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

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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 therapeutics, Rituxan® (Roche/Genentech, Inc.) showed worldwide sales in 2009 of $5.6 billion (Genew News Highlights, 2011). This, among others including Trastuzumab (Herceptin®), Fluconazole and Rinocin (Roche), have shown great promise for treatment of patients with life-threatening diseases. As a result, it is believed that development of these antibodies will continue to increase. Despite this, 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 Vinoper have set to together to develop a biosimilar to Rituxan (Genew News Highlights, 2011). As a result, assays that can assess the ability of a biosimilar to act in a similar manner 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 flow cytometry of the actual assay. Not only is this time consuming, but involves the use and eventual 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 is measured by an increase in the light produced by luciferase expressed in the target cell. The proper antibody also plays a role in the ADCC assay. A successful ADCC assay depends upon the ability of the test antibody to kill the target cells by interacting with the target cell and with the effector cell. The accuracy of the ADCC assay also depends on the purity and homogeneity of the test antibody.

Materials
Cells – Target Cells: Human Daudi cell line derived from African-American Burkitt's Lymphoma (ADCC, Catalog No. CCL-21). Human NK cell lines (500,000 cells/mL) (Stemcell Technologies, Catalog No. 4781). Media – Daudi Cell Propagation Media: RPMI 1640 (Life Technologies, Catalog No. 11875), FBS, 10% (Life Tech., Catalog No. 10378), ADCC Assay Media: RPMI 1640 (Life Technologies, Catalog No. 11875), Ultra-Low Isg FBS, 10% (Life Tech., Catalog No. 11422), NEAA, 1X (Life Technologies, Catalog No. 11140), Pen-Strep-Glutamine, 1X (Life Tech., Catalog No. 10378). Antibody – Rituxan (Genentech, South San Francisco, CA)

aCella-TOX Assay Chemistry – aCella-TOX™ Bioluminescence Cytotoxicity 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 a luminescent signal. No target cell preparation time is necessary, and shortened incubation times can be used if the procedure can be completed in half the time of the original procedure, with no impact to the assay's validity and flexibility of the method.

Automated ADCC Assay Procedure
Daudi target cells, at a concentration of 2x105 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 incubate for 15 minutes at 37°C. NK effector cells, at a concentration of 5x104 to 2x105 cells/mL, were then added (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 1.5 hours. The plate was then removed from the 37°C incubator and added to room temperature for 5-10 minutes. The target cells in the maximum lysin control were then lysed by adding 10 μL of the lysin buffer, and the plate was incubated for an additional 5 minutes. 125 μL of ADCC media containing 5% FBS was then added to each well, resulting in a total volume of 200 μL. The plate was then incubated for an additional 3 hours. The plate was then transferred to the Luminoskan (Thermo, Vantaa, Finland). The plate was read using the luminoskan H4 at 5 minute intervals. The results were then compared to the standard curve. The assay was performed multiple times with different donor cells, multiple runs were performed with separate blood donors to validate the repeatability of the robotic process.

Conclusions
The ability of the automated method to generate results that are 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 described. 20:1 and 10:1 effector:target cell ratios demonstrated that efficient cell lysis is seen with a two hour lysis incubation period. The curve shape, and similarities of EC50 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 51Cr assay. The difference in absolute lysin, seen with donor 1, may be due to differences associated with the cell labeling that takes place during the 5 Cr assay. However, the lack of variation in the curves is similar, confirming the similarity of the data.

Manual/Automated Assay Comparison
The ability of the automated method to generate results that are 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 described. 20:1 and 10:1 effector:target cell ratios demonstrated that efficient cell lysis is seen with a two hour lysis incubation period. The curve shape, and similarities of EC50 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 51Cr assay. The difference in absolute lysin, seen with donor 1, may be due to differences associated with the cell labeling that takes place during the 5 Cr assay. However, the lack of variation in the curves is similar, confirming the similarity of the data.

Cell Comparison
The final test of the assay included 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 cell to cell. This is witnessed in figures 5 and 6. Cryopreserved 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 evaluated.

Figure 7 – EC50 values and cytotoxicity curves generated with the aCella-TOX assay and cryopreserved NK cells. The similarity between the rituxan cytotoxicity curves and EC50 values generated using cryopreserved NK cells, to those generated using freshly isolated NK cells (Figure 6), confirm that the aCella-TOX assay procedure can deliver equivalent data using either cell format.