DiscoverX

User Manual

PathHunter®

β-Arrestin eXpress GPCR Assay

For Chemiluminescent Detection of β-Arrestin Recruitment in Human, Ortholog, and Orphan Cells

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For additional information or Technical Support, see contact information below.

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Overview

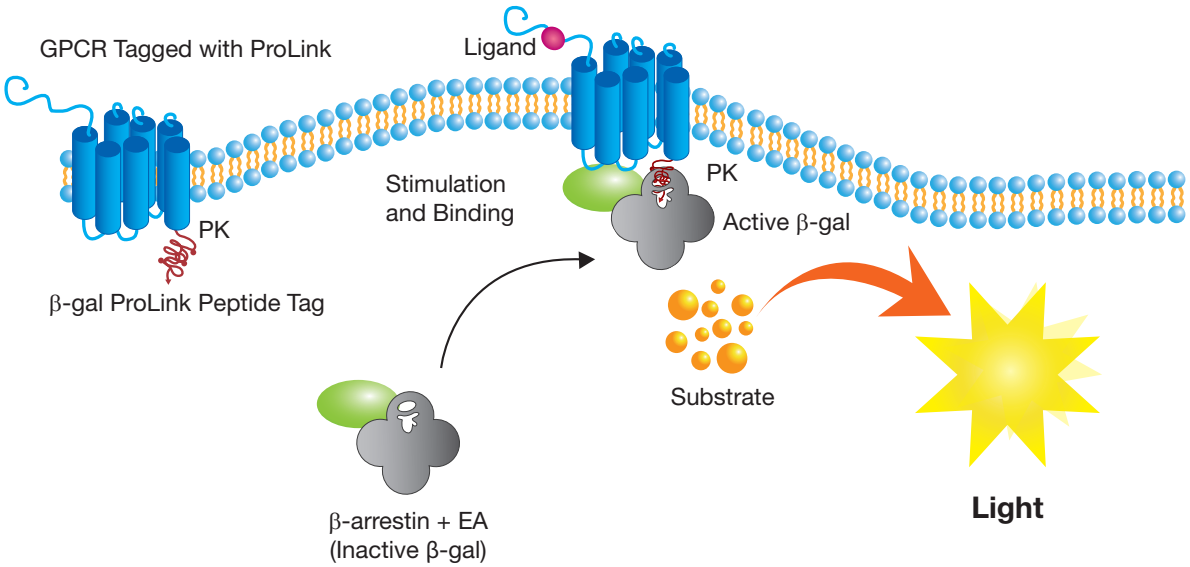
PathHunter β-Arrestin cell lines are stable clonal cell lines that expedite drug discovery and development by providing robust response to over 90% of all known G-protein coupled receptor (GPCRs), with accurate pharmacology. This assay measures an essential pathway in GPCR activation, i.e., β-arrestin recruitment to activated GPCRs, enabling scientists to screen for and profile functional agonists and inhibitors of GPCRs. These assays are successfully used to identify and optimize biologics and small molecule drugs. Since β-arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation. The PathHunter eXpress GPCR Assay can be used for small or large molecules. The kits provide a robust, highly sensitive and easy-to-use assay for monitoring G-protein coupled receptor (GPCR) activity by detecting the interaction of β-arrestin with the activated GPCR.

Technology Principle

GPCR activation following ligand binding leads to β-arrestin recruitment to the GPCR receptor. This assay measures the activation status of the target GPCR by detecting this β-arrestin recruitment using a homogeneous, easy-to-use, gain-of-signal assay based on Enzyme Fragment Complementation (EFC) technology.

The PathHunter β-Arrestin GPCR Assay uses a β-galactosidase (β-gal) enzyme split into two fragments, the Enzyme Donor (ED) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, in solution they can be brought together and complement to form an active β-gal enzyme.

Here, the target GPCR is tagged with the small fragment of β-gal called ProLink™ (PK), a low-affinity version of ED, and co-expressed in cells stably expressing β-arrestin tagged with EA. Activation of the GPCR stimulates binding of β-arrestin to the ProLink-tagged GPCR, forcing complementation of PK and EA, resulting in the formation of an active β-gal enzyme. The resulting active enzyme hydrolyzes substrate present in the PathHunter detection reagents to generate light.



PathHunter β-Arrestin GPCR Assay

PathHunter eXpress GPCR Assays

PathHunter eXpress β-Arrestin kits are complete, assay-ready kits that provide a robust and easy-to-use, cell-based functional assay to study GPCR activity through β-arrestin recruitment. The pre-validated, frozen eXpress cells have been manufactured for single-use and are provided in a convenient, ready-to-screen format that eliminates the need for lengthy, expensive and time-consuming cell culture, making functional testing fast and convenient.

The eXpress kit contains everything needed to perform a functional whole-cell GPCR assay in live cells. It includes single-use vials of frozen cells stably expressing the GPCR of interest, optimized cell plating reagent, chemiluminescent detection reagents and 96-well assay plates. After plating and stimulation of cells, simply follow the easy-to-follow protocol for adding detection reagents to the cells. The kits are available in multiple sizes that include enough cells and detection reagents for 200 or 1000 data points (based on a 96-well format). Please refer to the eXpress kit-specific datasheet for the recommended assay conditions, including incubation times and temperature. The datasheet also indicates the number of cells that will be added to the wells of the assay plate.

This flexible assay system has been designed to test agonists, antagonists or allosteric modulators in a 96-well format, and can be easily adapted to a 384-well plate format.

Materials Provided

PathHunter eXpress GPCR kits are offered in three kit configurations based on the number of cell line vials provided.

List of Components	2-Plate Kit	10-Plate Kit
PathHunter eXpress β-Arrestin GPCR cells	2 vials	10 vials
AssayComplete™ Cell Plating Reagent (mL)	1 x 100 mL	2 x 100 mL
PathHunter Detection Kit: - Substrate Reagent 1	3 mL	15 mL
- Substrate Reagent 2*	0.6 mL	3 mL
- Cell Assay Buffer	11.4 mL	57 mL
96-well white-walled clear-bottom tissue culture treated plate**	2 plates	10 plates

*Centrifuge vial before opening to maximize recovery.
**This assay can be run in a 384-plate format. The 384-well plates are not included in this kit, but can be purchased separately.

Storage Conditions

PathHunter β-Arrestin GPCR Cells

Cells must arrive in a frozen state on dry ice. These should be immediately transferred to vapor phase of liquid nitrogen for long-term storage. If cells will be thawed for use within 24 hours, they can be stored temporarily at -80°C.

Safety Warning: A face shield, gloves and lab coat should always be worn when handling frozen vials. The cryovials should be stored in the vapor phase of the liquid nitrogen. Upon thawing, if liquid nitrogen is present in the cryovial, it rapidly converts back to its gas phase, which can result in the vial exploding as it warms up.

PathHunter Detection Kit

The detection reagents include three components; Substrate Reagents 1 and 2, and Cell Assay Buffer. Upon arrival, store reagents at -20°C. The detection kit is stable until the expiration date indicated on the kit box outer label. Thaw reagents at room temperature before use. After thawing, reagents can be stored for up to 1 month at 2-8°C. For longer term storage, aliquots of all the components may be re-frozen once in opaque containers and stored at -20°C.

Cell Plating Reagent

Store reagents at -20°C. Thaw contents at room temperature and mix well by gently inverting the bottle prior to use. Once thawed, store at 4°C for up to 4 weeks. Avoid multiple freeze/thaw cycles.

96-well Tissue Culture-Treated Plates

Store at room temperature.

Additional Materials Required

Material	Ordering Information
Green V-bottom PP Ligand Dilution Plate	92-0011
PathHunter Anti-PK/PL Antibody *	92-0010
Control ligands	discoverx.com/controlligands
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 (or similar)
15 mL polypropylene tubes and 1.5 mL microtubes	
Tissue culture disposables pipettes (1 mL to 25 mL)	
Single and multi-channel micro-pipettors and pipette tips (10 µL to 100 µL)	
Humidified tissue culture incubator (37°C, 5% CO_2)	
Multimode or Luminescence plate reader	Refer to instrument compatibility chart at discoverx.com/instrument-compatibility

* Recommended for PathHunter eXpress β-Arrestin Orphan GPCR cells.

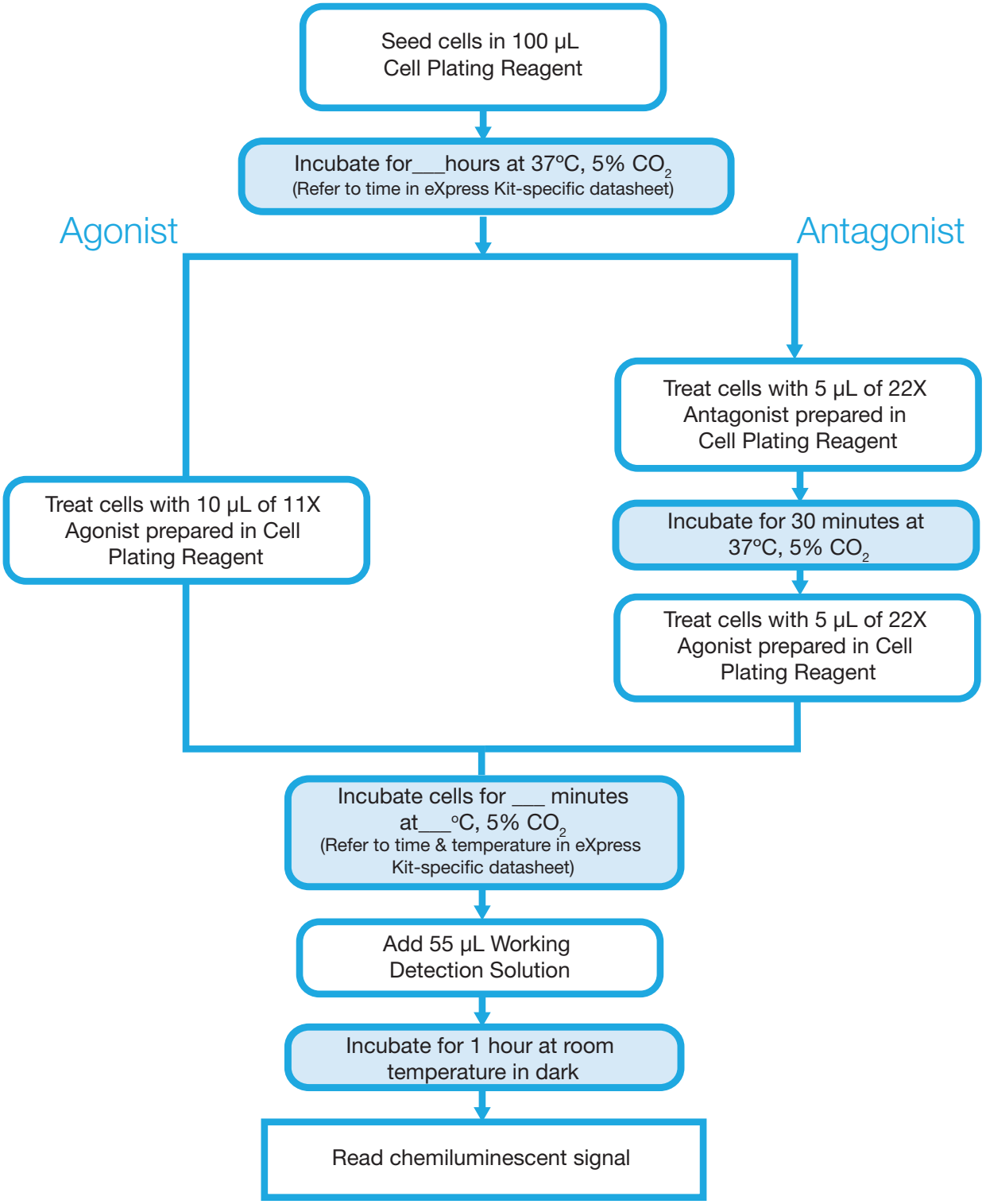
Protocol Schematic

Tip: *Use this sheet to note your assay specific conditions. Post it on your bench to use as a quick reference guide.*

Assay Name:_____ Date: _____

Product Details: _____

Quick-Start Procedure: In a white-walled 96-well tissue culture-treated plate provided in the kit, perform the following steps:



Detailed Assay Protocol (Agonist, 96-well)

The following detailed protocol is specific for detecting GPCR activation through β-arrestin recruitment in cells stimulated with an agonist in a 96-well assay plate.

Reagent	Volume per Well
AssayComplete™ Cell Plating Reagent (µL)	100
Agonist (µL)	10
Working Detection Solution (µL)	55
Total Assay Volume (µL)	**165**

Section I: Cell Preparation and Plating

The following steps outline the procedure for thawing and plating frozen PathHunter eXpress β-Arrestin GPCR cells from cryogenic vials. Each vial contains sufficient cell numbers for one 96-well microplate prepared at the seeding density described.

Do not substitute Cell Plating Reagent from an alternate kit at any time.

Do not expose cell vials to room temperature at any point leading up to the cell thawing steps. The vials contain only 100 µL of frozen cells, making the cells very prone to rapid, premature thaw, which will severely compromise cell viability and may result in assay failure.

1. Pre-warm AssayComplete Cell Plating Reagent in a 37˚C water bath for 15 minutes.
2. Transfer 11.5 mL of the pre-warmed AssayComplete Cell Plating reagent to a sterile, 15 mL polypropylene tube.
3. Remove the PathHunter eXpress β-Arrestin GPCR cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them in dry ice prior to thawing.

Safety Warning: When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate before opening the vial. Do not touch the bottom of the vials at any time to prevent inadvertent thawing of the cells.

4. Decontaminate the outside surface of the vial by spraying and wiping it with 70% ethanol. From this step onwards, all procedures should be carried out under aseptic conditions in a tissue culture hood.
5. Immediately transfer 0.5 mL of the pre-warmed AssayComplete Cell Plating Reagent to the cell vial to thaw the cells. Pipette up and down gently several times to ensure that cells are thawed and evenly suspended.
6. Transfer 0.6 mL of the thawed cell suspension to the 15 mL tube containing the 11.5 mL of pre-warmed AssayComplete Cell Plating Reagent. Gently mix the cells, then transfer them to a sterile disposable reagent reservoir.

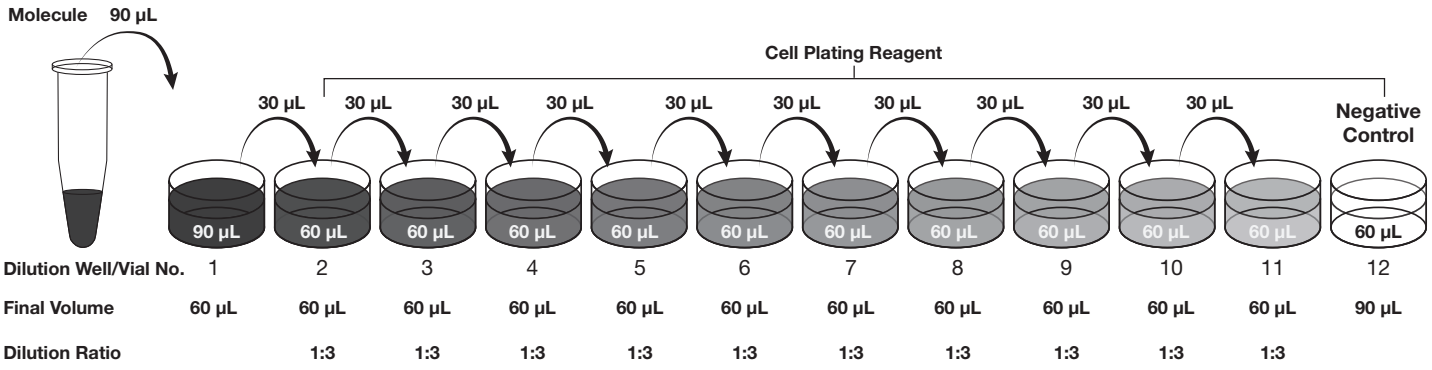
Do not thaw vials in 37˚C water bath. Do not centrifuge.

7. Transfer 100 µL of the cell suspension to each well of a 96-well assay plate.
8. Incubate the assay plate according to the time and conditions indicated in the assay kit-specific datasheet. Typically, plate incubation is done at 37°C and 5% CO_2, but the incubation time is specific for each kit.
9. Proceed to molecule preparation and addition.

Section II: Molecule Preparation

The following is a procedure for setting up agonist dose response dilutions.

1. Prepare molecule (agonist) serial dilutions in a separate dilution plate (not included in the eXpress kit) in an 11-point series of 3-fold dilutions of the molecule in Cell Plating Reagent as shown in the workflow below.

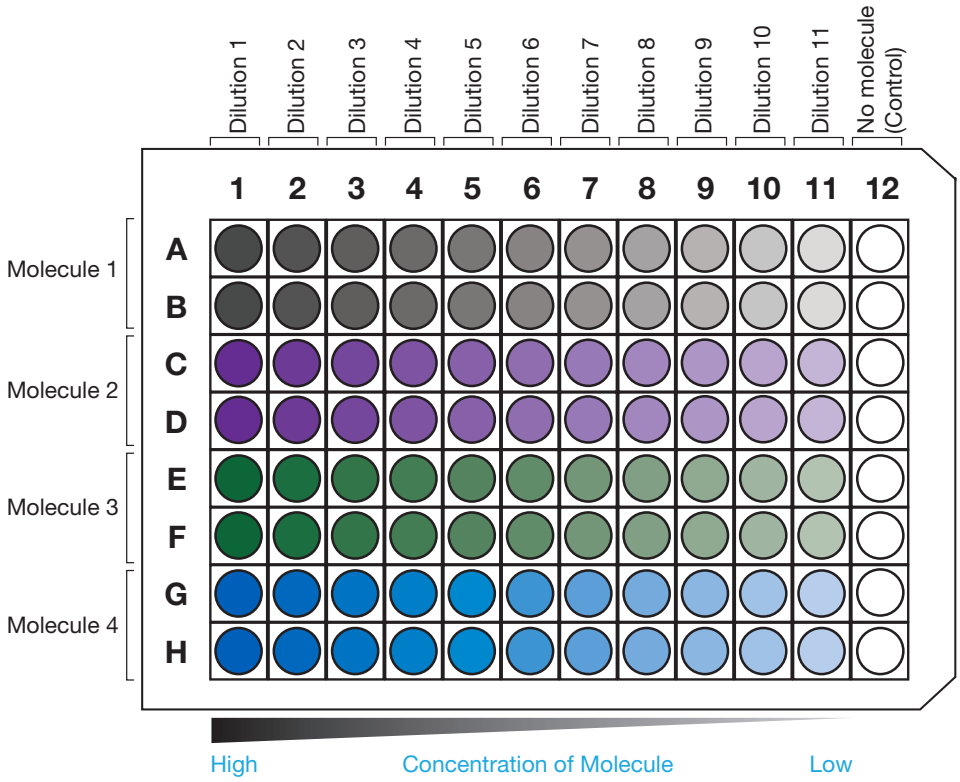


Molecule serial dilutions: Make 11 three-fold serial dilutions of the molecule in a dilution plate or tubes (not included in the kit).

2. The concentration of each dilution should be prepared at 11X the final screening concentration.
   a. For each molecule, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
   b. Add 60 µL of Cell Plating Reagent to dilution wells No. 2 through No. 12. This volume is enough for two rows of wells for each concentration. The volume may be adjusted according to the number of wells desired.
   c. Prepare the highest concentration of molecule in Cell Plating Reagent. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the molecule. Therefore, prepare a working concentration that is 2750X the expected EC_{50} per well to get an 11X working molecule concentration. For example, for an expected EC_{50} of 1 nM, prepare the highest working concentration at 2750 nM. This is 11X the screening or final highest concentration of 250 nM, and the expected EC_{50} will lie near the center of the dose response curve.
   d. Add 90 µL of the highest concentration of molecule to well No. 1 (see figure below).
   e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.
   f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.
   g. Repeat this process until well No. 11 is reached. Do not add molecule to well No. 12, which is for the negative control.
3. Set up additional serial dilutions for additional molecules in a similar manner.

Section III: Molecule Addition

The following is a procedure for adding the agonist dose response dilutions to the assay plate.



Agonist Assay Plate Map: Create 11-point dilutions with four different molecules in duplicate.

1. Add 10 µL of each 11X agonist serial dilution in duplicate to the designated agonist rows (e.g. agonist 1 in rows A and B; agonist 2 in rows C and D; agonist 3 in rows E and F, and agonist 4 in rows G and H) of the assay plate as indicated on the assay plate map.

2. Incubate the assay plate at the time and temperature specified in the datasheet for the eXpress kit.

Section IV: Detection Reagent Addition and Plate Reading

At this point, the agonist stimulation step has been completed. The following section contains procedures for adding the PathHunter Detection Reagent and reading the assay plate on a luminometer.

Detection reagents must be prepared as a working solution prior to use. The Working Detection Solution is stable for up to 24 hours at room temperature with no adverse impact on assay performance.

1. Prepare a stock of Working Detection Solution in a separate 15 mL polypropylene tube or reagent reservoir by mixing 19-parts of Cell Assay Buffer, 5-parts of Substrate Reagent 1, and 1-part Substrate Reagent 2, as shown in the table on page 8.

Components	Volume Ratio	Volume per Plate (mL)
Cell Assay Buffer	19	4.75
Substrate Reagent 1	5	1.25
Substrate Reagent 2	1	0.25
Total Assay Volume		**6.5**

2. Add 55 µL of Working Detection Solution to all wells of the assay plate.
3. Incubate assay plate for 1 hour at room temperature in the dark.
4. Read assay plate on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imagers. In general, the signal continues to increase and reaches a maximum approximately 3-4 hours after the last incubation step. The actual signal characteristics may be affected over time by lab conditions, such as temperature, and the user should establish an optimal read time for the assay. Luminescence readers usually collect signal from all wavelengths. Some instrument manufacturers may include a cut-off filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

Use the working detection solution within 24 hours.

Do not agitate or vortex plates after adding detection reagents.

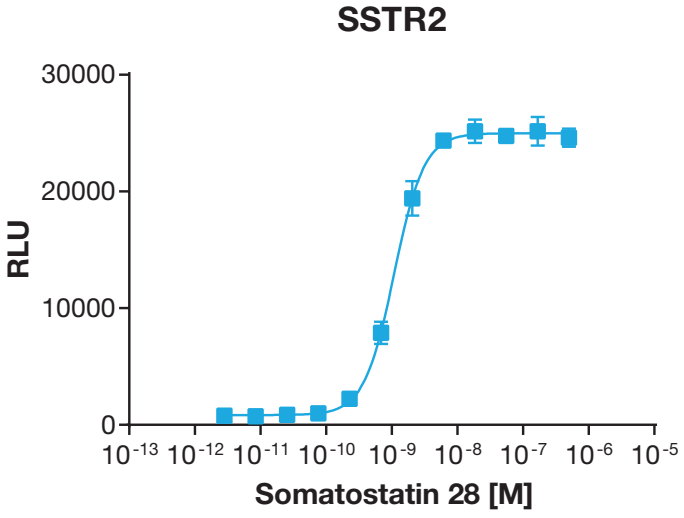
Working Detection Solution is light sensitive, thus incubation in the dark is necessary.

The assay plate should be read within 2 hours after adding the detection reagent solution.

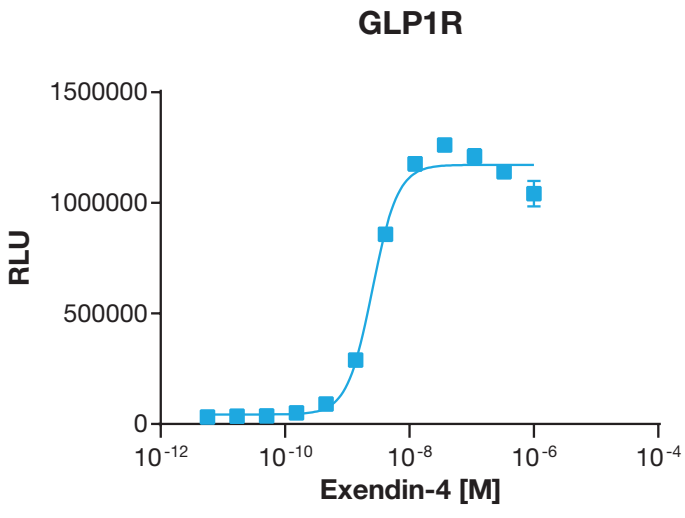
5. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Instruments Gen5, Microsoft Excel, etc.).

Typical Results

Shown below are typical results for the PathHunter eXpress GPCR Assay using the PathHunter eXpress CHO-K1 Somatostatin Receptor 2 (SSTR2) cells (left) and Glucagon-like Peptide Receptor 1 (GLP1R) cells (right).



EC_{50}	S/B
1.09 nM	29.7



EC_{50}	S/B
2.54 nM	37.3

Detailed Assay Protocol (Antagonist, 96-well)

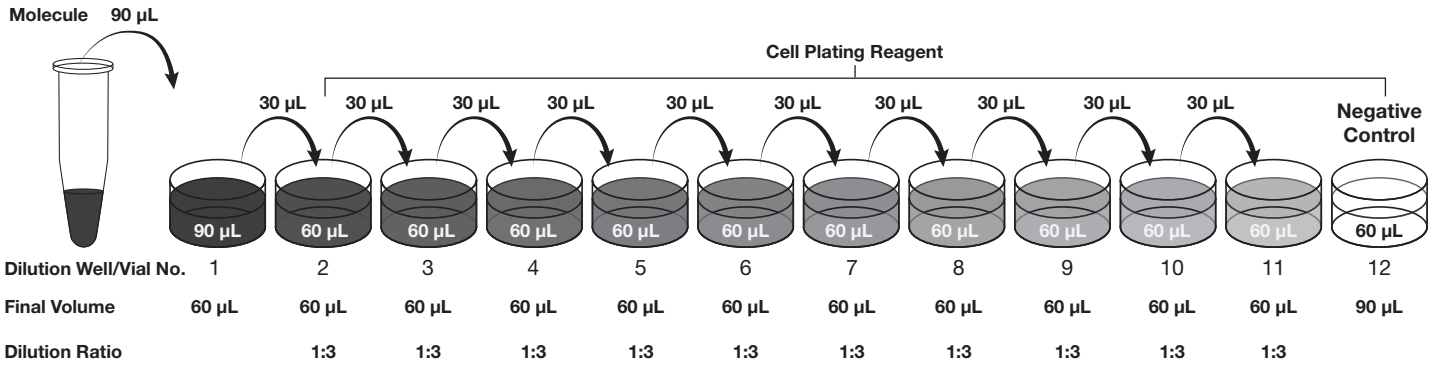
Antagonist tests are typically run by pretreating the target cells with antagonist, followed by stimulation of unoccupied receptors with a dose of a receptor agonist. Receptors not occupied by antagonists can be bound by agonists, which will activate the receptors. Receptors that are occupied by antagonist cannot bind agonists and will remain inactive.

The following procedure outlines the steps for testing for a dose-dependent antagonist inhibition in a 96-well assay plate.

Reagent	Volume per Well
AssayComplete™ Cell Plating Reagent (µL)	100
Antagonist (µL)	5
Agonist EC_{80} (µL)	5
Working Detection Solution (µL)	**55**

Section I: Antagonist Preparation

1. Prepare an 11-point series of 3-fold molecule (antagonist) serial dilutions in Cell Plating Reagent in a separate dilution plate or tubes (not included in the eXpress kit), as shown in the workflow below.



Molecule serial dilutions: Make 11 three-fold serial dilutions of the molecule in a dilution plate or tubes (not included in the kit).

2. The concentration of each dilution should be prepared at 22X the final screening concentration.

   a. For each molecule, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.

   b. Add 60 µL of Cell Plating Reagent to dilution wells No. 2 through No. 12. This volume exceeds what is required for two rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

   c. Prepare the highest concentration of molecule in Cell Plating Reagent. We recommend preparing a final screening concentration that is 250X the expected IC_{50} of the molecule. Therefore, prepare a working concentration that is 5500X the expected IC_{50} per well to get a 22X working molecule concentration. For example, for an expected IC_{50} of 1 nM, prepare the highest working concentration at 5500 nM. This is 22X the screening or final highest concentration of 250 nM, and the expected IC_{50} will lie near the center of the dose response curve.

   d. Add 90 µL of the highest concentration of molecule to well No. 1.

   e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

g. Repeat this process until well No. 11 is reached. Do not add molecule to well No. 12, which is for the negative control.

3. Set up serial dilutions for any additional molecules in a similar manner.

Section II: Agonist EC_{80} Challenge Preparation

The following is a protocol for preparing an agonist challenge dose that will be added to the cells to stimulate receptors that are not occupied by antagonists.

1. First determine the agonist EC_{80} either by visually estimating it from the reference curve provided on the specific eXpress kit datasheet or experimentally by following the steps below:

   a. Follow the Detailed Protocol for the Agonist assay.

   b. Plot the agonist response data using a variable slope sigmoidal curve function.

   c. Determine EC_{50} and Hill Slope.

   d. Calculate EC_{80} value (refer to the FAQ section for EC_{80} calculation).

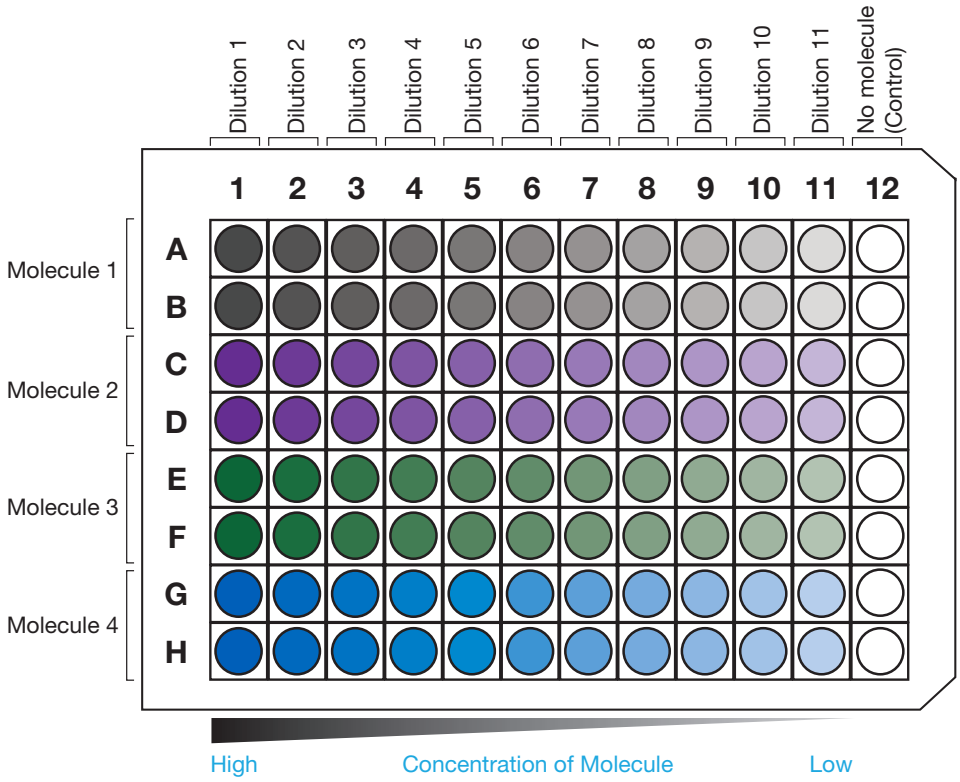
2. Prepare an agonist EC_{80} dilution in Cell Plating Reagent in a separate tube to a concentration that is 22X the final desired agonist dose.

3. Aliquot equal volumes of 22X agonist EC_{80} into wells No. 1 through No. 12 of an empty row in a molecule dilution plate.

Section III: Antagonist and Agonist EC_{80} Addition

The following is a procedure for adding the antagonist dose response dilutions followed by addition of the agonist EC_{80} dose.

1. Add 5 µL of each 22X antagonist serial dilution in duplicate to the designated antagonist rows (e.g. antagonist 1 in rows A and B; antagonist 2 in rows C and D). Repeat for the remaining molecules as indicated on the molecule assay plate map.



Antagonist Assay Plate Map: Create 11-point dilutions with four different molecules in duplicate.

2. Incubate assay plate for 30 minutes at 37°C and 5% CO_2.
3. Add 5 µL of the 22X agonist EC_{80} dilution to each assay well.
4. Incubate the assay plate at the time and temperature specified in the datasheet for the eXpress kit.

Section IV: Assay Detection and Plate Reading

Follow the instructions in Section IV of the Agonist protocol.

Detailed Assay Protocol (Anti-Ligand Antibody, 96-well)

Anti-ligand tests are typically run by pre-incubating the agonist with the anti-ligand antibody prior to loading the test sample onto the cell assay. Agonist occupied by the anti-ligand antibody will be unable to bind to and activate the receptor.

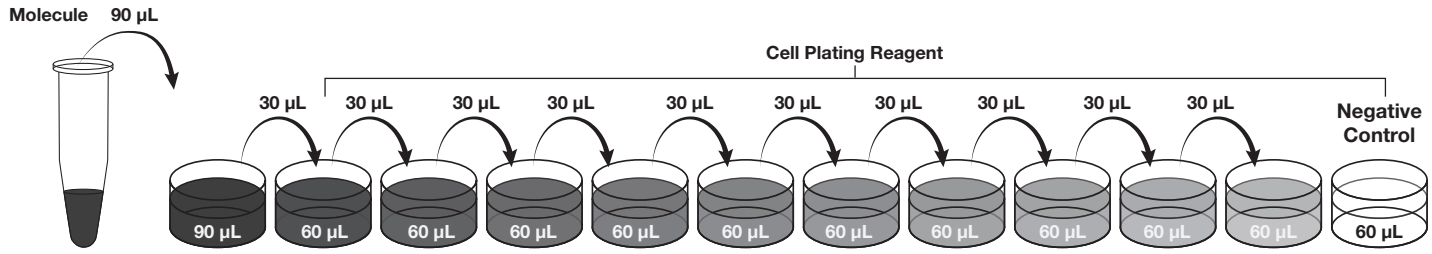
The following is a procedure for testing for a dose-dependent inhibition of an agonist by an anti-ligand antibody in the 96-well assay plate.

Reagent	Volume per Well
AssayComplete™ Cell Plating Reagent (µL)	100
Anti-Ligand Antibody + Agonist EC_{80} Mix (µL)	10
Working Detection Solution (µL)	55
Total Assay Volume (µL)	**165**

Section I: Anti-Ligand Antibody Preparation

The following is a procedure for setting up an anti-ligand antibody dose response curve.

1. Prepare an 11-point series of 3-fold dilutions of molecule (anti-ligand antibody) serial dilution in row A of a separate dilution plate or tubes (not provided in the kit) using Cell Plating Reagent, following the workflow below.



Molecule serial dilutions: Make 11 three-fold serial dilutions of the molecule in a dilution plate or tubes (not included in the kit).

2. The concentration of each dilution should be prepared at 22X the final screening concentration.
   a. For each molecule, label the wells in row A of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
   b. Add 60 µL of Cell Plating Reagent to dilution wells No. 2 through No. 12. This volume exceeds what is required for two rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

c. Prepare the highest concentration of molecule in Cell Plating Reagent. We recommend preparing a final screening concentration that is 250X the expected IC_{50} of the molecule (so that the expected IC_{50} will be near the center of the dose response curve). Therefore, prepare a working concentration that is 5500X the expected IC_{50} per well to get a 22X working molecule concentration. For example, for an expected IC_{50} of 1 nM, prepare the highest working concentration at 5500 nM. This is 22X the screening or final highest concentration of 250 nM.

d. Add 90 µL of the highest concentration of molecule to well No. 1.

e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

g. Repeat this process until well No. 11 is reached. Do not add molecule to well No. 12, which is for the negative control.

3. Set up serial dilutions for any additional molecules in a similar manner.

Section II: Agonist EC_{80} Challenge Preparation

The following is a protocol for preparing an agonist challenge dose that will be mixed with the anti-ligand antibody dose-response dilutions.

1. First determine the agonist EC_{80} either by visually estimating it from the reference curve on the specific eXpress kit datasheet or experimentally by following the steps below:

   a. Follow the Detailed Protocol for the Agonist assay.

   b. Plot the agonist response data using a variable slope sigmoidal curve function.

   c. Determine EC_{50} and Hill Slope.

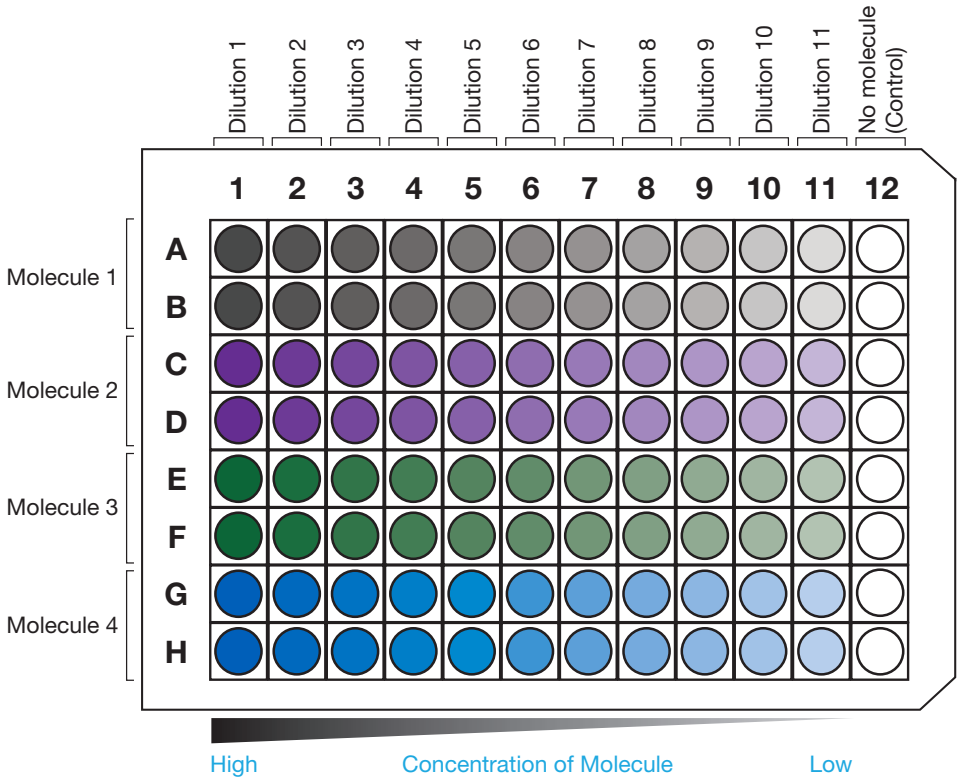
   d. Calculate EC_{80} value (refer to the FAQ section for EC_{80} calculation).

2. Prepare an agonist EC_{80} dilution in a separate tube, to a concentration that is 22X the final desired agonist dose.

   a. In row B of molecule dilution plate, aliquot 30 µL of the 22X agonist EC_{80} dilution into wells No. 1 through No. 12.

Section III: Anti-Ligand Antibody/Agonist Pre-Incubation and Addition

The following is a procedure for the mixing and pre-incubation of the anti-ligand antibody and agonist EC_{80} dose. Add the dilutions into specific wells as per the assay plate map shown below.



Anti-Ligand Antibody Assay Plate Map: Create 11-point dilutions with four different molecules in duplicate.

1. Using a multi-channel pipet, transfer 30 µL of 22X anti-ligand antibody dilution from the wells No. 1 through No. 12 of row A into the wells No. 1 through No. 12 of row B, which contain the 30 µL of agonist EC_{80} dilution, on the dilution plate. Gently mix by pipetting up and down. The result is a mix of 11X anti-ligand antibody and 11X agonist EC_{80}.
2. Pre-incubate the anti-ligand and agonist mix for at least 15 minutes. The optimal pre-incubation time and temperature should be determined empirically.
3. Add 10 µL of each 11X anti-ligand antibody/agonist mix in duplicate to the designated antibody rows (e.g. antibody/agonist mix 1 in rows A and B; antibody/agonist mix 2 in rows C and D). Repeat for the remaining antibody/agonist mix as indicated in the assay plate map.
4. Incubate the assay plate at the time and temperature specified in the respective datasheet for the eXpress kit.

Section IV: Assay Detection and Plate Reading

Follow the instructions in Section IV of the Agonist protocol.

Supplemental Information

Allosteric Modulators

For positive allosteric modulators (PAMs), refer to the detailed assay protocol for testing antagonists, but use an EC_{20} agonist challenge concentration instead of an EC_{80}.

For negative allosteric modulators (NAMs), follow the detailed assay protocol for testing antagonists with no changes.

Crude Biologic Samples

The PathHunter eXpress β-Arrestin GPCR Assay kits typically can be run in the presence of high levels of serum or plasma without adversely impacting assay performance. Therefore, samples can be prepared in neat serum or plasma, and added directly to cells without further dilution. For best results, the optimized minimum required dilution of crude samples should be determined empirically.

Also, after sample treatment, it may be necessary to remove the biologic test sample from the cells, and replace it with 110 µL fresh Cell Plating Reagent prior to the addition of the Working Detection Solution. The necessity of adding such a step must be determined empirically. High levels of protein in the wells may interfere with the EFC reaction or the optics of the plate reader.

Running Assay in a 384-well Plate

The eXpress kits are configured to run assays in a 96-well plate. The assay can be easily modified to run these in a 384-well plate, by adjusting the volumes using the following guidelines:

1. Suspend the cells in Cell Plating Reagent to a final volume of 10 mL.

2. Adjust volumes for the assay reagents for each protocol as follows:

For Testing Agonists	
Reagent	**Volume per Well**
AssayComplete™ Cell Plating Reagent (µL)	20
Agonist (µL)	5
Working Detection Solution (µL)	12.5
Total Assay Volume (µL)	**37.5**

For Testing Antagonists	
Reagent	**Volume per Well**
AssayComplete Cell Plating Reagent (µL)	20
Antagonist (µL)	2.5
Agonist EC_{80} (µL)	2.5
Working Detection Solution (µL)	12.5
Total Assay Volume (µL)	**37.5**

For Testing Anti-Ligand Antibodies	
Reagent	**Volume per Well**
AssayComplete Cell Plating Reagent (µL)	20
Anti-ligand Antibody + Agonist EC_{80} Mix (µL)	5
Working Detection Solution (µL)	12.5
Total Assay Volume (µL)	**37.5**

FAQs

How do you determine EC_{80} from the agonist reference curve?

- Determine the EC_{80} value and the Hill Slope from the agonist reference curve.
  - Use an online EC_{80} calculator like QuickCalc by GraphPad (http://www.graphpad.com/quickcalcs/Ecanything1.cfm), or
  - Use the formula below where F is the percent response and H is the Hill Slope from the agonist reference curve:

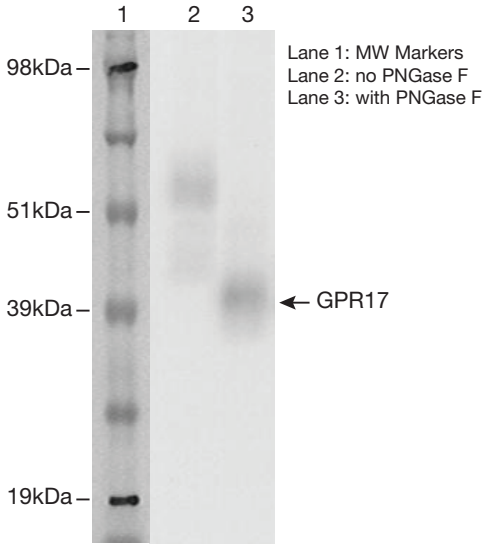
$$EC_F = \left(\frac{F}{100\text{-}F}\right)^{\frac{1}{H}} EC_{50}$$

An example of EC_{80} calculation:

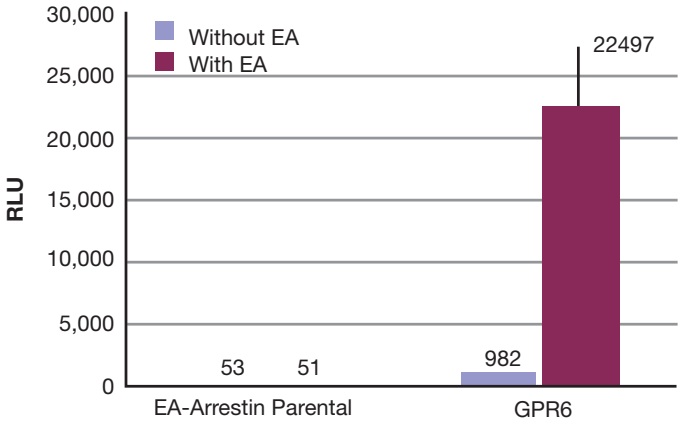
$$EC_{80} = \left(\frac{80}{100\text{-}80}\right)^{\frac{1}{H}} EC_{50}$$

How do you characterize Orphan GPCRs with this kit?

- PathHunter eXpress β-Arrestin Orphan GPCR cells are validated using the following criteria:
  - Confirmation of proper GPCR expression at the predicted molecular weight (Left);
  - *in vitro* complementation studies to measure basal activity and GPCR-PK expression (Right) and,
  - Cell viability after overnight incubation in the appropriate Cell Plating Reagent.



Cell lysates prepared from PathHunter eXpress β-Arrestin Orphan GPCR cells were treated with PNGase F (Glyko: Cat. No. GKE- 5003), run on a SDS-PAGE gel and analyzed. Alternatively, lysates can be analyzed by running a western blot using PathHunter Anti-PK/ PL Antibody (DiscoverX, Cat. No. 92-0010) and a commercially-available secondary antibody. Untreated lane resolves a band of appropriate size corresponding to GPCR-PK fusion protein and the PNGase F treated lane resolves a deglycosylated band indicative of proper expression and folding of GPCR protein.



PathHunter eXpress β-Arrestin Orphan GPCR cells were analyzed for basal activity as well as GPCR-ProLink expression by comparing the ratio of signal between untreated cells and cells treated with saturating amounts of exogenous EA, using ProLink™ Detection Kit (DiscoverX, Cat. No. 92-0006). Signal from complementation of ProLink and EA fragments correlates to the amount of GPCR-PK expression in the cell line.

What if there is no or low signal?

- Visually inspect the cells before and after running the assay in a clear bottom plate to ensure that they are healthy. Make sure proper cell plating procedure is followed to ensure assay performance.
- Make sure detection reagents are stored and prepared properly.
- Make sure the proper assay mode is used (agonist mode or antagonist mode).
- Make sure the plate reader is setup properly to read luminescence.
- High levels of protein in the wells may interfere with enzyme fragment complementation. If high levels are present, a media exchange step could be performed just prior to the detection reagent addition. A mild detergent may also help decrease protein aggregation.
- White-walled assay plates should be used since black-walled plates may decrease signal.

What if the response is lower than expected (lower than expected S/B)?

- Make sure that the ligand is prepared properly, take extra care to observe ligand solubility.
- Make sure DMSO and other solvent concentrations are not too high (not more than 1% final concentration).
- When testing ligands at high concentrations, make sure the vehicle is compatible.
- Make sure ligand is incubated for the designated time and at the designated temperature.
- Make sure plates are protected from light during incubation.

What if the EC_{50} does not match reported values?

- Make sure ligands are incubated at the proper temperature.
- The source of a ligand, particularly for biologics, can have a large effect on the observed pharmacology.
- Use fresh pipette tips during serial dilutions to help avoid carryover.

What if the variability between replicates is high?

- Make sure dispensing equipment is properly calibrated and proper pipetting technique is used.
- Make sure to avoid cell clumps when plating. Uneven number of cells in the wells can cause high variability.

Does the expression level of receptor affect the molecule response?

- No. The β-arrestin system is a stoichiometric system. Receptor expression levels do not distort the response to molecule.

Can I use bacterial lysate samples?

- Yes. Our assays can tolerate bacterial lysates. We have tested up to 17% lysate concentrations with no change in assay performance. It is best to use an *E. coli* strain with little to no *LacZ* expression since our readout is β-galactosidase complementation. We also recommend using a non-detergent or very light detergent lysis of the bacteria.

Can the cells provided in this kit be propagated?

- No. These cells provided in the kit are single-use only. These are manufactured to be division-arrested and cannot be propagated or sub-cultured.

Can I use my molecule, which is in a media containing high concentration of serum?

- Typically, our assays are highly tolerant to high serum content. However, there may be other ligands present in the serum that may raise the assay background, which can be target specific.
- We recommend that you aspirate the high serum media prior to adding detection reagents. Aspirating the media can help increase S/B, but it may not affect altered potency from ligands present in the serum or elevated background.

For additional information or Technical Support, see contact information below.

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