Applications of EFC-based Protein-Protein Interaction Assays for Gi, Gs, and Gq-coupled GPCR Screening

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Abstract

GPCR targets can be addressed with a variety of technologies, and they are increasingly being evaluated with multiple different assay approaches. DiscoveRx has validated over 60 GPCR targets (Gi, Gs, Gq, Type A & Type B) in a novel, generic cell-based assay format utilizing protein-protein interaction between the GPCR C-terminus and β-arrestin2. Unlike reporter gene assays or imaging approaches, this assay provides a rapid, direct readout on the interaction between the GPCR of interest and β-arrestin, and can be detected as soon as 30 seconds after compound treatment. This approach offers a unique enzyme amplification step during signal generation, resulting in very robust performance metrics and S:B ratios that can exceed 70-fold, which has proven particularly effective in addressing certain difficult GPCRs and Gi-coupled receptors in general. We demonstrate that the assay can be used in both Flash and Glow luminescence formats, as well as fluorescence detection. Furthermore, by using a rapid Flash detection, we can monitor calcium transients using standard techniques, as well as β-arrestin translocation in the same assay well. Because of the highly sensitive nature of the assay, we have also been able to detect GPCR-arrestin interaction for a receptor that was previously thought to have no such interaction (ADORA3). Overall, we have shown that the assay can be applied to a wide range of GPCRs spanning all receptor subtypes, highlighting the generic nature of the approach, and present data outlining some of the key features and benefits provided for primary screening, secondary screening, and GPCR profiling applications.

Introduction

The DiscoveRx PathHunter cell-based assay system allows users to create simple, homogeneous assays for monitoring complex cell signaling pathways. For analysis of GPCR signaling, we have applied our EFC technology to a protein-protein interaction assay that monitors β-arrestin binding to activated GPCRs. This approach provides a generic, gain-of-signal assay format for virtually any GPCR, irrespective of the associated G-protein coupling. Data is shown below for examples of GPCRs from a variety of families, coupling mechanisms, and sub-classes. We will also highlight the utility of the assay for high throughput screening, introducing a Flash luminescence PathHunter detection system that can monitor GPCR activity in 30 seconds.

Figure 1. The DiscoveRx β-Arrestin Assay. EFC utilizes two complementing fragments of β-Gal termed EA (enzyme acceptor) and a short sequence called ProLink (donor peptide). ProLink has low binding affinity for EA, but when the two fragments are attached to interacting proteins, those proteins will drive complementation. In this assay, the ProLink peptide is added to the extreme C-terminus of an exogenously expressed GPCR and the larger EA fragment is fused C-terminally to human β2-arrestin and expressed stably in clonal cell lines. Upon GPCR activation, a cascade of events ensues that results in translocation of arrestin to the activated GPCR and generation of either a Glow or Flash luminescence signal.

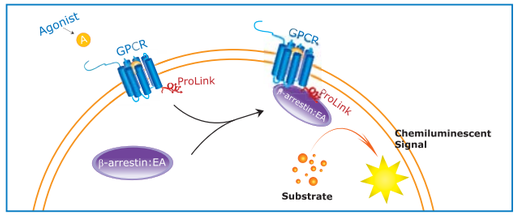
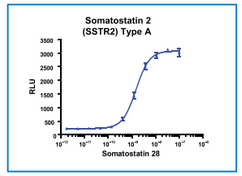


Figure 2. Broad GPCR Application & Good Performance in Stable Pools. The PathHunter Arrestin approach has been successfully applied to a very wide range of receptors of both Type A and Type B classes. As shown below, the Gi-coupled SSTR2 receptor shows a S:B ratio > 15-fold and agonist EC_{50} of ~ 2 nM. The Gs-coupled SCTR receptor shows a S:B ratio > 25-fold and agonist EC_{50} of ~ 60 nM. The majority of testing has been performed in stable cell pools as shown here, but clonal derivatives have also been tested, with even better results.



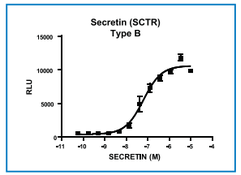
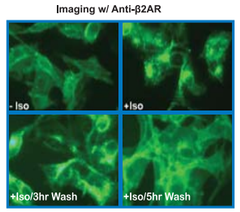


Figure 3. Biological Integrity of the EFC Arrestin Assay. The ProLink tag was designed for weak binding and for monitoring protein-protein interaction naturally and reversibly in a cell-based assay format ideal for HTS applications. Using imaging, receptor behavior can be monitored in time course assays before and after stimulation, and even following compound washout (below left). One primary benefit of the EFC approach is that the same biology can be measured without the need for imaging in a simple microplate assay with a chemiluminescence readout (shown below, right).



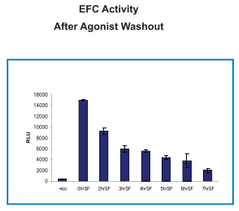
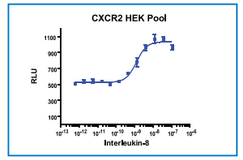
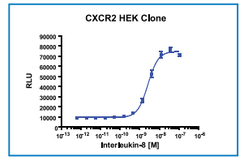


Figure 4. Assay Performance in Pools Versus Clones. Stable pools have now been compared to clonal isolates for approximately 10 targets. In this case, the CXCR2 pool showed very low overall expression, and a S:B ratio of 2-fold (below left). Following clonal selection, one clone showed nearly the same EC_{50} for agonist, and much better performance (below right).

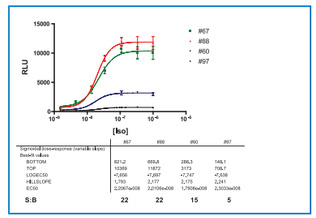


EC_{50} = 1.8 nM; S/B = 2.1



EC_{50} = 2.6 nM; S/B = 7.8

Figure 5. Evaluation of Receptor Expression Level. For the CXCR2 receptor a dramatic improvement in expression seemed apparent in one of the clones isolated. For the ADBR2 target, we isolated a larger number of clones and compared them for pharmacology, S:B ratio and qualitative analysis of expression level (below right). Interestingly, pharmacology was virtually identical in all the clones irrespective of expression, but overall S:B ratio correlated with GPCR expression.



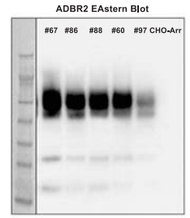


Figure 6. Performance Enhancement and Increased Speed Using Flash Detection. DiscoveRx has recently developed a PathHunter detection assay that utilizes Flash luminescence. Using one of the ADBR2 clones described previously, we have shown that significant signal can be detected in as little as 30 seconds after detection reagent addition, with S:B ratio up to 60-fold.

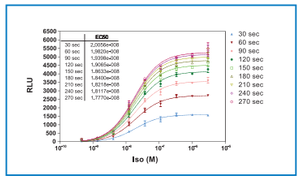
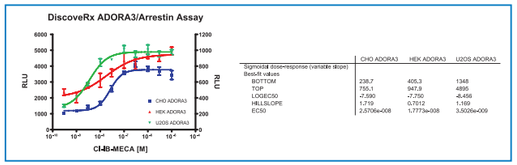
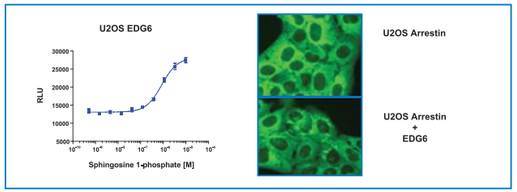


Figure 7. EFC Offers High Sensitivity Compared to Imaging. It had previously been reported through the use of imaging techniques that the ADORA3 receptor did not show detectable interaction with β2-arrestin (J. Cell Sci 113, 2463-2470, 2000). We looked at this receptor in our assay format and found that we were able to detect a response, and that the response did vary slightly across three cell types tested. The results suggest that EFC offers enhanced sensitivity compared to imaging approaches.



Sigmoidal dose-response (variable slope) Best-fit values	CHO ADORA3	HEK ADORA3	U2OS ADORA3
BOTTOM	238,7	405,3	1348
TOP	765,1	947,9	4895
LOGEC50	-7,590	-7,750	-8,456
HILLSLOPE	1,719	0,7012	1,169
EC50	2,570fe+008	1,777 3e+008	3,5026e+009

Figure 8. Analysis of Challenging Receptors. A number of receptors can be challenging to work with due to levels of constitutive activity. One such receptor in our experience has been EDG6. As shown below, the cells have very high background complementation. However, it has been possible to generate a response > 2-fold even in the presence of this background. The images below were generated using an antibody to the EA-Arrestin and show that the EDG6 expressing cells do show an activated arrestin phenotype even in the absence of compound or serum, consistent with the EFC results.



Summary - PathHunter β-arrestin Assays

FEATURE	BENEFIT
• Homogeneous, single-addition assay	• Simple automation for HTS
• Flash luminescence capability	• Ability to detect transient interactions, and improve screening throughput
• Generic GPCR functional assay	• Same assay can be used for any GPCR irrespective of G-protein coupling, no force coupling required, and can also be applied to orphan targets
• Large signal-to-background ratios	• Ability to screen for range of compouncd affects, and re-address hard targets
• Gain-of-signal readout for any target	• Simplified assay interpretation, and no requirement for forskolin stimulation
• Rapid, direct measure of GPCR activation state	• No waiting for reporter genes, and minimized potential for false positives from pathway interference