APPLICATION NOTE

Enabling Functional Therapeutic Antibody Research Using PathHunter® β-Arrestin Technology

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Here we show how PathHunter® β-Arrestin assays can be used to detect and characterize functional therapeutic antibodies targeting GPCRs or their ligands. By providing a simple, scalable, mix-and-read whole-cell assay, which is tolerant to high concentrations of serum and plasma, PathHunter® assays are the ideal quantitative tool to enable the discovery and characterization of potent, selective and high affinity biotherapeutics.

Introduction

GPCRs represent the largest class of cellular targets being investigated for the treatment of solid tumors, hematological malignancies, cardiovascular disease, diabetes, asthma, and many other diseases (1). In addition to small molecules that target GPCRs, antibodies can be selected that recognize the same binding site that a small molecule drug recognizes. Due to their specific antigen binding properties and the ability to genetically engineer antibodies that bind with high affinity and specificity to the target of interest, biotherapeutics are now one of the fastest growing segments of pharmaceutical research (2).

Our studies demonstrate that PathHunter Arrestin assays are able to detect both anti-ligand and anti-receptor antibodies designed to neutralize or block the biological activity associated with the binding of endogenous ligand to the cell surface receptor. Traditionally, enzyme linked immunosorbent assays (ELISAs) have been used for screening biotherapeutics. Although reliable and accessible in most laboratories, these assays are time-consuming and labor intensive, requiring multiple wash steps and reagent additions that limit the scalability and automation required for high throughput screening. Moreover, since these assays typically detect binding of peptides at the receptor site instead of binding to the full length receptor in the context of a whole cell, they are not good predictors of the actual mechanism by which the antibody molecules will exert their effects *in vivo*.

To demonstrate that the PathHunter Arrestin technology can be used as a powerful tool to characterize potent, specific and high affinity functional antibodies, we validated our PathHunter Arrestin cell lines using commercially available antibodies. The cell lines express full length GPCR receptors on the cell surface and both anti-ligand and anti-receptor antibodies can be detected even in the presence of high levels of complex biological samples such as serum and human plasma. Moreover, PathHunter Arrestin assays can be used to measure endogenous GPCR ligands that are upregulated in certain disease states, making this a powerful platform for studying mechanism of action and predicting the effect of a novel therapeutic *in vivo*.

Finally, we show that our frozen, ready to assay kits are ideally suited for quality control testing of biotherapeutics as they provide robust and reliable results from experiment to experiment and batch to batch. Thus, DiscoveRx's comprehensive menu of PathHunter β-Arrestin assays can provide high-value functional information at every stage of therapeutic antibody discovery.

Materials and Methods

Reagents

- Recombinant human CCL20/MIP-3 alpha (Cat. #92-1005)
- Recombinant human CCL3 (Cat. #92-1002)
- Gastric Inhibitory Peptide, GIP (Cat. #92-1078)
- Cell Detachment Reagent (Cat. #92-0009)
- PathHunter® β-Arrestin cells expressing: CXCR2 (Cat. #93-0202E2CP0M), EDG2 (Cat. #93-0644C2), CCR6 (Cat. #93-0194E2CP0M), GIPR (Cat. #93-0299E2CP2M) and CCR5 (Cat. #93-0224E2CP0M)
- PE-conjugated anti-CXCR2 antibody (Cat. #FAB331P) and mouse anti-human CCL20/MIP-2 alpha (Cat. #MAB360) were purchased from R&D Systems (Minneapolis, MN)
- Mouse anti-human CD195/CCR5 (Cat. #555991) was purchased from BD Pharmingen (San Diego, CA)
- Carageenan (Cat. #22049-5G-F) was purchased from Sigma Aldrich (St. Louis, MO)
- Female C57Bl/6 mice 6-8 weeks of age purchased from Harlan Laboratories (Placentia, CA)

PathHunter® β-Arrestin GPCR Assays

In the PathHunter β-Arrestin system, the GPCR of interest is fused in frame with the small, 42 amino acid fragment of β-gal (called ProLink™, PK) and co-expressed in cells stably expressing a fusion protein of β-Arrestin and the larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). Activation of the GPCR stimulates binding of β-Arrestin to the PK-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β-gal enzyme. This action leads to an increase in enzyme activity that can be measured using a chemiluminescent substrate. For all 96-well assays from frozen cells, vials were pre-warmed in a 37°C water bath and suspended in 12 mL of the appropriate Cell Plating Reagent (CPR). 100 µL of cells (10,000 cells per well) were seeded and incubated for 48 hours at 37°C, 5% CO_2. Following incubation, the cells were exposed to control agonists for 90 minutes at 37ºC, 5% CO_2 prior to running the PathHunter

assay. For the PathHunter® assay, 55 µl of PathHunter Detection Reagents was added and the plates were incubated for 60 minutes at room temperature. Data was read on a multimode plate reader (chemiluminescence) and analyzed using GraphPad Prism® 4 (La Jolla, CA).

Cell Surface Expression

Cultured PathHunter CXCR2 β-Arrestin cells were harvested at 70-80% confluency, centrifuged at 500 x g for 5 minutes and washed three times in an isotonic PBS buffer supplemented with 0.5% BSA (PBS-BSA). Cells were then resuspended in PBS-BSA to a final concentration of 2 x 10^6 cells/mL and 25 µL (5 x 10^4 cells) was transferred to a 96-well plate. Cells were stained with 20 µL of PE-conjugated anti-CXCR2 reagent and incubated for 30 minutes at 4°C. Following incubation, cells were washed twice in 200 µL of PBS-BSA. Following the final wash, the cell pellet was resuspend in 200 µL of PBS-BSA and analyzed by flow cytometry. As a control for analysis, CHO-K1 cells were also stained with a PE-conjugated mouse isotype control antibody.

Neutralizing Antibody Assays

PathHunter eXpress CCR6 β-Arrestin cells were seeded in 100 µL of Cell Plating 0 (CP0) Reagent and incubated for 48 hours. Assays were run in agonist mode by incubating in the presence of increasing concentrations of control CCL20 agonist or in antagonist mode by incubating with increasing concentrations of the anti-CCL20 mAb and an EC_{80} of the control agonist. Similarly, PathHunter eXpress CCR5 β-Arrestin cells were seeded in 100 µL of CP0 Reagent for 48 hours. Assays were run in agonist mode by incubating in the presence of increasing concentrations of control CCL3 agonist or in antagonist mode by incubating with increasing concentrations of the anti-CCR5 mAb. Following incubation with control ligand or antibodies, cells were incubated for 90 minutes at 37ºC, 5% CO_2. After the incubation, medium was aspirated and replaced with fresh PathHunter® Detection Reagents. Following 60 minutes at room temperature, chemiluminescence was measured on a multimode plate reader and data was analyzed.

Serum and Plasma Tolerance

PathHunter eXpress CCR6 cells were seeded in 100 µL of CP2 Reagent. After 24 hours, CP2 was aspirated and replaced with 100 µL of fresh CP2 containing 0-80% FBS and cells were incubated with varying doses of control agonist for 90 minutes at 37ºC, 5% CO_2. For the plasma experiment, samples of either heparinized, undiluted (neat) human plasma containing human CCL5, or Ham's F12 medium containing human CCL5 were added directly to PathHunter eXpress CCR5 cells and incubated for 90 minutes at 37ºC, 5% CO_2. Following agonist stimulation for both the serum and plasma tests, the serum or plasma containing samples were removed, PathHunter Detection Reagents were added and cells were incubated for 60 minutes at room temperature before chemiluminescence was measured.

Carageenan-Air Pouch Acute Model of Inflammation

Six female C57Bl/6 mice were anaesthetized with isoflurane and injected subcutaneously with 5 mL of sterile air into the intrascapular area of the back of the mouse to generate air pouches. On day 6, 1 mL of a 1% suspension of carrageenan (3 mice, Group 1) or saline only (3 mice, Group 2) was injected into the air pouch. Three hours post injection, the pouch fluid was collected by lavage with 1 mL of cell culture medium. Samples were centrifuged at 500 g for 10 minutes and undiluted (neat) lavage fluid containing endogenous lysophosphatidic acid (LPA) was added directly to PathHunter EDG2 β-Arrestin cells and incubated for 60 minutes at 37ºC, 5% CO_2. Following stimulation, the samples were removed, PathHunter® Detection Reagents were added and cells were incubated for 60 minutes at room temperature before chemiluminescence was measured.

Reproducibility Assays

To analyze reproducibility in EC_{50} and signal-to-noise ratios, 3 vials from the same lot (L#08J1108) of PathHunter GIPR cells were thawed and tested weekly according to the recommended protocol.

Results and Discussion

The PathHunter β-Arrestin system combines engineered clonal cell lines stably expressing full length cell surface GPCR receptor and optimal assay performance using a simple, mix-and-read chemiluminescent detection system (Fig. 1). In contrast to other traditional screening methods such as ELISAs, which are expensive and labor intensive to run, these homogenous assays are simple and easy to use and can enable screening of libraries of hundreds of biotherapeutic candidates in a fast, easy and cost-effective manner. Each β-Arrestin cell line is engineered to overexpress a unique membrane-bound cell surface GPCR receptor. The physical association of the ProLink™ and Enzyme Acceptor complementation partners is necessary for signal generation. Moreover, the Arrestin measurement is proximal to receptor activation. By monitoring the receptor directly and maintaining the requirement for physical contact of the EA and ProLink, the PathHunter system is highly specific and the data not only indicates specific binding of the antibody but it also directly correlates with the ability of the therapeutic to modulate GPCR receptor signaling.

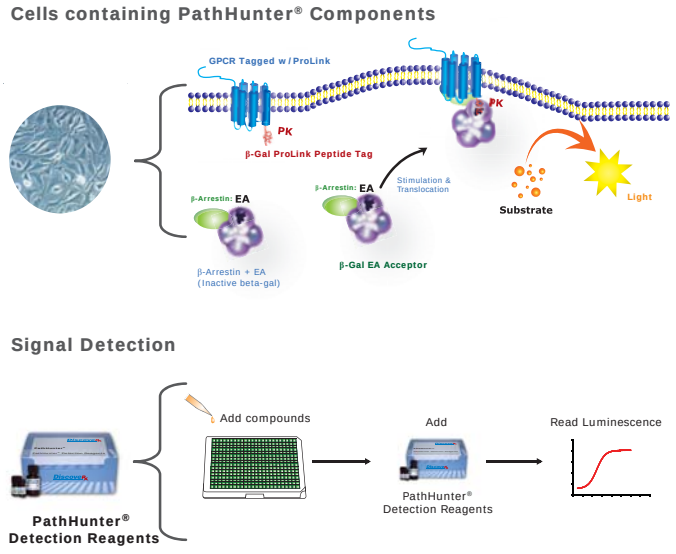

Figure 1. The PathHunter® β-Arrestin system combines engineered clonal cell lines validated for stable GPCR receptor expression and optimal assay performance using chemiluminescent PathHunter® Detection Reagents.

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Cell Surface Receptor Expression

Figure 2 shows cell surface GPCR expression using a representative PathHunter® CXCR2 β-Arrestin cell line. To quantitatively determine the percentage of cells bearing the cell surface receptor CXCR2 and to qualitatively determine the density of this receptor on cell surface, PathHunter cells were stained with CXCR2 antibody (green) or an isotype control (blue). Parental CHO-K1 cells lacking the CXCR2 receptor were used as a negative control in the study (red). These data demonstrate that PathHunter β-Arrestin cell lines express high levels of the cell surface receptors. These cell lines can be used to screen for biologically relevant therapeutic antibodies that target native epitopes and/or binding sites within the full length receptor. With over 160 GPCR targets available in this format, DiscoveRx provides a simple, cell-based tool to determine specificity, binding affinity, and rank ordering of a novel therapeutic antibody, making the study of biotherapeutics easy and accessible.

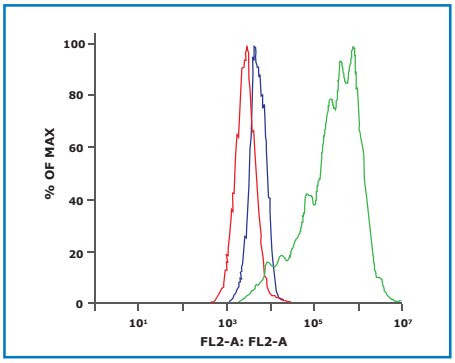
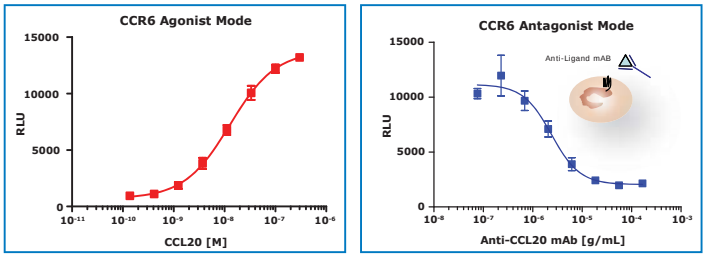


Figure 2. Flow cytometric analysis of PathHunter® cells stably expressing the human CXCR2 chemokine receptor were stained with an anti-CXCR2 antibody demonstrating surface expression of the CXCR2 receptor. Histograms: anti-CXCR2 antibody (green); native CHO-K1 cells (red); isotype control (blue).

Detection of Neutralizing Antibodies

Due to the high levels of non-specific binding of certain peptide ligands, activation of associated cell surface receptors can be difficult to study using traditional methods. Figure 3 demonstrates that PathHunter β-Arrestin cell lines can be used to establish two highly specific competitive assay formats to selectively screen anti-GPCR neutralizing antibodies that block the ligand binding site - one assay where the target antibody binds to the ligand and one where the antibody binds the specific receptor. Using PathHunter CCR6 β-Arrestin cells incubated in the presence of increasing concentrations of a monoclonal antibody against the CCL20 ligand, neutralization of CCL20 bioactivity was observed (Fig. 3A). Similarly, the PathHunter CCR5 β-Arrestin cell line was able to detect a dose dependent neutralization of CCR5 receptor signaling by incubation with increasing concentrations of a monoclonal antibody that binds directly to the CCR5 receptor and blocks CCL3 ligand binding (Fig. 3B). Together these data demonstrate that functional cell-based assays are powerful tools for large molecule therapeutic discovery and development since they can detect both anti-ligand and anti-receptor antibodies and the activity measured can mimic the mechanism by which these molecules exert their effect is *in vivo*.

A. Anti-Ligand Antibody



B. Anti-Receptor Antibody

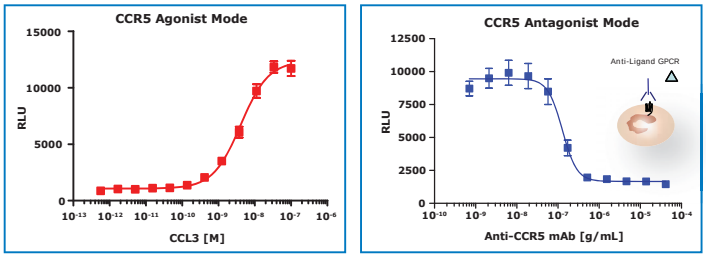


Figure 3. PathHunter® cells expressing the human CCR6 (top) and CCR5 (bottom) chemokine receptors were incubated with an increasing concentration of antibody in the presence of the appropriate control ligand. Both anti-ligand and anti-GPCR neutralizing antibodies were detected.

Tolerance to High Serum and Plasma Levels

Identification of GPCR modulating antibodies in serum or human plasma using functional assays can be problematic due to off-target effects of contaminating serum and cell supernatant components. And since the majority of drug candidates are synthetic analogs of endogenous ligands, neutralizing antibody responses to the drug are often observed which bind to and clear drugs from circulation. Figure 4 demonstrates that PathHunter β-Arrestin GPCR assays show a remarkable tolerance to high concentrations of serum and human plasma. The sensitivity of ligand binding was not significantly altered in tissue culture media containing up to 80% serum (Fig.4A) or in samples of undiluted (neat), heparinized human plasma (Fig. 4B). Because of the specific nature of the assay and high tolerance to serum, these assays provide a highly robust, sensitive and standardized method to determine the activity of functional antibodies in a variety of complex biological samples including hybridoma supernatants, bacterial lysates, and human and animal serum.

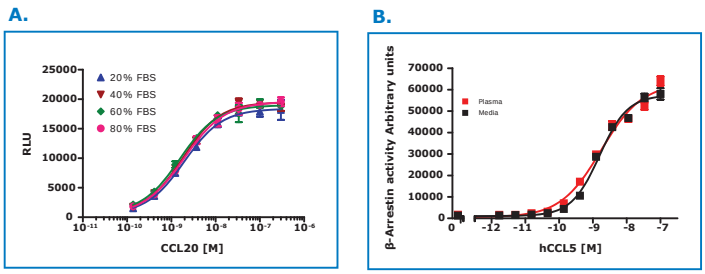


Figure 4. PathHunter® β-Arrestin cells are able to detect ligand binding. (A) PathHunter cells expressing human CCR5 receptor in the presence of high concentration of serum. (B) PathHunter cells expressing human CCR6 in the presence of neat human plasma.

In vivo Efficacy Models

Although binding and specificity of therapeutic antibodies are important to the success of any large molecule discovery program, accurately predicting the behavior of the biotherapeutic *in vivo* is critical to understanding its overall clinical effect in various disease states. Therefore, an assay system is required that can detect the upregulation of possible biomarkers in disease (ie. the GPCR ligand) to enable accurate and reliable efficacy determinations of candidate biotherapeutic molecules.

PathHunter® β-Arrestin assays were applied to the measurement of endogenously secreted lysophosphatidic acid (LPA) using an *in vivo* mouse model of acute inflammation (Fig. 5). PathHunter EDG2 β-Arrestin cells were able to detect secreted LPA found in lavage fluids isolated from mouse air pouches following treatment with Carageenan. Saline solution served as a negative control in this experiments. In contrast to other standard methods used to measure lipids, which require expensive instrumentation (mass spectrometry) and highly skilled scientists to purify the ligands (HPLC), the PathHunter β-Arrestin technology can be used as a simple and reliable platform to monitor the ability of a therapeutic antibody to block receptor signaling thus predicting the effect of a novel therapeutic *in vivo*.

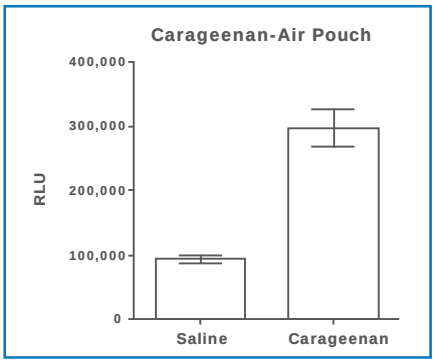


Figure 5. PathHunter® β-Arrestin cells are detect endogenously secreted LPA from mouse lavage fluid in response to Carageenan injections in an acute mouse model of inflammation.

Functional Quality Control Testing

Figure 6 demonstrates that PathHunter® β-Arrestin GPCR assays show excellent reproducibility from experiment to experiment. Using frozen, assay ready cells that express the GIPR receptor, we demonstrate that these kits are ideally suited for quality control testing of your biotherapeutic as they provide robust and reliable results from experiment to experiment. Since the assays are compatible with most standard instrumentation, this technology provides both convenience and cost-savings for standard antibody production labs.

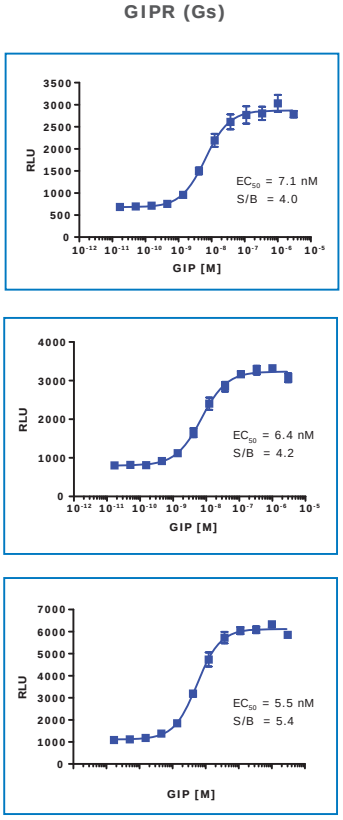


Figure 6. Frozen, ready-to-assay PathHunter® eXpress GIPR cells show predictable pharmacology and reproducible results from experiment to experiment.

Summary

DiscoveRx has the broadest portfolio of functional cell-based assays consisting of more than 500 human, mouse and rat GPCR targets for discovery and analysis of novel biotherapeutics. Regardless of whether you are doing basic research, screening therapeutic candidates, performing preclinical testing or involved in clinical development, the PathHunter® β-Arrestin technology can meet the diverse needs of the GPCR research community. With its unparalleled specificity and ease of use, the PathHunter β-Arrestin technology can detect novel therapeutic antibodies even in the presence of complex biological samples such as serum and plasma. Importantly, these assays are able to provide the best *in vivo* quantitative estimate of the potential therapeutic efficacy of antibodies, other proteins, or small molecules affecting activity of known and orphan GPCRs. Scalable PathHunter β-Arrestin assays are available as frozen cell lines and bulk reagents, convenient ready-to-use kits. In addition, you can perform customized profiling against a panel of GPCRs through our outsourced screening services.

References

1. Kerr, E. Refining GPCR discovery approaches. *Gen Eng Biotech News* 31(8): 14, 16-18, 2011.

2. The Future of the Biologicals Market: Market overview, innovations and company profiles. *Business Insights.* March 1, 2010.

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