

Introduction

Receptor tyrosine kinases (RTK) are cell-surface receptors that are critical in normal cell signaling processes and are implicated in a variety of disease areas such as cancer, inflammation, diabetes, CNS and others. Mutations leading to overexpression of Epidermal Growth Factor Receptor (EGFR), in particular, have been linked to colon and lung cancers, among others. Monoclonal antibodies against receptor tyrosine kinases have revolutionized cancer therapeutics due to their highly selective therapeutic effect while having minimal off-target effects (Imai and Takaoka 2006). Many FDA-approved monoclonal antibodies, whose mechanisms of action target kinases, exist as therapeutic agents. However, various low throughput strategies such as ELISA or complex in-vivo models are often utilized to identify anti-ligand, and anti-receptor antibodies. Therefore, functional cell-based assays that can detect antagonistic activity of blocking antibodies and instrument platforms that can automate and demonstrate sensitive detection are highly desirable in therapeutic antibody discovery and characterization.

In this study we describe a novel, target-specific cell-based assay platform that can be used to detect and differentiate potential inhibitors of Epidermal Growth Factor receptors. The assay uses a full-length receptor protein allowing for detection of antibody inhibitors for ligands of membrane bound receptors and is based on a proprietary Enzyme Fragment Complementation (EFC) Assay Technology denoted as PathHunter[®] Receptor Tyrosine Kinase Functional assay. The PathHunter assay enables antibody interrogation and characterization in a cellular context. The assay was automated in 384-well format using simple, modular contact and non-contact instrumentation for fast and efficient creation of dose-response curves, as well as cell and reagent dispensing. Detection of the chemiluminescent signal was carried out using a multi-mode microplate reader with integrated data analysis. Optimization, validation, and pharmacology data demonstrate how the combination of assay technology and instrumentation create an ideal method for the detection of blocking antibodies in a convenient, reproducible manner that can save valuable time and translates routine bench top assays to high throughput methodologies.

PathHunter Receptor Tyrosine Kinase Assay Principle & BioTek Instrumentation

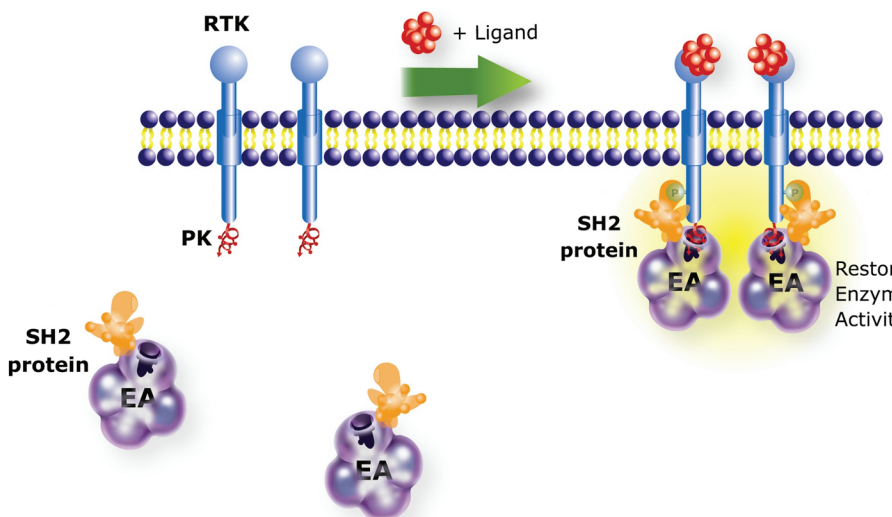


Figure 1 – The engineered cell line incorporates Enzyme Fragment Complementation (EFC) technology in which the β -galactosidase (β -gal) enzyme has been split into two inactive fragments. A small peptide epitope (ProLink) is expressed recombinantly on the intracellular C-terminus of the Receptor Tyrosine Kinase (RTK). SH2 domains are co-expressed with a larger sequence, termed enzyme acceptor (EA). Activation of receptor tyrosine kinase (RTK) results in dimerization and phosphorylation of RTK with subsequent interaction with the SH2 protein. This interaction generates an active β -galactosidase enzyme (complementation of β -gal fragments PK and EA), which is detected using a chemiluminescent substrate.

Instrumentation

Precision[™] Microplate Pipetting System. The instrument combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to perform serial dilutions of ligands and test antibodies in 96-well format, and transfer 5 μ L aliquots into the 384-well assay plates.

MultiFlo[™] Microplate Dispenser. The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 μ L. The instrument was used to dispense cells, EC₅₀ concentrations of ligand, as well as detection reagent in 20 μ L, 5 μ L, and 15 μ L volumes, respectively.

Synergy[™] H4 Hybrid Multi-Mode Microplate Reader. The reader combines a filter-based and monochromator-based detection system in one unit. A dedicated luminescence detection system was used to quantify the chemiluminescent signal from each assay well.

Materials and Methods

Materials

U2OS cells expressing ErbB1 (Catalog No. 93-0681C3), ErbB2/ErbB3 (Catalog No. 93-0535C3), or ErbB4 (Catalog No. 93-0465C3) receptors, Cell Detachment Reagent (Catalog No. 92-0009), PathHunter Cell Plating 16 Reagent (Catalog No. 93-0446RP16), PathHunter Detection Kit (Catalog No. 93-0001), PathHunter select U2OS Cell Culture Kit-K (92-0018GK3), PathHunter select U2OS Cell Culture Kit-L (92-0018GL3) recombinant human EGF (Catalog No. 92-1113), Heregulin- β 1 (Catalog No. 92-1031), and recombinant human Neuregulin-1 β 2 were provided by DiscoverX Corporation (Fremont, CA). Epidermal Growth Factor Receptor (EGFR) Ab-1 (Clone 528) (Catalog No. MS-268-P0) and Ab-2 (Clone 225) (Catalog No. MS-269-P0) were purchased from Thermo Scientific (Fremont, CA). EGFR Mouse anti-Human Monoclonal (Azide-free) (528) (Catalog No. LS-C87999), (225) (Catalog No. LS-C88001), and (11.6) (Catalog No. LS-C88141) antibodies were purchased from Lifespan Biosciences (Seattle, WA). Dulbecco's Phosphate Buffered Saline (DPBS), 1X (Catalog No. 14190) was purchased from Life Technologies (Carlsbad, CA). Bovine Serum Albumin (BSA) (Catalog No. A3294) was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Methods

Cell Propagation: U2OS ErbB1, ErbB2/3, or ErbB4 cells were propagated in complete medium, as described by the manufacturer. Medium information, including supplier and catalog numbers, can be found in the product insert for each cell line included in the test. Cell recovery is greatly improved when selection antibiotics are omitted for the first 24 hours. After that, cells were maintained in selective media at all times to maintain expression of fusion proteins.

Ligand/Antibody Preparation: Ligands were titrated in DPBS with 0.1% BSA to create a 12-point dose response curve. Serial 1:4 dilutions were performed with a starting 1X concentration of 2,000 ng/mL for EGF and Neuregulin-1 β 2, and 100 ng/mL for Heregulin- β 1. Antibodies were also titrated in DPBS with 0.1% BSA to create an 11-point dose response curve. Serial 1:2.5 dilutions were carried out with a starting concentration of 40 μ g/mL.

Cell Preparation: PathHunter U2OS cells are propagated in complete medium, as previously described. At the time of assay, cells are transferred into PathHunter Cell Plating 16 Reagent and diluted to the proper concentration.

Automated RTK Assay Procedure

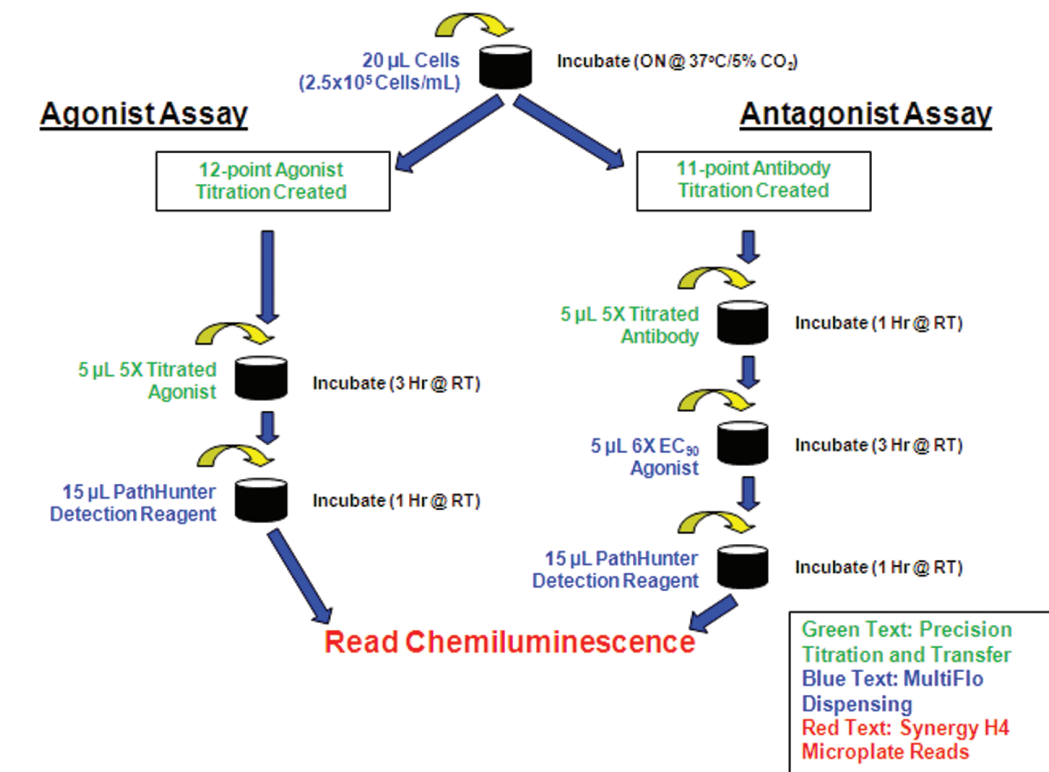


Figure 2 – Automated Assay Procedure. A thaw-plate-treat-read protocol is described here. U2OS ErbB1, ErbB2/3, or ErbB4 cells are added to the assay plate and incubated overnight in a 37°C/5% CO₂ cell incubator. Following incubation, compound additions (agonist or antagonist) are carried out. The remaining steps of each assay procedure are then performed. EC₅₀ agonist concentrations used in the antagonist assay are determined from previously generated ligand dose response curve data.

Assay/Reader Sensitivity Test

The automated assay procedure was tested using three different cell types expressing either the EGFR receptors ErbB1, ErbB2/ErbB3, or ErbB4. The agonist assay format was run using known ligands for each receptor (EGF:ErbB1; Heregulin- β 1:ErbB2/3; Neuregulin-1 β 2:ErbB4). A cell concentration of 10,000 cells/well was originally tested. The sensitivity of the assay and reader were further tested by comparing the original data to that generated using a cell concentration of 5,000 cells/well.

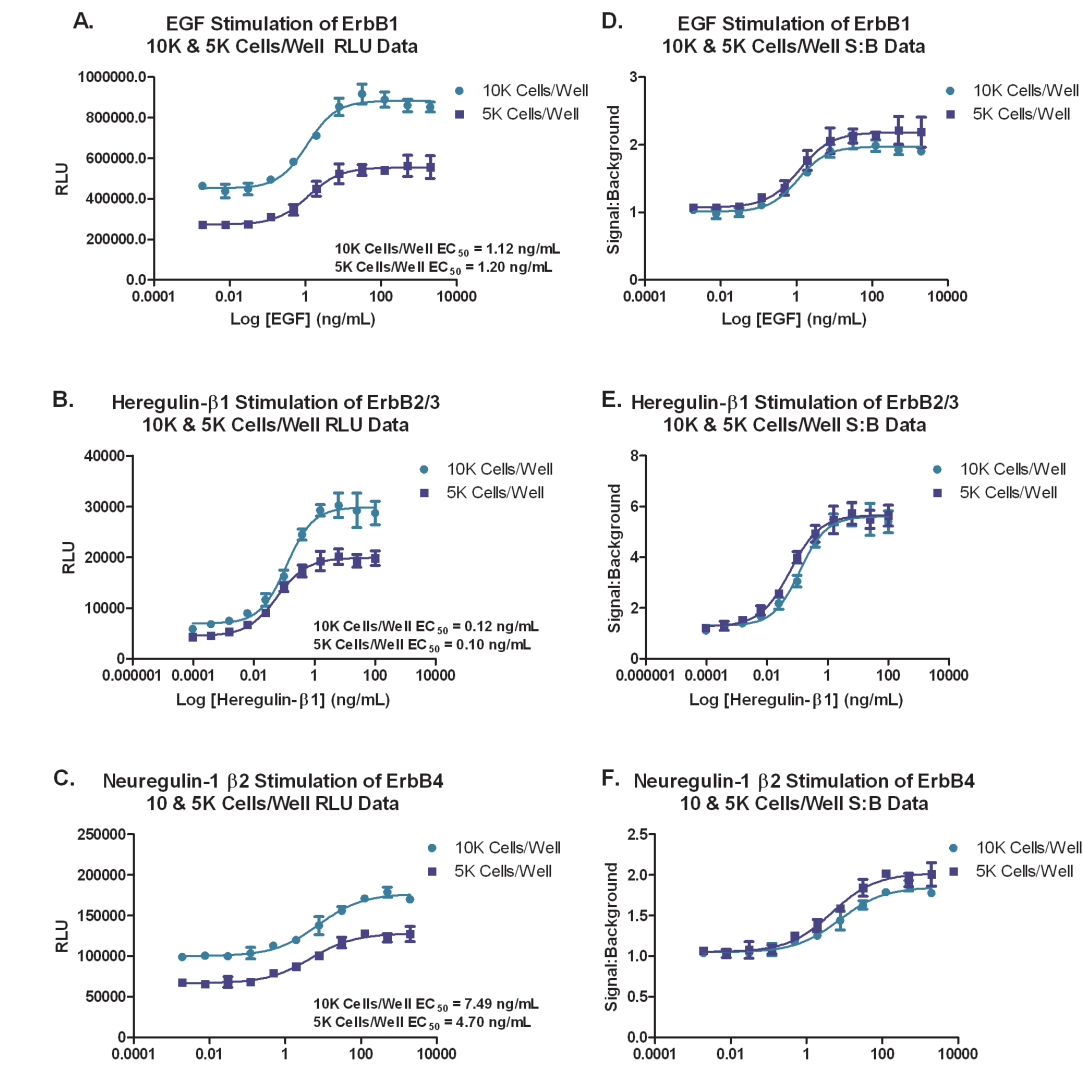


Figure 3 – Sensitivity Test Results. Dose response curves for each EGFR receptor assay, using the known ligand, are shown here. RLU values for each concentration tested (Fig 3A, 3B and 3C) as well as signal:background (Fig 3D, 3E and 3F) are shown.

By comparing the signal:background values for the dose response curves generated using either 10,000 cells/well or 5,000 cells/well, it can be seen that the same assay window is generated even when using a lower cell concentration. This confirms the sensitivity of the reader to be able to accurately detect the chemiluminescent signal from receptor stimulation using fewer cells/well. Use of lesser cells in the assay can lower the amount of time, labor, and supplies needed to propagate the number of cells required to run each test, thereby lowering the price per well of the overall assay.

Automated Assay Procedure Validation

Multiple runs were performed with each assay, using the 5,000 cells/well concentration determined previously, in order to ensure that the automated process was able to generate data that was repeatable and also agreed with values generated previously using manual pipetting (data not shown).

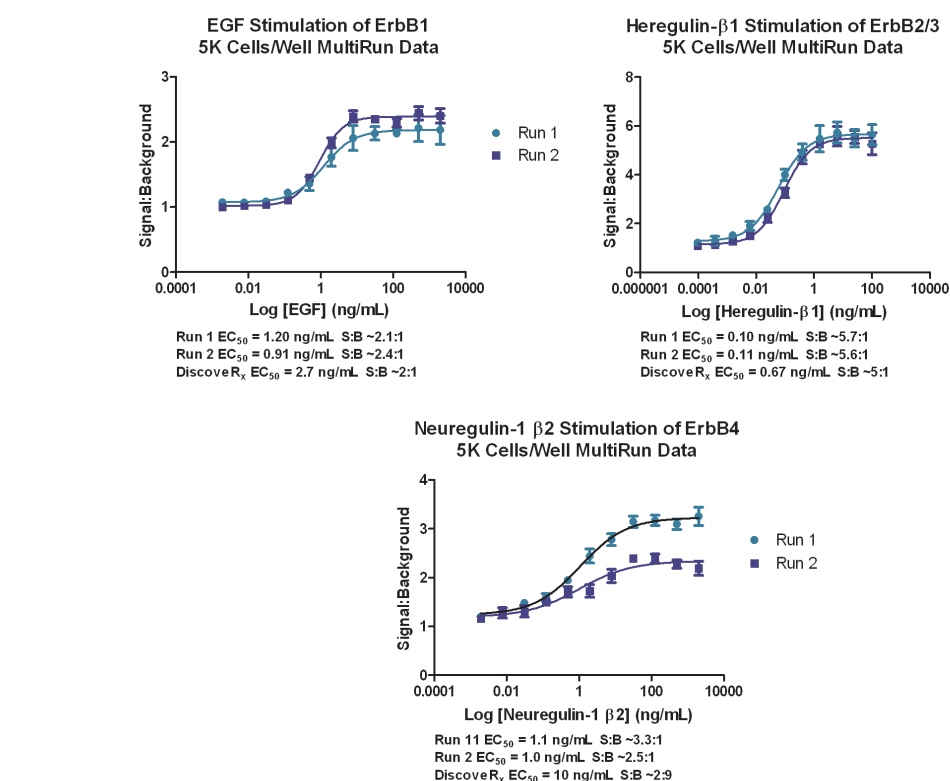


Figure 4 – Multi-Run Data. Dose response curves and determined EC₅₀ and S:B values for n=2 runs with each receptor assay. Previously generated results using the manual method also listed.

The similar shape to the dose response curves, as well as the agreement of the generated EC₅₀ and signal:background values between the two runs, and when compared to previous results, demonstrates that the automated procedure generates accurate, repeatable results.

EGFR (ErbB1) Blocking Antibody Test

The ability of the PathHunter EGFR receptor assays and automated process to accurately detect the antagonistic effect of blocking antibodies was then assessed. A total of five antibodies with known ability to prevent blocking of the EGF ligand to EGFR (ErbB1) were included. An antibody known to bind to the CXCR4 G protein-coupled receptor was also included as a negative control. Dose response curves were generated for each antibody and tested with the ErbB1 RTK assay using the automated antagonist assay procedure. A previously generated EC₅₀ concentration of EGF was used to stimulate receptor activity.

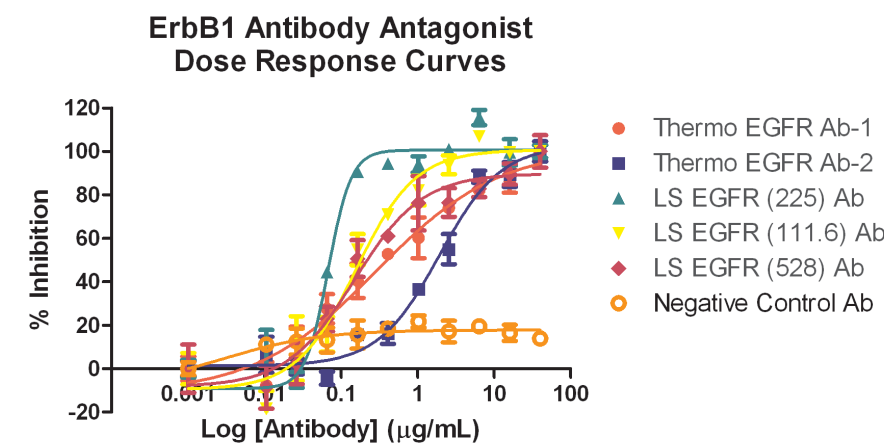


Figure 5 – Blocking Antibody Inhibition. Dose response curves showing inhibition of EGF binding to EGFR across multiple antibody concentrations.

ErbB1 Antibody Antagonist IC ₅₀ Values (µg/mL)			
Thermo EGFR Ab-1	0.29	LS EGFR (111.6) Ab	0.16
Thermo EGFR Ab-2	1.92	LS EGFR (528) Ab	0.15
LS EGFR (225) Ab	0.10	Negative Control Ab	NA

Table 1 – Blocking Antibody IC₅₀ values. IC₅₀ values generated using automated processing of test antibodies with the ErbB1 assay.

Inhibition of EGF binding to the ErbB1 receptor by blocking antibodies was easily and accurately assessed using the automated 384-well procedure. The values obtained in the PathHunter assay correlate with those listed by each antibody supplier, which cite blocking ability at 0.5 μ g/mL. No discernible inhibition was seen with the negative control antibody, confirming that changes in luminescent signal were due to blocking of ligand:receptor binding.

Antibody Selectivity Confirmation

Antibody dose response curves were also run with the ErbB2/ErbB3 and ErbB4 assays in order to further confirm the ability to detect blocking of specific ligand:receptor binding. EC₅₀ concentrations of Heregulin- β 1 and recombinant human Neuregulin-1 β 2 were used to stimulate receptor activity of ErbB2/3 and ErbB4, respectively.

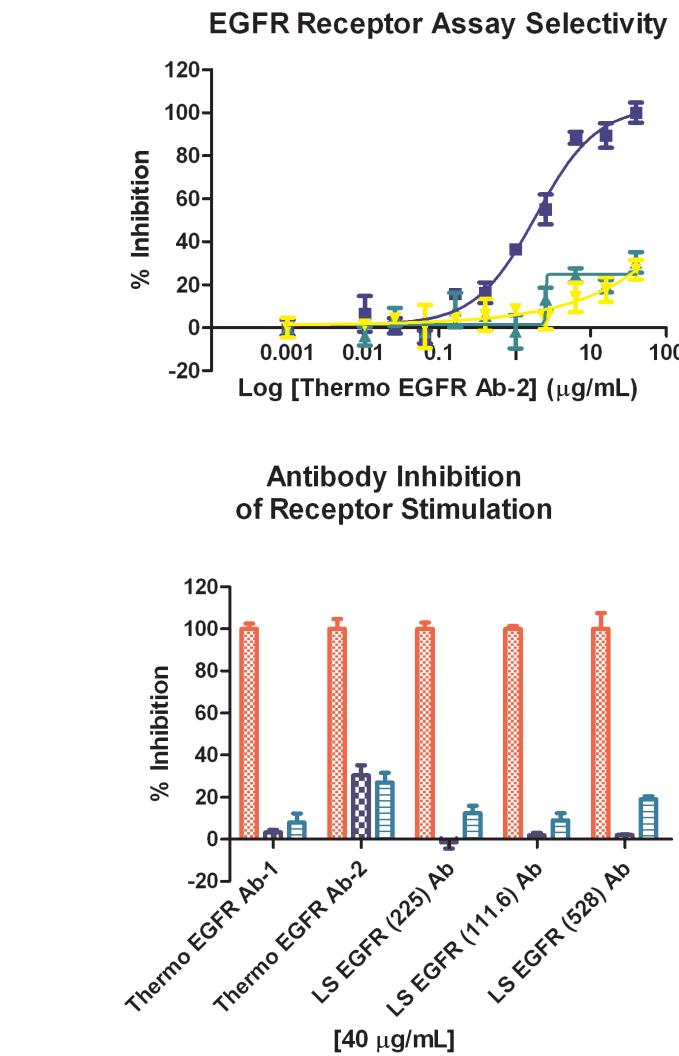


Figure 6 – Antibody Selectivity Results. A. Inhibition by Thermo EGFR Ab-2 of EGF:ErbB1, Heregulin- β 1:ErbB2, and Neuregulin-1 β 2:ErbB4 binding. B. Antibody inhibition of ligand binding for each ligand:receptor combination. % inhibition shown caused by the uppermost concentration of antibody tested.

Inhibition curves generated with each antibody:receptor assay combination illustrates that blocking of ligand binding is specific to the EGF:ErbB1 combination. Blocking of binding for other ligands is negligible and only increases at the highest concentrations tested.

Conclusions

- The PathHunter Receptor Tyrosine Kinase assays provide an easy-to-use, sensitive, functional cell-based system for the assessment of blocking antibodies.
- The inclusion of engineered cell lines, expressing the receptor of interest, reduces the complexity of the assay, offers convenience, and eliminates the time required for assay development.
- The assay procedure can be easily automated in 384-well format using contact and non-contact dispensing.
- The sensitivity of the Synergy Multi-Mode Microplate Reader provides the opportunity to reduce cell numbers used in the assay, thereby saving time and cost/well for each experiment performed.
- The combination of cells, assay, and liquid handling and detection instrumentation create an ideal solution to screen for blocking antibodies and can be routinely used in antibody characterization.