


Novel, Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assays



Brad Larson¹, Sumant Dhawan², Shalini Wadwani², Nicolas Pierre³,  Cell Technology, Inc.
Stéphane Martinez², Francois Degorce³, and Peter Banks¹

¹BioTek Instruments, Inc., Winooski, Vermont, USA • ²Cell Technology, Inc., Mountain View, California, USA • ³Cisbio US, Inc., Bedford, Massachusetts, 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 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 ⁵¹Cr. 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.

A number of new cell-based technologies have been, and continue to be, developed that are easier to use than ⁵¹Cr, less time consuming, and do not include the use of radioactivity. These include high-throughput methods that separately assess antibody binding to target as well as CD16 receptors, as well as medium-throughput methods that assess this binding in one assay. Each has its own strengths, and can be the assay of choice depending on the needs of the researcher, or the point in development at which the testing is taking place. In addition, these new assay chemistries are amenable to automated processing and can be detected using microplate readers. Here we describe the automation of two such chemistries. The first targeted towards early identification of lead antibody candidates, and the second ideal for confirmation of final therapeutic formulations using actual human natural killer (NK) cells. Results demonstrate how the combination of assay and instrumentation create robust, efficient, and easy-to-use methods for the identification and validation of antibodies being considered for use in ADCC applications.

BioTek Instrumentation

Precision™ Microplate Pipetting System

The Precision 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 polypropylene plate, transfer samples from plate to plate, as well as dispense assay components for the aCella-TOX™ assay. For the HTRF® target-based and CD16a assays, the instrument was used for ligand and antibody titration and transfer to the LV384-well assay plates.

MultiFlo™ Microplate Dispenser

The dispenser offers fast, accurate non-contact plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 µL. The instrument was used to dispense cryopreserved cells, media, EC₅₀ concentrations of test antibodies, as well as labeled acceptor antibodies in the higher throughput HTRF assays.

Synergy™ H4 Hybrid Multi-Mode Microplate Reader

The hybrid reader combines a filter-based and monochromator-based detection system in one unit. The HTRF certified reader uses the filter-based system and Xenon flash lamp to detect the 665 nm and 620 nm fluorescent emissions from this chemistry using Ex: 330/80 nm; Em: 620/10 and 665/8 nm filters, and a 365 nm cutoff dichroic mirror. A dedicated luminescence detection system was used to quantify the luminescent signal from the aCella-TOX assay chemistry. The plates for this assay were read in kinetic mode to capture the signal every 5 minutes.

aCella-TOX ADCC Assay

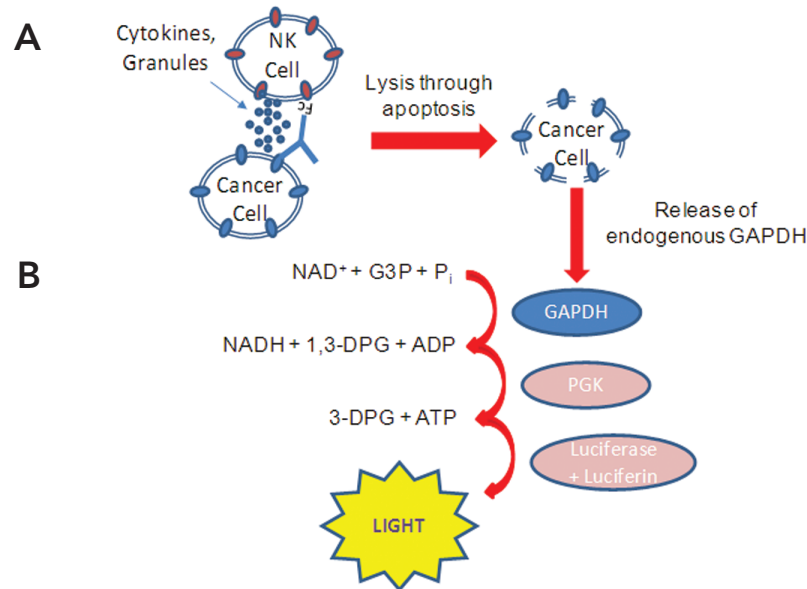


Figure 1 – A. Effector cells cause lysis of target cells through the antibody-dependent cell-mediated cytotoxicity process. **B.** GAPDH is released from dying cells, leading to ATP production. The ATP produced is then coupled to a luciferase/luciferin reaction producing light.

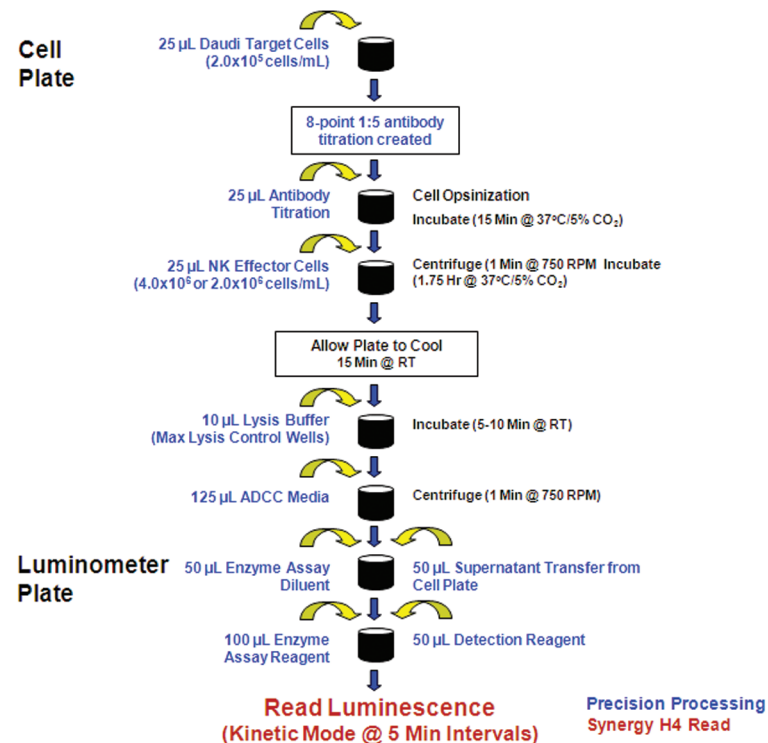


Figure 2 – Automated Assay Procedure

% 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 ADCC Assay (Continued)

Manual/Automated Assay Comparison

The automated method, using the Precision, was tested to confirm its ability to generate results that were similar to those generated manually. All steps of the assay were performed either robotically, using the Precision, or by hand using the procedure previously explained. Multiple individual runs were performed with separate blood donors to validate the repeatability of the robotic process. 20:1 and 10:1 effector to target cell ratios were tested in order to determine the sensitivity of the assay and Synergy H4. NK effector cells were isolated from whole blood, and rituximab was used as the control test antibody.

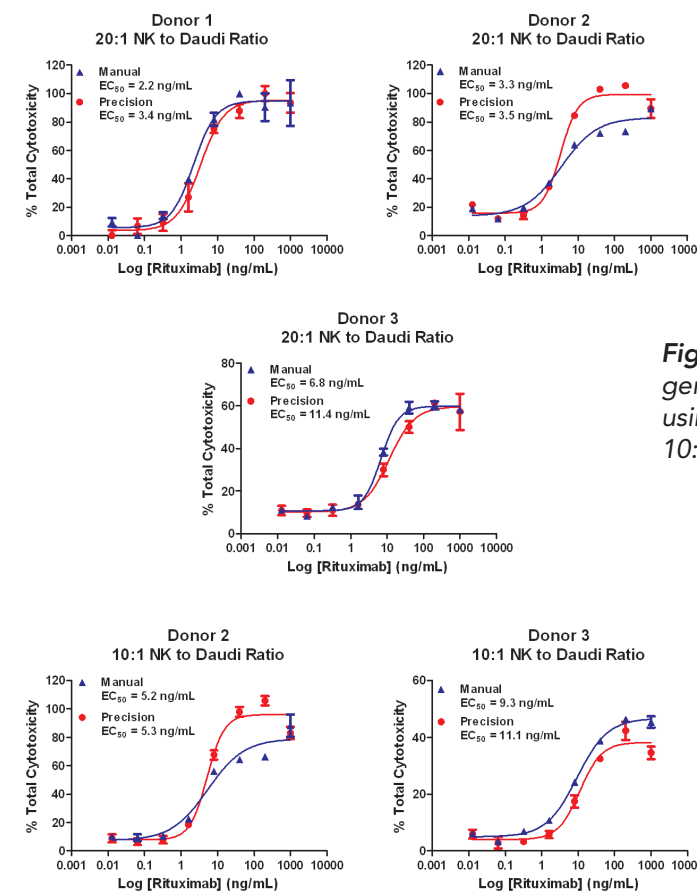


Figure 3 – Robotic and manually generated cytotoxicity curves, using rituximab and a 20:1 or 10:1 effector:target cell ratio.

The similarity in cytotoxicity curves and EC₅₀ values demonstrate that robotic processing can generate data that is equivalent to that which was produced manually. It is also evident from the 10:1 E:T ratio data that the assay and microplate reader are able to easily quantify the signal using a lower concentration of effector cells. Finally, repeatable results from multiple different donors also shows that the procedure is robust.

aCella-TOX ADCC Assay Data using Cryopreserved NK Cells

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 each blood donor, as witnessed in Figure 3. 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 manufacturer recommendations. 20:1 and 10:1 effector to target cell ratios were examined.

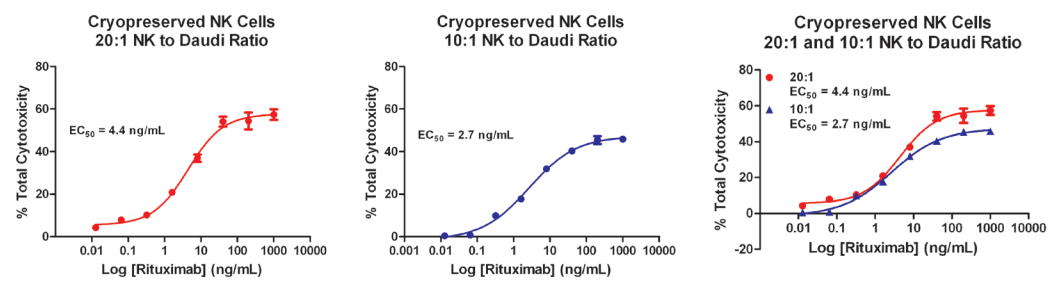


Figure 4 – EC₅₀ values and cytotoxicity curves generated with the aCella-TOX assay and cryopreserved NK cells.

The similarity between the rituximab cytotoxicity curves and EC₅₀ values generated using cryopreserved NK cells, to those generated using freshly isolated NK cells (Figure 3), confirm that the automated aCella-TOX assay procedure can deliver equivalent data using either cell format.

HTRF Target Receptor Binding Affinity Assay

The assay described here monitors the ability of test antibodies to bind to a target antigen on the receptor of choice, such as a Receptor Tyrosine Kinase, which has been cloned into a HEK293 cell line, expressed, and labeled with SNAP-Tb. EGFR-An ErbB family receptor targeted by immunotherapeutics such as Cetuximab, is the receptor of choice shown here.

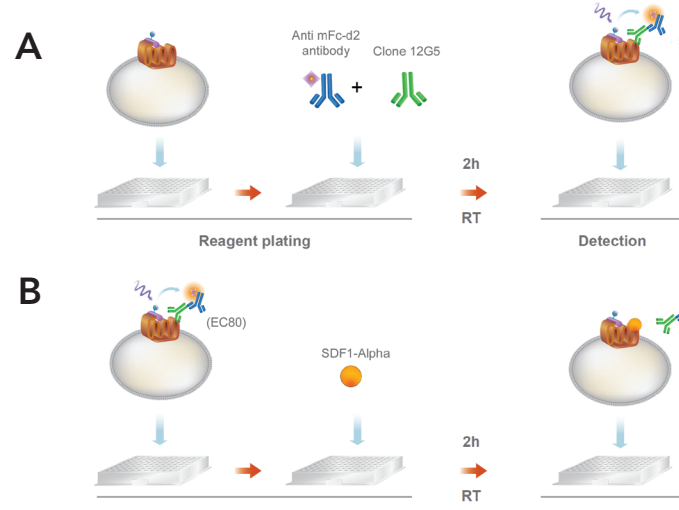


Figure 5 – Binding Measurement. A. Indirect Binding Binding of antibodies to the target antigen is measured through the use of an anti-species specific Fc-d2 labeled antibody. The target antibody binds to the SNAP-Tag labeled receptor antigen (EGFR). Upon addition, the secondary labeled antibody binds to the primary, unlabelled antibody, causing an increase in HTRF signal. **B. Competitive Binding** Antibodies known to bind to the target receptor (Ex: EGF:EGFR) are then added. As the proteins bind to the receptor the primary:secondary antibody complex is dissociated, causing a decrease in HTRF signal.

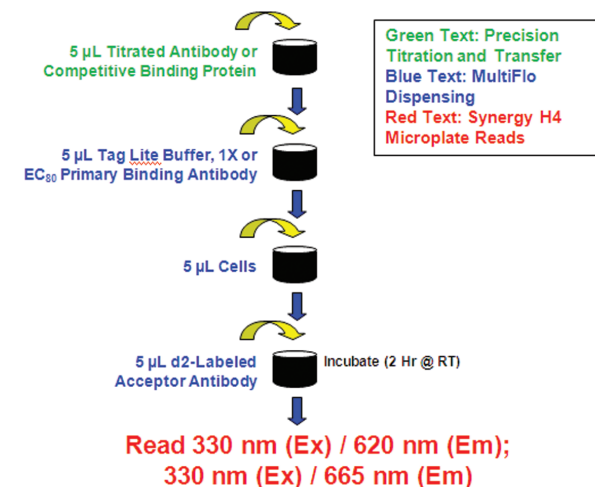


Figure 6 – Titration and transfer of antibody or competitive binding proteins accomplished via the Precision. Subsequent antibody, cell, and media additions can be accomplished on the MultiFlo using single or multiple addition methods.

HTRF Target Receptor Binding Affinity Assay (Continued)

To gain insight into the binding characteristics of Cetuximab to EGFR, a binding experiment was performed in an indirect format through the use of a secondary labeled human anti-EGFR antibody. Competitive binding experiments were also performed using EGF as the known EGFR receptor ligand.

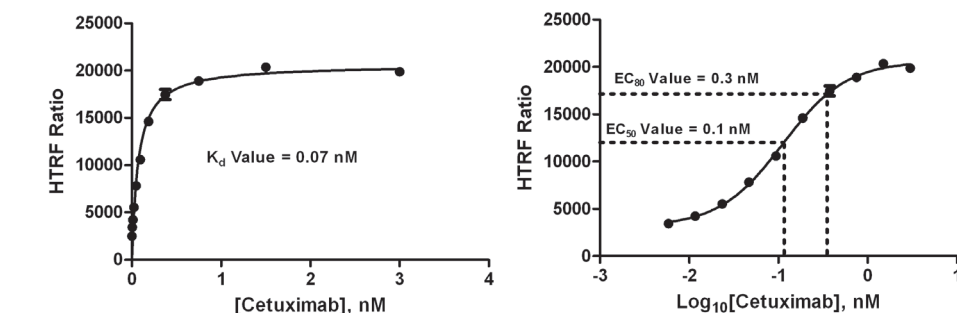


Figure 7 – Cetuximab Binding. A. K_d Determination A Cetuximab dose response curve was created using a 1:2 dilution scheme, starting at 3 nM. Cryopreserved cells were resuspended at a concentration of 1x10⁵ cells/mL. [Anti-human Fc-d2 Ab] equaled 30 nM. The K_d value was determined using the Nonlinear Regression Michaelis-Menten curve fit. **B. EC₅₀ Calculation** The EC₅₀ value was determined from the EC₅₀ and hill slope values, using a Nonlinear Regression Sigmoidal Dose-Response (variable slope) curve fit.

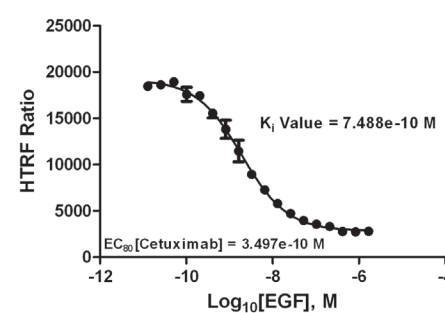


Figure 8 – Competitive Binding of Cetuximab and EGF to EGFR. An EGF dose response curve was created using a 1:2 dilution scheme, starting at 1.67 µM. Cetuximab was added at the EC₅₀ concentration previously determined. Cell and Fc-d2 antibody concentrations were as previously described.

HTRF Fc Fragment:CD16a Binding Affinity Assay

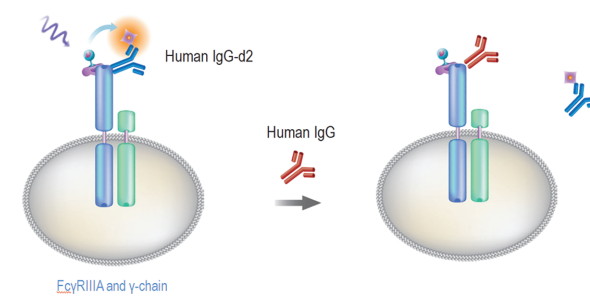


Figure 9 – Fc Binding on CD16a. Human IgG labeled with the d2 acceptor binds to HEK293 cells expressing the FcγRIIIA and gamma chain labeled with SNAP-Tb, generating a specific HTRF signal. Unlabeled IgG test antibodies can then be assessed for their ability to bind to the FcγRIIIA and gamma chain, which subsequently displaces the labeled IgG antibody, causing a decrease in HTRF signal.

IgG Binding Affinity

The automated CD16a assay was validated using two different methods. The first included testing various IgG isotypes for their affinity to bind CD16a and recruit effector cells. Results were compared to values generated manually, as well as to those previously reported in the literature².

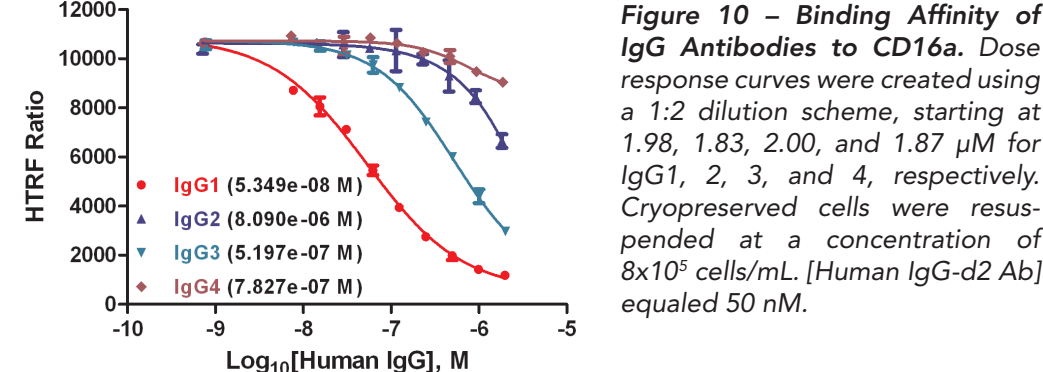


Figure 10 – Binding Affinity of IgG Antibodies to CD16a. Dose response curves were created using a 1:2 dilution scheme, starting at 1.98, 1.83, 2.00, and 1.87 µM for IgG1, 2, 3, and 4, respectively. Cryopreserved cells were resuspended at a concentration of 8x10⁵ cells/mL. [Human IgG-d2 Ab] equaled 50 nM.

By comparing the IC₅₀ values found here to those generated previously using the manual method; 6.738e-08 M (IgG1), 4.225e-05 M (IgG2), 9.127e-07 M (IgG3), and 8.008e-05 M (IgG4), it is apparent that the rank order of IgG binding is accurately calculated, and also agrees with that reported in the literature.

Therapeutic Ab Binding Affinity

Cetuximab, a chimeric mouse-human antibody, is known to inhibit binding of activating ligands to EGFR. However, recent studies have shown that the therapeutic antibody also has potential ADCC activity against EGFR-expressing lung cancer cell lines. (Kurai et. al; 2007) Therefore, the antibody was included in the second test, along with other with other antibodies with known binding affinity to CD16a, to ensure that the binding and effector cell recruitment of newly developed therapeutic antibodies could be accurately assessed with the automated assay procedure.

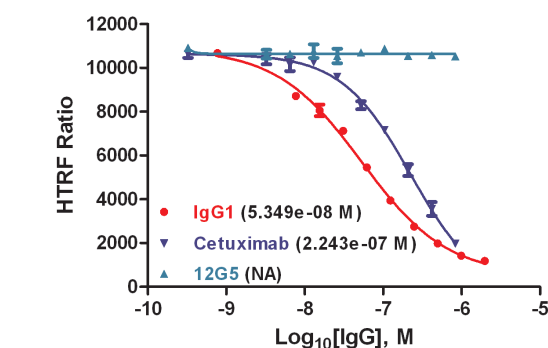


Figure 11 – Therapeutic Antibody Binding Affinity to CD16a. Dose response curves were created using a 1:2 dilution scheme, starting at 1.98 µM for IgG1, and 0.83 µM for Cetuximab and 12G5. Cryopreserved cell and Human IgG-d2 Ab concentrations were as previously described.

The IC₅₀ value for Cetuximab compared well to the manually generated value of 2.275e-07 M. This result, along with that from other human IgG antibodies, demonstrates that the automated assay is able to accurately assess binding of human IgGs to CD16a. As would be expected, the mouse 12G5 antibody does not dissociate the IgG-d2 antibody from the CD16a Fc receptor.

Conclusions

- The aCella-TOX cell-based ADCC assay provides a simplified procedure when using Natural Killer cells, that is more reproducible and can be performed in a shorter time period when compared to the ⁵¹Cr assay.
- The HTRF receptor binding assay is able to assess indirect binding of IgG antibodies, as well as competitive binding of unlabeled proteins or ligands, to target receptors, while the CD16a assay is able to accurately detect the binding of test human IgG antibodies to CD16a Fc receptors
- The assay technologies can be easily automated, which can save valuable resource time, and provide greater reproducibility when compared to manual processing.
- The Precision can also consistently, and accurately titrate test ligands and antibodies using a simple format.
- The Synergy H4 is able to accurately quantify the luminescent and HTRF signals from each assay well in 96- or LV384-well formats.
- The equivalent dose response curves, binding, and EC₅₀ 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.