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

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Here we demonstrate the automation of a low-volume 384-well method to rapidly and easily characterize potential new antibody therapeutics and biosimilars for their utility in Antibody-Dependent Cell-Mediated Cytotoxicity. Two separate HTRF® assays are incorporated which monitor binding of the Fab portion of the antibody to the target of interest, as well as binding of the Fc portion to the CD16a receptor. The assay chemistries can be easily automated using contact and non-contact dispensing instrumentation to create a high-throughput solution to predict ADCC assay efficiency.

Introduction

Since the end of the 1990's, the pharmaceutical industry has increased their interest in biologics, especially in oncology and inflammation therapeutics. The selection of potent and selective 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 immune system effector cells, a process known as Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC). The ability to promote ADCC is an important attribute of successful candidates, and should be sought in the final drug design.

Here we present the automation of two HTRF® assays for the characterization and selection of potent antibody drug candidates. 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, the combined 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 and 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.

Materials and Methods

Materials

Assay chemistries, cryopreserved cells, and the therapeutic antibody Cetuximab were provided by Cisbio. SDF1a Human (Catalog No. chm-262) and EGF Mouse (Catalog No. CYT-554) were purchased from Prospec. Anti-hCXCR4 Clone 12G5 (Catalog No. MAB170) was purchased from R&D Systems. IgG1 (Catalog No. I5154), IgG2 (Catalog No. I5404), IgG3 (Catalog No. I5654), and IgG4 (Catalog No. I4639) were purchased from Sigma. 384-well low volume, white, round-bottom, non-binding surface plates (Catalog No. 3673) were purchased from Corning Life Sciences.

Instrumentation

The 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 known receptor ligands, and transfer aliquots into the LV384-well assay plates.

The 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, EC_{80} concentrations of test antibodies, as well as labeled acceptor antibodies.

The Synergy™ H4 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.

Assay Chemistries

Target Receptor Binding Assay

The first assay quantifies a test antibody's binding ability to a target antigen on a receptor, such as a Receptor Tyrosine Kinase or GPCR, which has been cloned into HEK293 cells, 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. 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 (Figure 1a. CXCR4; Figure 1b. EGFR). Upon addition, the secondary labeled antibody binds to the primary, unlabelled antibody, causing an increase in HTRF signal.

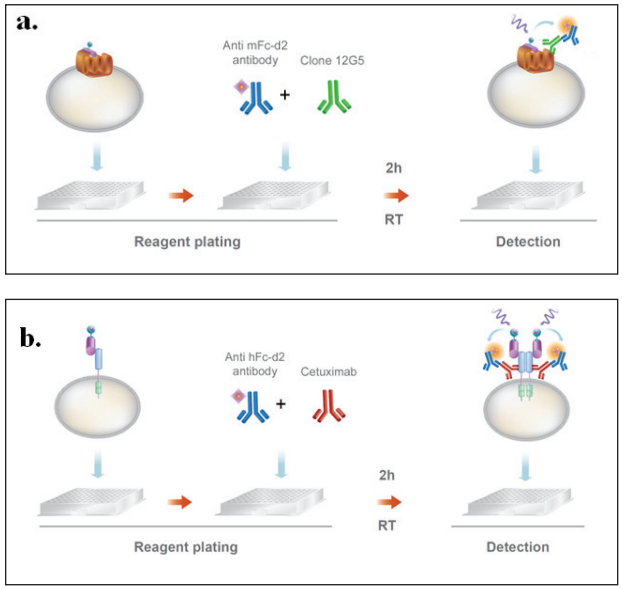

Figure 1. Target receptor indirect binding measurement.

Competitive binding between known ligands and test antibodies can also be measured. An EC_{80} concentration of test antibody is added, followed by various concentrations of other binding proteins to the target receptor (Figure 2a. SDF1-Alpha:CXCR4; Figure 2b. 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.

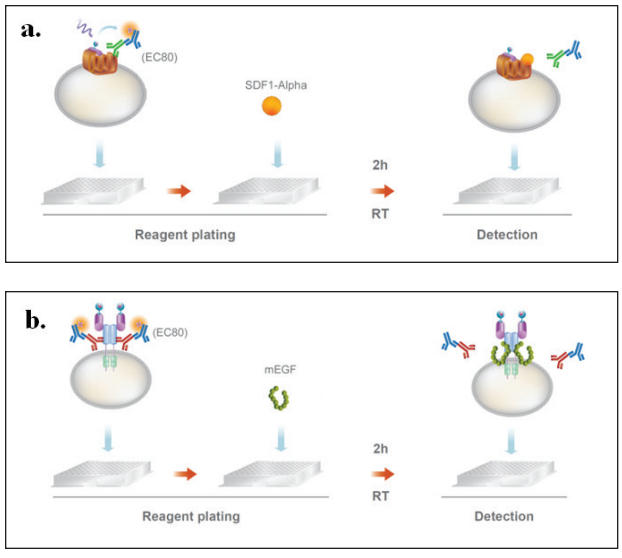

Figure 2. Target receptor competitive binding measurement.

CD16a Cellular Binding Assay

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 (Figure 3). 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.

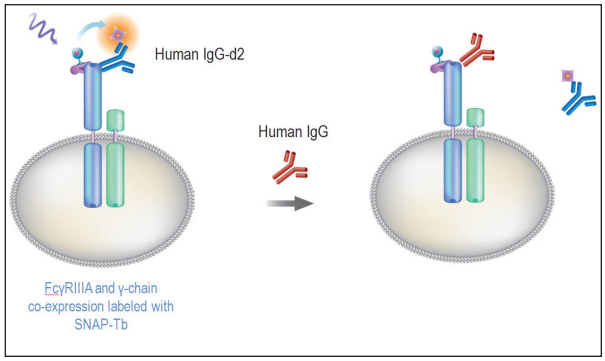

Figure 3. Fc binding on CD16a.

Automated Procedure

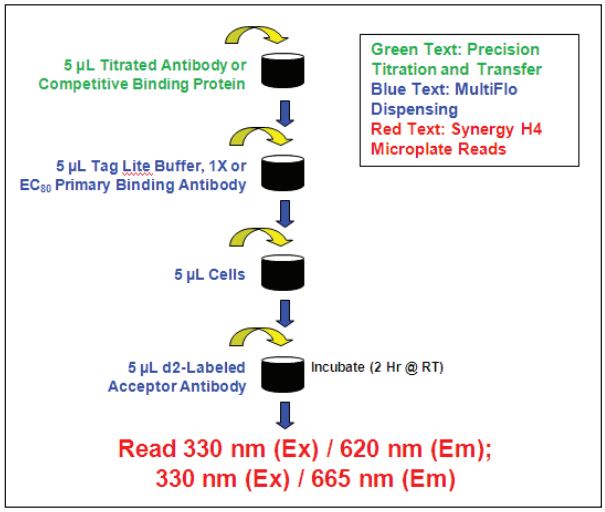

Figure 4. Titration and transfer of antibody or competitive binding proteins initially accomplished. Subsequent antibody, cell, and media additions can be accomplished on the MultiFlo™ using a single or multiple addition methods.

Results and Discussion

Target Receptor Binding

To gain insight into the binding characteristics of Cetuximab to EGFR and 12G5 to CXCR4, and validate the automated target receptor binding assay process, experiments were performed in an indirect format through the use of secondary labeled anti-species specific Fc-d2 labeled antibodies.

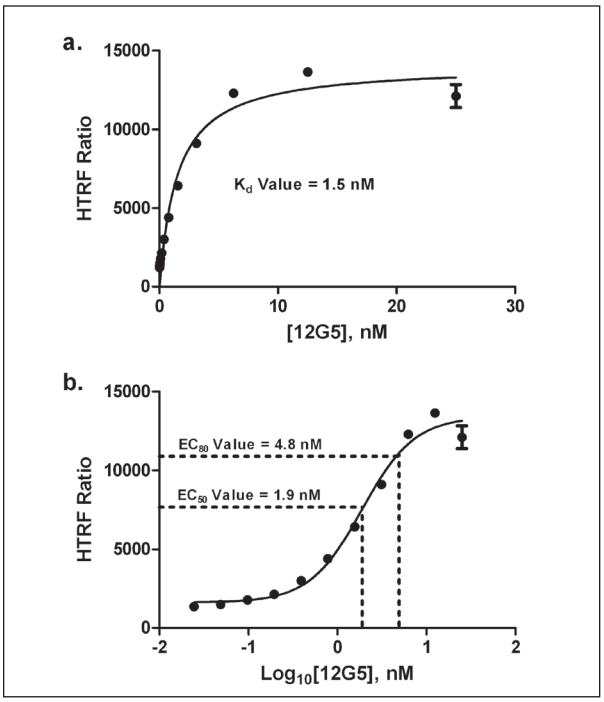

Figure 5. A 12G5 dose-response curve was created using a 1:2 dilution scheme, starting at 25 nM. Cryopreserved cells were resuspended at a concentration of $8x10^5$ cells/mL. [Anti-mouse Fc-d2 Ab] equaled 100 nM. **a.** The K_d value was determined using the nonlinear regression Michaelis-Menten curve fit. **b.** The EC_{80} value was determined from the EC_{50} and hill slope values, using a nonlinear regression sigmoidal dose-response (variable slope) curve fit.

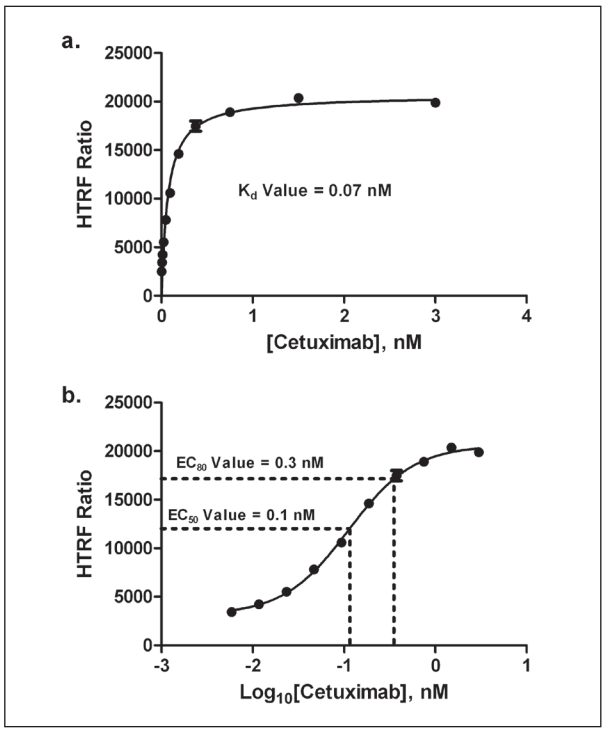

Figure 6. A Cetuximab dose-response curve was created using a 1:2 dilution scheme, starting at 3 nM. Cryopreserved cells were resuspended at a concentration of $1x10^6$ cells/mL. [Anti-human Fc-d2 Ab] equaled 30 nM. **a.** The K_d value was determined using the nonlinear regression Michaelis-Menten curve fit. **b.** The EC_{80} value was determined from the EC_{50} and hill slope values, using a nonlinear regression sigmoidal dose-response (variable slope) curve fit.

12G5 and Cetuximab demonstrated the ability to bind to their target receptors. Cetuximab, in particular, 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 literature[1].

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

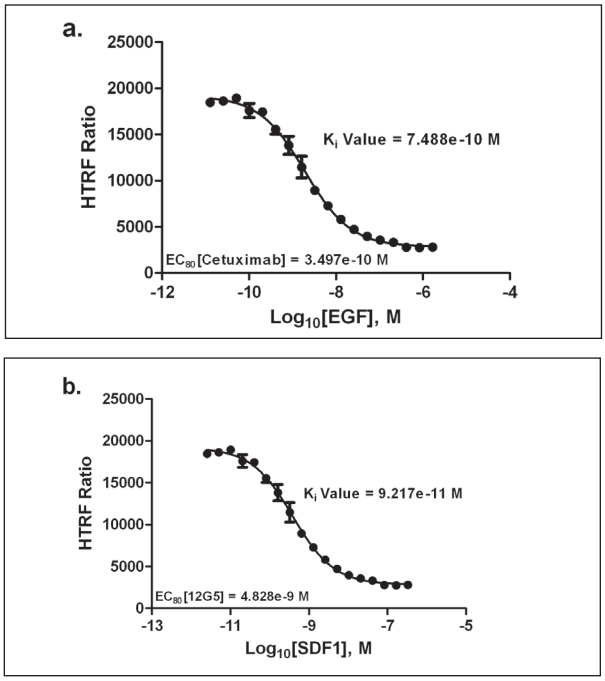

Figure 7. **a.** An EGF dose-response curve was created using a 1:2 dilution scheme, starting at 1.67 µM. Cetuximab was added at the EC_{80} concentration previously determined. **b.** An SDF1 dose-response curve was created using a 1:2 dilution scheme, starting at 0.33 µM. 12G5 was added at the EC_{80} concentration previously determined.

Dose-response curves and K_i inhibition constants were generated for each ligand. The curves clearly show inhibition of antibody binding with increasing ligand concentration. Using this system, binding affinity of unlabeled ligand/proteins to the receptor of interest can be quantified.

CD16a 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 literature[2].

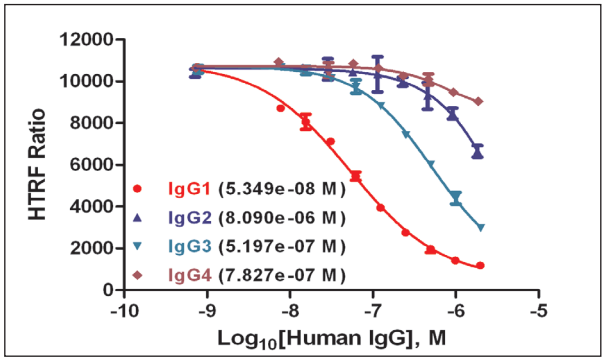

Figure 8. Binding Affinity of IgG Antibodies to CD16a. Dose-response curves were created using a 1:2 dilution scheme, starting at 1.98, 1.83, 2.00, and 1.87 µM for IgG1, 2, 3, and 4, respectively. Cryopreserved cells were resuspended at a concentration of $8x10^5$ cells/mL. [Human IgG-d2 Ab] equaled 50 nM.

By comparing the IC_{50} 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.

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.

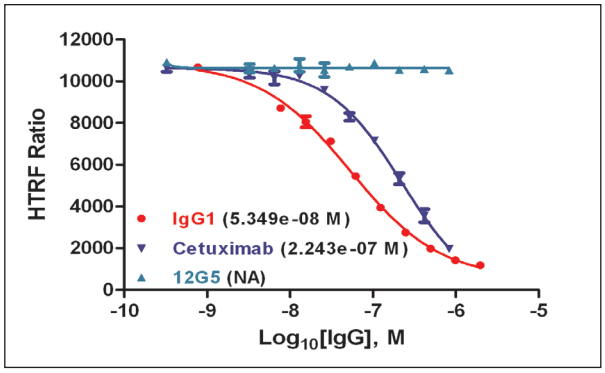

Figure 9. Therapeutic Antibody Binding Affinity to CD16a. Dose-response curves were created using a 1:2 dilution scheme, starting at 1.98 µM for IgG1, and 0.83 µM for Cetuximab and 12G5. Cryopreserved cell and Human IgG-d2 Ab concentrations were as previously described.

The IC_{50} 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 data demonstrates that 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 also able to easily detect the fluorescent signals from each assay in a LV384-well format. The combination of assay chemistries and instrumentation create a perfect combination for high-throughput detection of therapeutic antibody binding in order to predict ADCC assay efficiency.

References

1. Kamat V et al, Cancer Biol Ther 2008,7(5):726-33.
2. Bruhns P. et al., Blood 2009, 113(16): 3716-3725.