Cr Comparison

A comparison of the optimized aCella-TOX assay to the $^{51}$Cr release assay (CRA) was then performed. This was done in order to ensure that the new assay technology would deliver results that were similar to those generated using an established assay. For the CRA, Daudi target cells were plated at a concentration of 10,000 cells/well, followed by various concentrations of rituxan. Following a 30 minute incubation at 37°C, freshly purified NK cells were added at an E:T ratio of 20:1. The plate was again incubated at 37°C for 4 hours, mixed, and spun for 5 minutes at 150 x g. Supernatant aliquots were transferred to a separate plate, and scintillation was then counted. The aCella-TOX assay was performed as previously described, also using freshly purified NK cells.

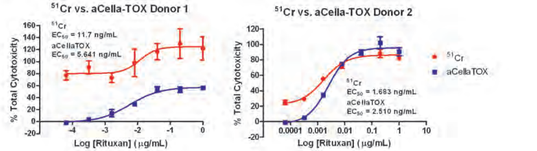


***Figure 5** – Rituxan cytotoxicity curves generated using either the $^{51}$Cr or aCella-TOX assays and two separate donors.*

The shape of the curve, and similarities of EC$_{50}$ values for both assays demonstrate that the aCella-TOX assay is able to deliver similar results for test antibodies, with similar or less variation than the $^{51}$Cr assay. The difference in absolute lysis, seen with donor 1, may be due to artifacts associated with the cell labeling that takes place during the $^{51}$Cr assay. However the inflection point of the curves is similar, confirming the similarity of the data.

## Manual/Automated Assay Comparison

The ability of the automated method to generate results that were similar to manual processing was then tested using the Precision. All steps of the assay were performed either robotically, using the Precision, or by hand using the procedure previously explained. 20:1 and 10:1 effector to target cell ratios were tested. NK effector cells were isolated from whole blood. Multiple individual runs were performed with separate blood donors to validate the repeatability of the robotic process.

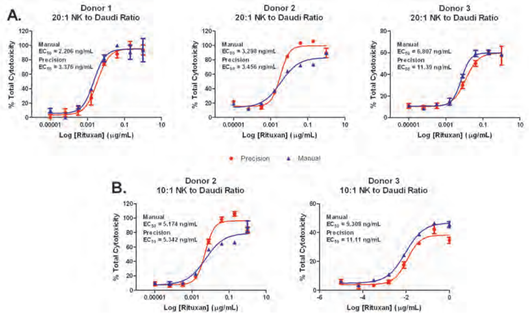


***Figure 6** – Robotic and manually generated cytotoxicity curves, using rituxan and a 20:1 (A.) or 10:1 (B.) effector:target cell ratio.*

The similarity in cytotoxicity curves and EC$_{50}$ values demonstrate that robotic processing can generate data that is similar to that generated by an experienced manual operator using either a 20:1 or 10:1 effector to target cell ratio. Repeatable results from multiple different donors show that the procedure is robust.

## Cell Comparison

The final test of the assay incuded the use of cryopreserved NK cells. Part of the difficulty with using freshly isolated NK cells is the variation that can be seen in cytotoxicity from each blood donor. This is witnessed in figures 5 and 6. Cyropreserved NK cell lots can include cells from multiple donors, which can eliminate this problem. For this test, the assay was once again run in an automated format. The cryopreserved cells were thawed and prepared per the manufacturers recommendations. 20:1 and 10:1 effector to target cell ratios were examined.

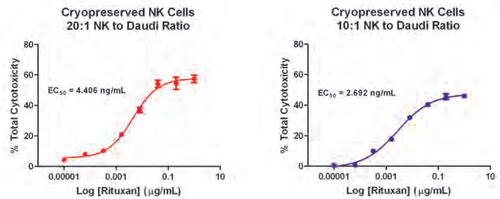


***Figure 7** – EC$_{50}$ values and cytotoxicity curves generated with the aCella-TOX assay and cryopreserved NK cells.*

The similarity between the rituxan cytotoxicity curves and EC$_{50}$ values generated using cryopreserved NK cells, to those generated using freshly isolated NK cells (Figure 6), confirm that the automated aCella-TOX assay procedure can deliver equivalent data using either cell format.

## Conclusions

1. The aCella-TOX cell-based ADCC assay provides a simplified procedure, that is more reproducible and can be performed in a shorter time period when compared to the $^{51}$Cr assay
2. The assay can be automated in 96-well format, which can save valuable resource time, and provides greater reproducibility when compared to manual processing
3. The Precision can consistently, and accurately titrate the test antibody, as well as dispense the other assay components, including target and effector cells
4. The Synergy H4 is able to accurately quantify the luminescent signal from each assay well using each plate type tested
5. Equivalent data can be generated using cryopreserved NK cells, providing a more consistent and readily available NK cell format
6. The equivalent cytotoxicity curves and EC$_{50}$ values from automated and manual processing demonstrate how the combination of assay, cells, and instrumentation provide a simple, yet robust solution for performing antibody-dependent cell-mediated cytotoxicity assays