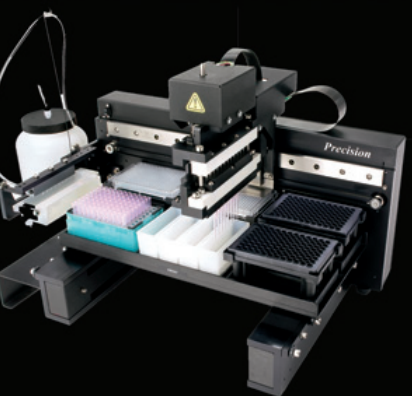


A Semi-Automated, Non-Radioactive Assay for the Detection of Antibody-Based Complement-Dependent Cytotoxicity

Brad Larson and Peter Banks, BioTek Instruments, Inc., Winooski, VT
Sumant Dhawan and Shalini Wadwani, Cell Technology, Inc., Mountain View, CA
Mark Rothenberg, Corning Life Sciences, Kennebunk, ME

Cell Technology, Inc.



We describe the use of a non-radioactive, bioluminescent antibody-based Complement-Dependent Cytotoxicity (CDC) assay. The simple protocol details the sequential addition of cells, antibody therapeutic, complement, and reagents that can be readily automated with a simple, inexpensive pipetting station. Here we demonstrate the semi-automation of the CDC assay using Daudi cells, rituximab (monoclonal antibody used in the drug *Rituxan*) and baby rabbit complement.

Introduction

Pharmaceutical companies are increasingly focused on biologic therapeutics rather than biochemical, chemical and radiological compounds for many cancer treatments. Biologic therapeutics only target and destroy specific cancer cells, thus preventing damage to surrounding, healthy cells and reducing unwanted cytotoxic events. The most prominent and successful example is Rituximab (*Rituxan*®; Roche/Genentech), a chimeric monoclonal antibody (mAb) therapy that is used for certain cancers and autoimmune disorders.

In spite of increased biologics research, specific mAb anti-tumor mechanisms remain undefined. Evidence is mounting to suggest that antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) both play a role in tumor cell lysis, but the importance of each, and any synergistic or antagonistic interrelation is as of yet unclear¹. ADCC is a prominent mechanism in the host immune defense where antibodies bind to a target cell and subsequently recruit effector cells, such as natural killer (NK) cells, to bind via Fc receptors. The NK cells then engulf and destroy the target cell. On the other hand, in the CDC mechanism, antibodies once again bind to a target cell through a specific antigen, but activate the complement cascade, a multi-pathway attack on the target cell (Figure 1), rather than recruiting NK cells.

Here, we present a non-radioactive, bioluminescent antibody-based CDC assay (Figure 2) that quantitatively measures release of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) naturally found in primary, mammalian and bacterial cells. In this manner, prelabeling, transfection, transformation and other methods to artificially introduce molecules into cells is negated. The reagents may be used for CDC and ADCC assays.

Adding automation to the assay process can reduce variability compared to manual methods. Additionally, automated instruments can increase throughput and decrease overall project time.

Key Words:

Biotherapeutics
Antibody
IgG
Rituximab
CDC
Immunotherapeutics
CD20
Luminescence
Automation
Liquid Handling

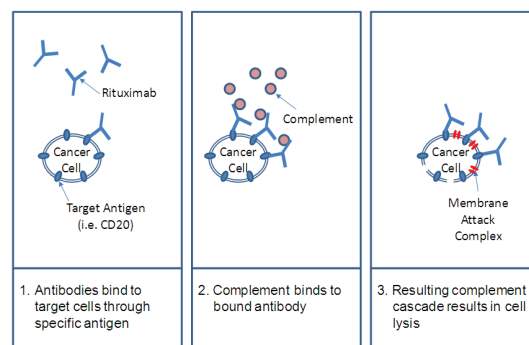


Figure 1. Principle of CDC specific to *Rituxan*.

As each mechanism may play a role in anti-tumor mechanisms, biologics research should not solely focus on ADCC assays, but should also include CDC assays to obtain a true mechanistic profile.

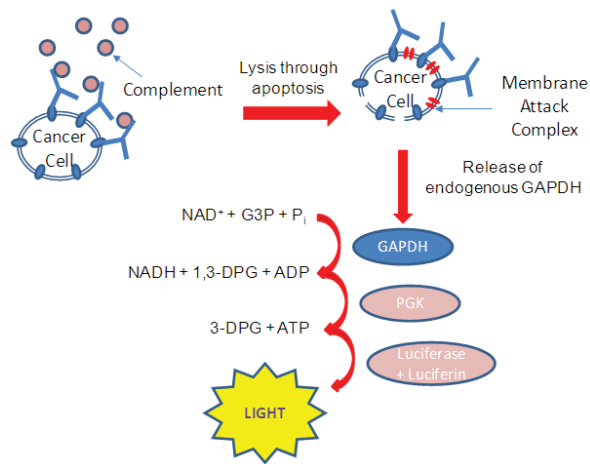


Figure 2. aCella-TOX CDC-based assay principle based on the release of endogenous Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). The assay kit contains the enzyme 3-Phosphoglyceric Phosphokinase (PGK) to produce ATP, which is then used in conjunction with luciferase and luciferin to generate luminescence.

Materials and Methods

Materials

Daudi cells (human Burkitt's lymphoma cell line, catalog number CCL-213) were obtained from ATCC (Manassas, Virginia) and used as target cells in the CDC assay. Rituximab, the monoclonal antibody in the drug *Rituxan*, and aCella™-TOX (catalog number CLATOX 100-3) were provided by Cell Technology, Inc (Mountain View, California). Baby rabbit complement (catalog number CL3441-S) was purchased from Cedarlane Laboratories (Burlington, North Carolina).

Daudi Cell Propagation Medium consisted of RPMI 1640 (catalog number 11875), Heat Inactivated Fetal Bovine Serum, 10% (catalog number 10082), NEAA, 1X (catalog number 11140), and Pen-Strep-Glutamine, 1X (catalog number 10378). The aforementioned reagents were supplied by Life Technologies Corporation, Grand Island, New York.

Clear, round-bottom TC-treated 96-well microplates (catalog number 3799) were used for antibody dilution and to perform the cell lysis portion of the assay procedure. 96-well white, flat bottom, PS, non-treated microplates (catalog number 3912) were then used for lysate transfer, aCella-TOX reagent addition, and luminescent signal detection. Both plate types were provided by Corning Life Sciences, Corning, New York.

Instrumentation

The Precision™ Microplate Pipetting System combines an eight-channel pipetting head and an eight-channel bulk reagent dispenser in one instrument.

The instrument was used to dispense all assay components, including target (Daudi) cells and complement, serially titrate antibody across a 96-well polypropylene plate, transfer samples from plate to plate, and dispense the aCella-TOX assay components.

The Synergy™ H4 Hybrid Multi-Mode Microplate Reader combines filter- and monochromator-based detection systems 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, using integrated Gen5™ Data Analysis Software, to capture the luminescent signal every five minutes.

Automated CDC Assay Procedure

20 μ L of Daudi target cells, at a concentration of 2×10^5 cells/mL were added to each well of the 96-well assay plate. A 10-point titration curve of the test antibody was then created using 1:2.5 serial dilutions beginning at 1 μ g/mL. 25 μ L of each antibody dilution was added to the plate to start the reaction. The plate was shaken for 30 seconds and then placed in a 37° C/5 % CO₂ incubator for 15 minutes to allow the cells to opsonize. Baby rabbit complement was then diluted in Daudi cell medium, and 25 μ L was added to the appropriate wells. Following shaking for 30 seconds, the plate was incubated at 37° C/5% CO₂ for 30 minutes. The plate was then removed from the 37° C incubator and allowed to cool to room temperature (RT) for 15 minutes. The target cells in the maximum lysis control wells were then lysed by adding 10 μ L of the Lysis Buffer, and the plate was incubated on the benchtop for an additional 5 minutes at RT. 125 μ L of Daudi Cell Medium was then added to each well to bring the volume to 200 μ L. The plates were centrifuged for one minute at 750 RPM. 50 μ L of Enzyme Assay Diluent was then transferred to the appropriate wells of an opaque white luminescence plate. 50 μ L of each reaction supernatant was transferred to wells containing the assay diluent. 100 μ L of 2X Enzyme Assay Reagent (containing G3P), followed by 50 μ L of 1X Detection Reagent was added to each diluted supernatant. The plates were again shaken for 30 seconds and immediately read in luminescence mode using the Synergy H4 at five minute intervals. The % Total Cytotoxicity values were graphed and 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 to correct for background interference. Average non-lysed target cell-only control well signal was then subtracted from all sample wells. % Total Cytotoxicity was then calculated by dividing adjusted sample well signal by the average maximum lysis signal, and multiplying the result by 100.

Results and Discussion

Automated vs. Manual Processing Comparison

Initial tests were performed to confirm the automated method's ability to generate results similar to manual processing (Figure 3). All assay steps were performed either robotically, using the Precision, or manually using the procedure previously explained. 5000 Daudi cells/well as well as a final 1X complement concentration of 5% were used for the test.

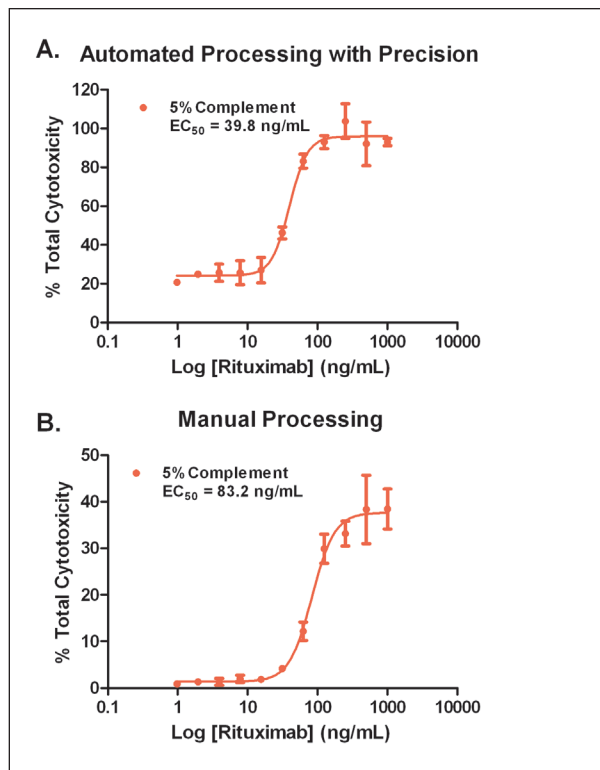


Figure 3. Dose response curves and EC₅₀ values generated using the Precision (A.) or manually (B.).

The cytotoxicity curve and EC₅₀ value similarities, when compared to manual data generated by Cell Technology, demonstrate that robotic processing can generate results equivalent to those produced manually. The differences seen in % Total Cytotoxicity are explained by the fact that different complement lots, and therefore different background levels, were seen in the assay.

Variable Assay Parameter Analysis

Assay parameter optimization is essential to ensuring appropriate data and the best possible assay window. Two specific parameters that may have a profound effect on data accuracy are the percentage of complement added to the target cells and antibody and the incubation time after all three components are combined. Therefore, it is highly recommended to optimize these conditions before proceeding with antibody testing and validation.

To accomplish this, we tested 2.5-15% complement concentrations (Figure 4), and incubation times of 30, 75, and 120 minutes (Figure 5). EC₅₀ values and signal:background (S:B) ratio were used to determine the most optimal conditions.

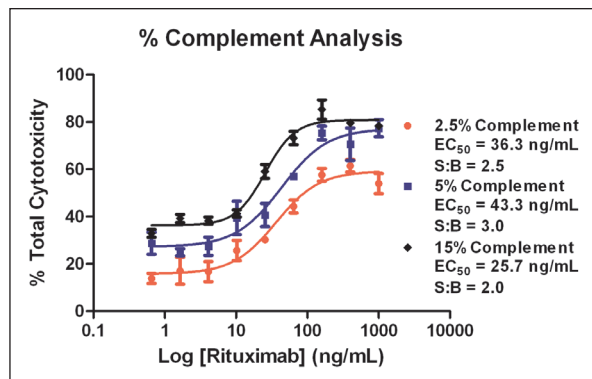


Figure 4. Variable complement concentration results. Final 1X concentrations of 2.5, 5, and 15 % tested. Assay incubation time equaled 30 minutes.

While the EC₅₀ values were equivalent with each test, maximum % cytotoxicity and background values varied with each complement concentration tested. Background values were lower using 2.5% complement, however a much lower maximum cytotoxicity was achieved. Equivalent higher values were seen using 5% and 15% complement, although background values increased to a greater extent using the highest percentage. Therefore, a final complement concentration of 5% was shown to yield the largest S:B ratio for the assay.

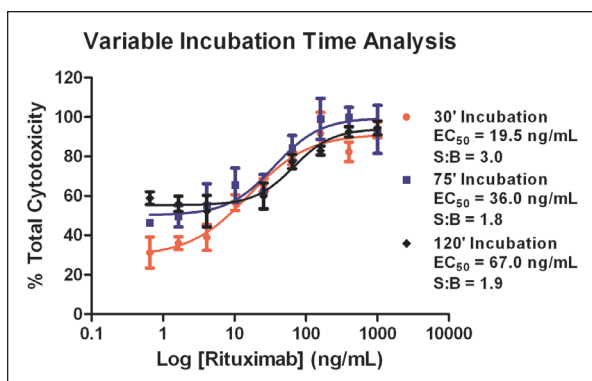


Figure 5. Variable assay incubation time results. Post complement addition incubation times included 30, 75, and 120 minutes. Final 1X complement concentration equaled 5 %.

Variable incubation test results demonstrate that while maximum % cytotoxicity values are similar with each incubation time, the background increases dramatically and EC₅₀ values are right-shifted with increased incubation. This not only affects the assay window, but can also cause a false decrease in apparent therapeutic potency.

Automated Assay Robustness Validation

Delivering robust, repeatable data is critical to automating an assay procedure. This was tested by performing multiple CDC assay runs using the same complement lot (Figure 6), as well as testing multiple individual complement lots (Figure 7). The previously optimized assay conditions of 5% (1X) complement and a thirty minute post-complement incubation time were incorporated into these tests.

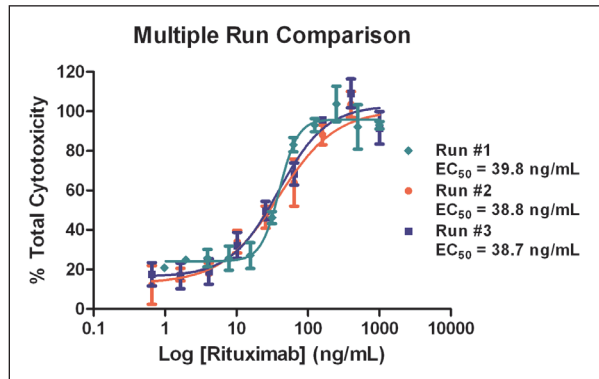


Figure 6. Results from three independent CDC assay runs using 5000 Daudi cells/well, and a 5% concentration of one individual complement lot.

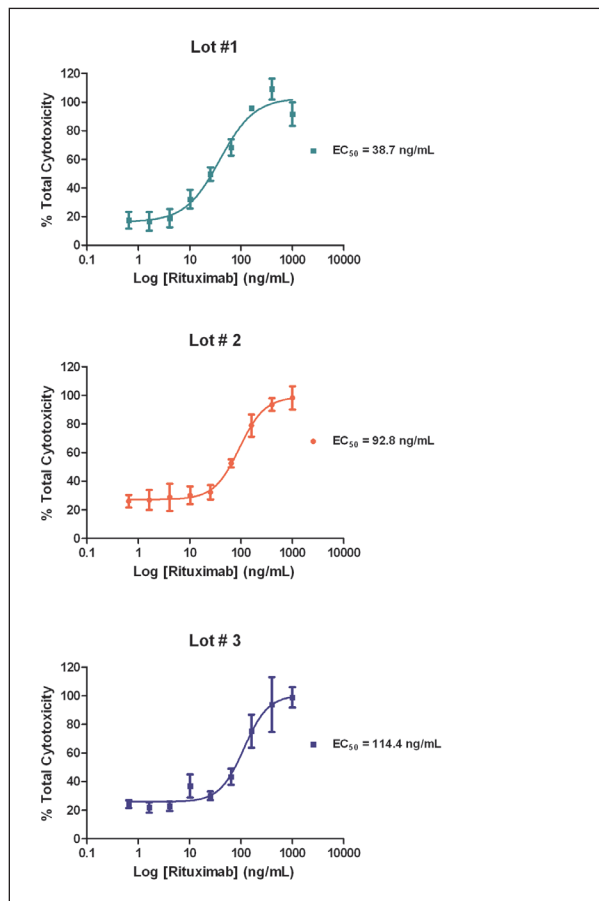


Figure 7. Results from a single CDC assay run using 5000 Daudi cells/well, and a 5% concentration of three individual complement lots.

The cytotoxicity curve similarities across the three runs using the same complement lot, as well as EC_{50} values, demonstrates that the Precision consistently titrates the antibody and dispenses assay components. This is further proven when examining results using separate complement lots. While the actual EC_{50} values vary slightly, which is explained by variations in the immune response from individual animals, a similar curve shape is seen, and there is little variation between replicates at each antibody concentration.

Conclusions

The optimization and validation results demonstrate that the automated procedure, using a microplate pipetting system, can produce an equivalent performance relative to manual processing using identical detection technology. However, the automated process can relieve the operator of tedious tasks, reduce human error associated with repetitive procedures, and provides the operator with time to commit to other more pressing tasks. In addition, the automated procedure can be conducted in laminar flow hoods to help maintain sterile conditions. Finally, the combination of assay chemistry, appropriate microtiter plates, and automation creates a robust process to help ascertain a clearer picture of what antibody-dependent cytotoxic processes are elicited to kill a target cancer cell.

References

1. Wang, S.Y.; Weiner, G. Complement and cellular cytotoxicity in antibody therapy of cancer. *Expert Opin Biol Ther.* **2008**, *8*(6), 759-768.