

Semi-Automation of a Nonradioactive Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay

Part I: Automation of a bioluminescent ADCC procedure with a microplate pipetting system

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Abstract

This application note describes the use of a nonradioactive, bioluminescent antibody-dependent cell-mediated cytotoxicity (ADCC) assay. The protocol is a simple sequential addition of reagents and cells that can be readily automated with a simple, inexpensive pipetting station. This application note demonstrates the semi-automation of the ADCC assay using Daudi cells, rituximab (monoclonal antibody used in the drug Rituxan), and freshly isolated primary human NK cells.

Introduction

ADCC is part of a host immune defense where an effector cell of the immune system is directed to lyse a target cell or pathogen. This is accomplished by specific antibodies raised against antigens on the target cell/pathogen surface. It is one of the mechanisms through which antibodies act to limit and contain infection. One of the most common effector cell types is natural killer (NK) cells. Antibodies recruit effector cells such as NK cells through their Fc portion. NK cells, for example, express CD16 or FcγRIII (CD16a) on their cell surface, which are Fc receptors that recognize the Fc portion of an antibody, such as IgG. Thus once the antibody binds to the target cell, it can bring NK cells in close proximity to the target cell through binding of its Fc receptors to the Fc portion of the IgG. Once this binding event occurs, the NK cell releases cytokines and cytotoxic granules that enter the target cell and promote apoptosis.

This principle is used in targeted immunotherapeutic intervention, particularly for oncology indications. Examples of immunotherapeutics that use this strategy are Rituxan and Herceptin. Combined, these two drugs generated over US \$11B in 2010 – clearly both are blockbuster drugs. Rituxan uses the monoclonal antibody rituximab to bind the antigen CD20 on B cells. In many leukemias and lymphomas, B cells are proliferating uncontrollably and the rituximab is used to recruit effector cells such as NK cells to control the population of B cells in peripheral blood. Herceptin also uses a monoclonal antibody, trastuzumab to bind to over-expressed HER2-neu, a phenotype of a subset of breast cancer patients. Similar to rituximab, the trastuzumab then recruits effector cells to induce apoptosis of the breast cancer cells. This principal is demonstrated in Figure 1.

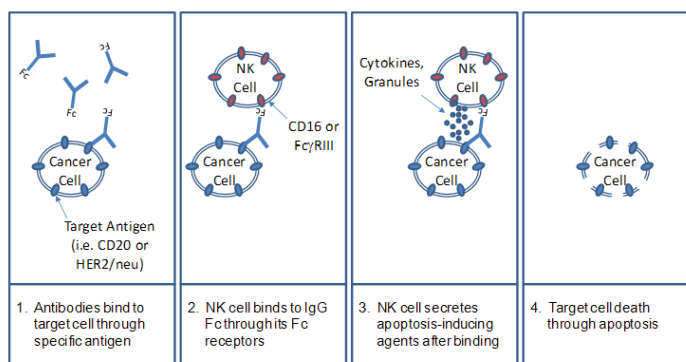


Figure 1. Principle of ADCC specific to Rituxan and Herceptin.

In vitro ADCC assays are common tools for immunotherapeutic drug discovery. In addition, with the blockbuster drugs Rituxan and Herceptin facing patent expiry in the coming years, efforts to develop biosimilar products by competitive companies are ramping up. A number of detection technologies exist for determining the efficacy of antibodies or effector cells in eliciting ADCC. These technologies include radiometric methods, such as chromium-51 [⁵¹Cr], europium [Eu] and sulfur-35 [³⁵S] release assays. In these assays, a target cell line expressing a certain surface-exposed antigen is labeled by uptake of the detection element, followed by incubation with antibody specific for that antigen.

After washing, effector cells expressing Fc receptor CD16 are co-incubated with the antibody-labeled target cells. Target cell lysis is subsequently measured by release of intracellular element by a scintillation counter (⁵¹Cr or ³⁵S) or spectrophotometry (Eu). These assays tend to be problematic. They either involve radio-isotopes, which are noted for costly required regulatory processes and disposal of waste and/or suffer from high background signals due to leakage of the element from the cell.

A nonradiometric ADCC assay that relieves these issues is described here. The bioluminescent assay does not require loading of reagent, but instead relies on the quantification of the release of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a natural component of cells. Figure 2 demonstrates the principal of the detection technology which results in the generation of luminescence from luciferin/luciferase.

The assay workflow is a sequential addition of assay components which is amenable to automation. This application note demonstrates the semi-automation of the workflow with a compact microplate pipetting station that fits in a laminar flow hood, helping to maintain sterile conditions for the assay.

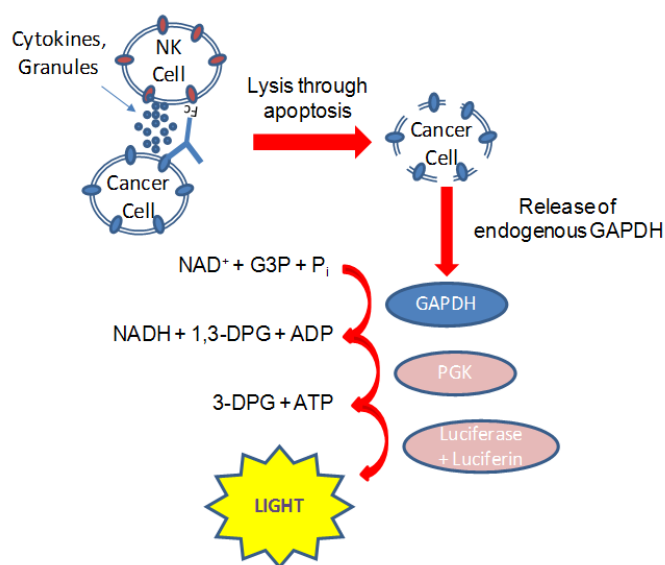


Figure 2. aCella-TOX assay principle based on the release of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The assay kit contains the enzyme 3-phosphoglyceric phosphokinase (PGK) to produce ATP which is then used in conjunction with luciferase and luciferin to generate luminescence.

Materials and methods

Materials

Daudi cells (human Burkitt's lymphoma cell line) were obtained from ATCC (part number CCL-213) and used as target cells in the ADCC assay. Rituximab, the monoclonal antibody in the drug Rituxan, and aCella-TOX (part number CLATOX 100-3) were provided by Cell Technology, Inc. Human primary NK cells were freshly isolated by Cell Technology for all experiments.

Daudi cell propagation medium consisted of RPMI 1640 (Life Technologies, part number 11875), FBS, 10% (Life Technologies, part number 10437), NEAA, 1x (Life Technologies, part number 11140), and pen-strep-glutamine, 1x (Life Technologies, part number 10378). ADCC assay medium consisted of the same components with the exception that ultra-low IgG FBS, 10% (Life Technologies, part number 16250), was substituted for the original FBS.

Instrumentation

The Agilent BioTek 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

(Daudi) and effector (NK) cells, serially titrate antibody across a 96-well PP plate, transfer samples from plate to plate, as well as dispense the aCella-TOX assay components.

The Agilent BioTek Synergy H4 hybrid multimode 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 2×10^5 cells/mL in 25 μ L were added to the 96-well assay plate. An 8-point titration curve was then created of the test antibody using serial 1:5 dilutions beginning at 1 μ g/mL. A 25 μ L amount of each antibody dilution was added to the plate to start the reaction. The cells were allowed to opsonize for 15 minutes at 37 $^{\circ}$ C. NK effector cells, at a concentration of 4×10^6 cells/mL, were then added (in 25 μ L) to give an E:T ratio of 20:1. The plate was centrifuged for 1 minute, and incubated at 37 $^{\circ}$ C for 1.75 hours.

The plate was then removed from the 37 $^{\circ}$ C incubator and allowed to cool to room temperature for 5 to 10 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 for an additional 5 minutes. A 125 μ L amount of ADCC assay medium was then added to each well to bring the volume to 200 μ L. The plates were centrifuged for 1 minute. 50 μ L of enzyme assay diluent was then transferred to the appropriate wells of an opaque white luminescence plate. A 50 μ L amount of each reaction supernatant was transferred to wells containing the assay diluent. A 100 μ L amount 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 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 nonlysed effector cell only and target cell only control well signal was then subtracted from all sample wells, while average nonlysed 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.

Results and discussion

Figure 3 depicts the comparative performance of using a manual pipette compared to the Precision automated pipetting system in the ADCC assay. There is no discernible difference in the analytical performance, yet the automated workflow relieves the tedious serial dilution of antibody and reagent addition steps for the operator.

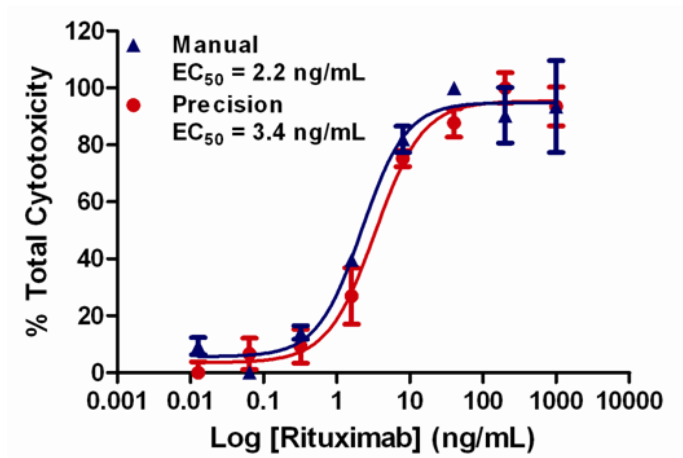


Figure 3. Comparative performance in manual and automated procedures in rituximab dose-response in aCella-TOX assay.

Table 1 depicts the range in EC_{50} typical from a number of NK cell donors quantified by the aCella-Tox kit or ^{51}Cr release assays with Daudi cells as target and rituximab as immunotherapeutic. Assay results indicate equivalent performance.

Table 1. Comparative performance in EC_{50} for manual and automated aCella-TOX assays and ^{51}Cr release assays.

	EC_{50} Results (ng/mL)		
	This Work	aCella-TOX Previous Donors	^{51}Cr Previous Donors
Manual	2.2	2.5 to 5.6	1.6 to 11.7
Automated	3.4	N/A	N/A

Conclusion

The data demonstrate that an automated procedure using a microplate pipetting system can produce an equivalent performance relative to both manual processing using the identical detection technology and gold standard radiometric detection. The automated procedure relieves the operator of tedious tasks, reduces human error associated with complicated procedures and provides the operator with time to commit to other more pressing tasks. In addition, the automated procedures can be conducted in laminar flow hoods to help maintain sterile conditions.

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