

Automated Solutions for Cellular Screening and Characterization of Therapeutic Antibodies for Antibody-Dependent Cellular Cytotoxicity Utility



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Introduction

Since the end of the 1990's, the pharmaceutical industry has seen an increased interest in biologics, especially in the therapeutic areas of oncology and inflammation. The selection of potent and selective mAbs (monoclonal antibodies) for specific target receptors, such as Receptor Tyrosine Kinase (RTK) and G protein-coupled receptors (GPCRs), is the first step in successfully developing an antibody based drug. In addition to potent binding, some antibodies have the ability to recruit the immune system effector cells, a process known as ADCC (Antibody Dependent Cell Cytotoxicity). The ability to promote ADCC is an important attribute of successful candidates and for that reason should be sought in the final drug design.

Here we present the automation of two assays for the characterization and selection of potent antibody drug candidates. Both assays rely on HTRF[®] detection. The first assay quantifies the binding affinity of antibodies to their target antigen, on live cells. The second assay measures the affinity of the antibody Fc portion to the CD16 receptor also on live cells. As Fc-CD16 binding affinity correlates well with effector function, this method is a simple, yet efficient and precise way of assessing biological activity through ADCC.

Each assay procedure has been automated through the incorporation of easy-to-use, robust instrumentation. An 8-channel liquid handler is used for antibody titration and transfer to the low-volume 384-well assay plates. Cell and reagent dispensing are then carried out using a non-contact liquid dispenser. The small footprint of the instrument, and ability to autoclave the dispensing pathway, allow for sterile manipulations of each component. Binding, as well as antibody competition experiments were performed to validate the automated assay procedure. Results demonstrate that the combination of assay and instrumentation create a powerful, easy to use and efficient method to screen and characterize antibodies being considered for use in ADCC applications.

BioTek Instrumentation



Figure 1 – 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 perform the serial dilutions of test antibodies and binding proteins, and transfer aliquots into the LV384-well assay plates.



Figure 2 – MultiFlo[™] Microplate Dispenser offers fast, accurate 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.



Figure 3 – Synergy[™] H4 Hybrid Multi-Mode Microplate 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.

Target Receptor Binding Affinity

The assays described here monitor the ability of test antibodies to bind to a target antigen on the receptor of choice, such as a Receptor Tyrosine Kinase or GPCR, which has been cloned into a HEK293 cell line, expressed, and labeled with SNAP-Tb. Two specific receptors were used in this project; EGFR-An ErbB family receptor targeted by immunotherapeutics such as Cetuximab, and CXCR4-A GPCR belonging to the chemokine family, and also a known anti-cancer therapy target.

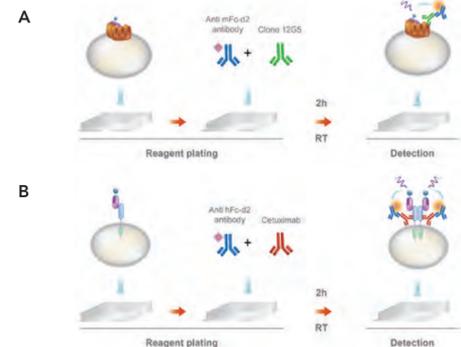


Figure 4 – Indirect Binding Measurement. Binding of antibodies to the target antigen is measured through the use of an anti-species specific Fc-d2 labeled antibody. The target antibody binds to the SNAP-Tag labeled receptor antigen (A.CXCR4; B.EGFR). Upon addition, the secondary labeled antibody binds to the primary, unlabelled antibody, causing an increase in HTRF signal.

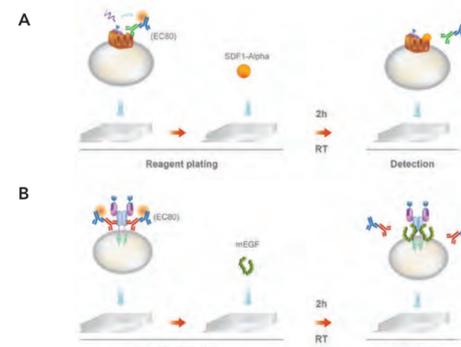


Figure 5 – Competitive Binding Assessment. Antibodies known to bind to the target receptor are added at an EC₅₀ concentration. Other binding proteins to the target receptor (A.SDF1-Alpha;CXCR4; B.EGF:EGFR) are then added. As the proteins bind to the receptor the primary:secondary antibody complex is dissociated, causing a decrease in HTRF signal.

Fc Fragment: CD16a Binding Affinity

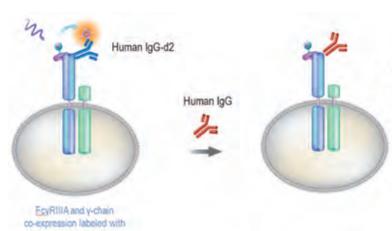


Figure 6 – Fc Binding on CD16a. Human IgG labeled with the d2 acceptor binds to HEK293 cells expressing the γRIIIA and gamma chain co-expression labeled with SNAP-Tb, generating a specific HTRF signal. Unlabeled IgG test antibodies can then be assessed for their ability to bind to the FcγRIIIA and gamma chain, which subsequently displaces the labeled IgG antibody, causing a decrease in HTRF signal.

Automated Assay Workflow

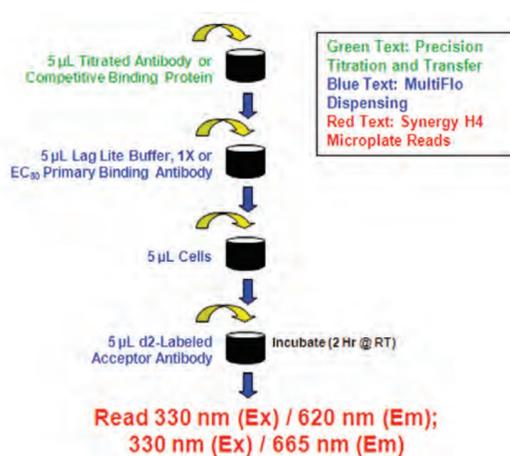


Figure 7 – Titration and transfer of antibody or competitive binding proteins initially accomplished. Subsequent antibody, cell, and media additions can be accomplished on the MultiFlo using a single or multiple addition methods.

Cetuximab: EGFR Binding Assessment

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 anti-human labeled antibody.

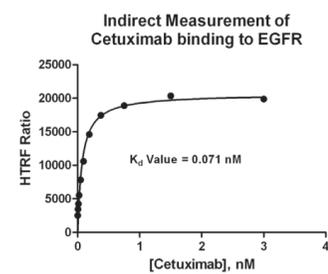


Figure 8 – Cetuximab Equilibrium Binding Constant K_d Determination. A Cetuximab dose response curve was created using a 1:2 dilution scheme, starting at 3 nM. Cryopreserved cells were resuspended at a concentration of 1x10⁶ cells/mL. [Anti-human Fc-d2 Ab] equaled 30 nM. The K_d value was determined using the Nonlinear Regression Michaelis-Menten curve fit.

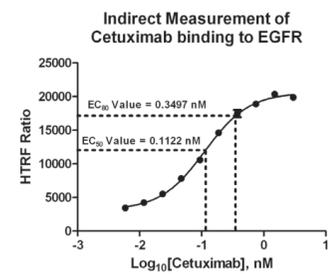


Figure 9 – Cetuximab Binding EC₅₀ Calculation. The EC₅₀ value was determined from the EC₅₀ and hill slope values, using a Nonlinear Regression Sigmoidal Dose-Response (variable slope) curve fit.

Cetuximab showed high affinity with a K_d in the subnanomolar range, which agrees with previously generated values of 0.03 nM, and those described in the literature¹.

12G5: CXCR4 Binding Assessment

The binding characteristics of 12G5 to CXCR4 were also assessed. Initial binding of 12G5 was performed once again using an indirect format through the use of a secondary labeled mouse anti-CXCR4 antibody.

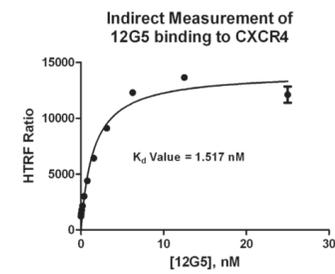


Figure 10 – 12G5 Equilibrium Binding Constant K_d Determination. A 12G5 dose response curve was created using a 1:2 dilution scheme, starting at 25 nM. Cryopreserved cells were resuspended at a concentration of 8x10⁵ cells/mL. [Anti-mouse Fc-d2 Ab] equaled 100 nM. The K_d value was determined using the Nonlinear Regression Michaelis-Menten curve fit.

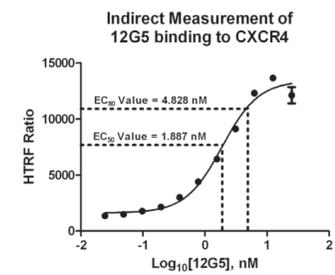


Figure 11 – 12G5 Binding EC₅₀ Calculation. The EC₅₀ value was determined from the EC₅₀ and hill slope values, using a Nonlinear Regression Sigmoidal Dose-Response (variable slope) curve fit.

Competitive Binding Evaluation

Competitive binding experiments were performed with the two systems previously described. Known ligands EGF and SDF1 were used to illustrate competitive binding with the EGFR and CXCR4 receptors, respectively.

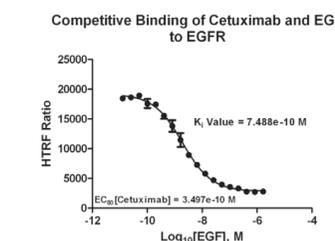


Figure 12 – Competitive Binding of Cetuximab and EGF to EGFR. An EGF dose response curve was created using a 1:2 dilution scheme, starting at 1.67 µM. Cetuximab was added at the EC₅₀ concentration previously determined. Cell and Fc-d2 antibody concentrations were as previously described.

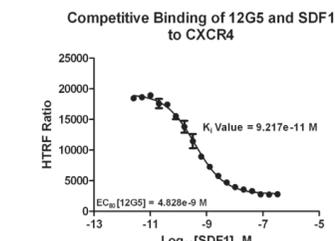


Figure 13 – Competitive Binding of 12G5 and SDF1 to CXCR4. An SDF1 dose response curve was created using a 1:2 dilution scheme, starting at 0.33 µM. 12G5 was added at the EC₅₀ concentration previously determined. Cell and Fc-d2 antibody concentrations were as previously described.

Dose response curves and K_d inhibition constants were generated for each ligand. The curves clearly show inhibition of antibody binding with increasing ligand concentration, confirming that both antibodies bind on the orthosteric site of the related receptors. Using this system, binding affinity of unlabeled ligand/proteins to the receptor of interest can be quantified.

CD16a Affinity Determination

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.

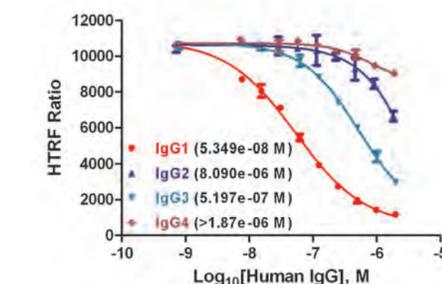


Figure 14 – Binding Affinity of IgG Antibodies to CD16a. Dose response curves were created using a 1:2 dilution scheme, starting at 1.98, 1.83, 2.00, and 1.87 µM for IgG1, 2, 3, and 4, respectively. Cryopreserved cells were resuspended at a concentration of 8x10⁵ cells/mL. [Human IgG-d2 Ab] equaled 50 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

The epidermal growth factor receptor (EGFR) inhibitor, Cetuximab, was also tested to ensure that the binding and effector cell recruitment of newly developed therapeutic antibodies could be accurately assessed with the automated assay procedure.

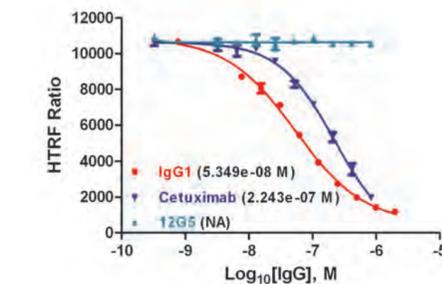


Figure 15 – Therapeutic Antibody Binding Affinity to CD16a. Dose response curves were created using a 1:2 dilution scheme, starting at 1.98 µM for IgG1, and 0.83 µM for Cetuximab and 12G5. Cryopreserved cell and Human IgG-d2 Ab concentrations were as previously described.

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 receptor binding assays are able to assess indirect binding of IgG antibodies, as well as competitive binding of unlabeled proteins or ligands, to target receptors
- The CD16a assay is able to accurately detect the binding of test human IgG antibodies to CD16a Fc receptors
- The Precision is capable of accurately creating and transferring dose response curves of antibodies and other competitive binding proteins, while the MultiFlo is able to rapidly dispense cells, media, and antibodies to LV384-well plates in a non-contact format
- The HTRF[®] certified Synergy H4 is able to easily detect the fluorescent signals from each assay in a LV384-well format
- The combination of assay chemistry and instrumentation creates a perfect combination for high-throughput detection of therapeutic antibody binding in order to predict ADCC assay efficiency