User Manual

PathHunter® eXpress β-Arrestin GPCR Assay

For the Chemiluminescent Detection of β-Arrestin Recruitment to Human, Ortholog, and Orphan GPCRs

Document Number 70-255 Revision 5

Please read the entire User Manual before proceeding with the assay.

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Overview

PathHunter® eXpress β-Arrestin GPCR Assays are single-use cell-based assays derived from clonal cell lines that can 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 β-Arrestin 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.

Assay Principle

GPCR activation following ligand binding leads to β-arrestin recruitment to the receptor. This assay measures the activation status of the target GPCR by detecting β-arrestin recruitment using a homogeneous, easy-to-use, gain-of-signal assay based on Enzyme Fragment Complementation (EFC) technology (Figure 1).

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.

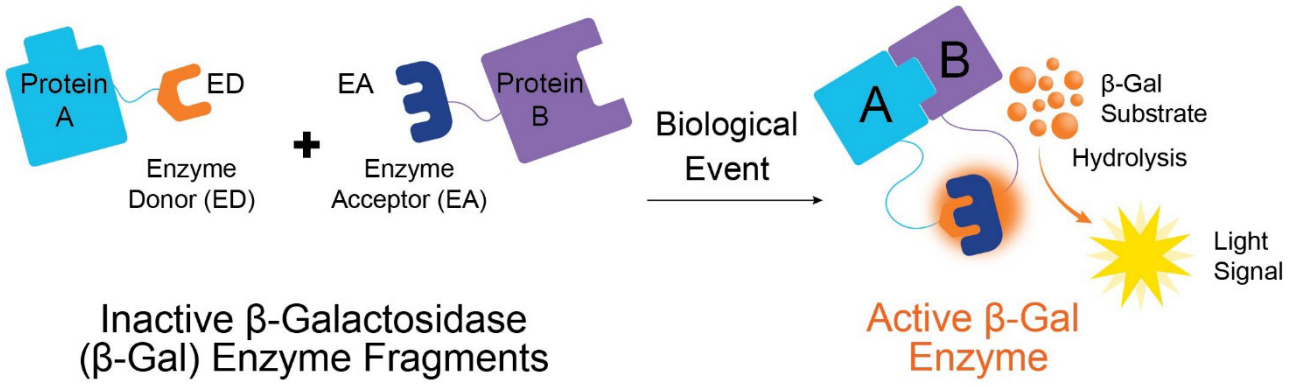


Figure 1. **Enzyme Fragment Complementation (EFC) technology**

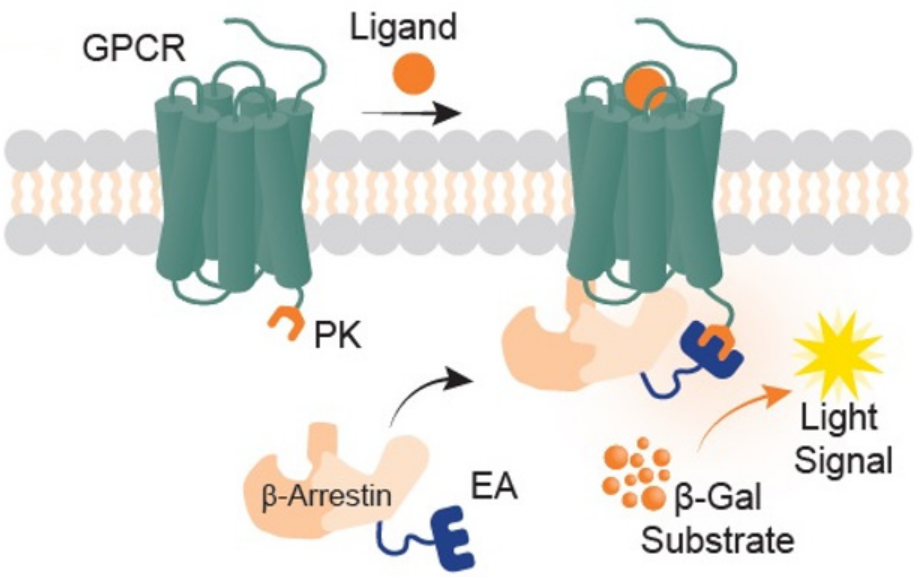


Figure 2. Assay Principle

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 (Figure 2). 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.

Assay Workflow

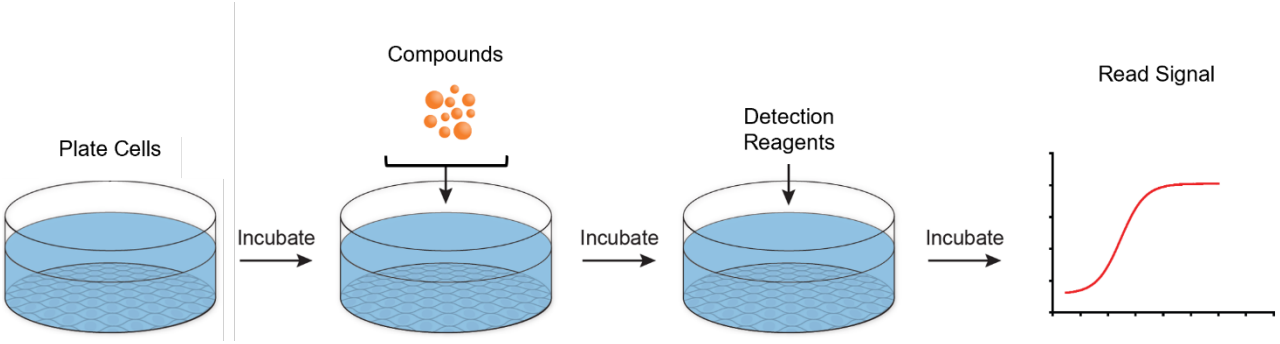


Figure 3. Assay Workflow

Please read the entire protocol completely before running the assay. Successful results depend on performing these steps correctly. The Assay Procedure sections, and Quick Start Guides contain detailed information about how to run the assays.

Assays should be run after plating and stimulating the cells. Following treatment of the cells with compound, GPCR activity is detected by adding a working solution of chemiluminescent PathHunter® Detection reagents using a simple, mix-and-read protocol.

Monitor GPCR activity with these PathHunter eXpress β-Arrestin GPCR Assay steps (Figure 3).

1. Plate cells.
2. Dilute and add compounds.
3. Perform functional assay in agonist, antagonist, or allosteric modulator mode.

Materials Provided

PathHunter eXpress GPCR kits are offered in two 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.

Table 1. Materials Provided

Storage Conditions

PathHunter® eXpress β-Arrestin GPCR Cells

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

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times 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

Materials	Ordering Information
Ligands	discoverx.com/product-category/control-ligands-inhibitors/
Sterile 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-pipettes and pipette tips (10 µL-1,000 µL)	
Humidified tissue culture incubator (37° C and 5% CO_2)	
Multimode or Luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/tools-resources/instrument-compatibility/

*Series refer to the different sizes available for that reagent or kit.

Table 2. Materials Required

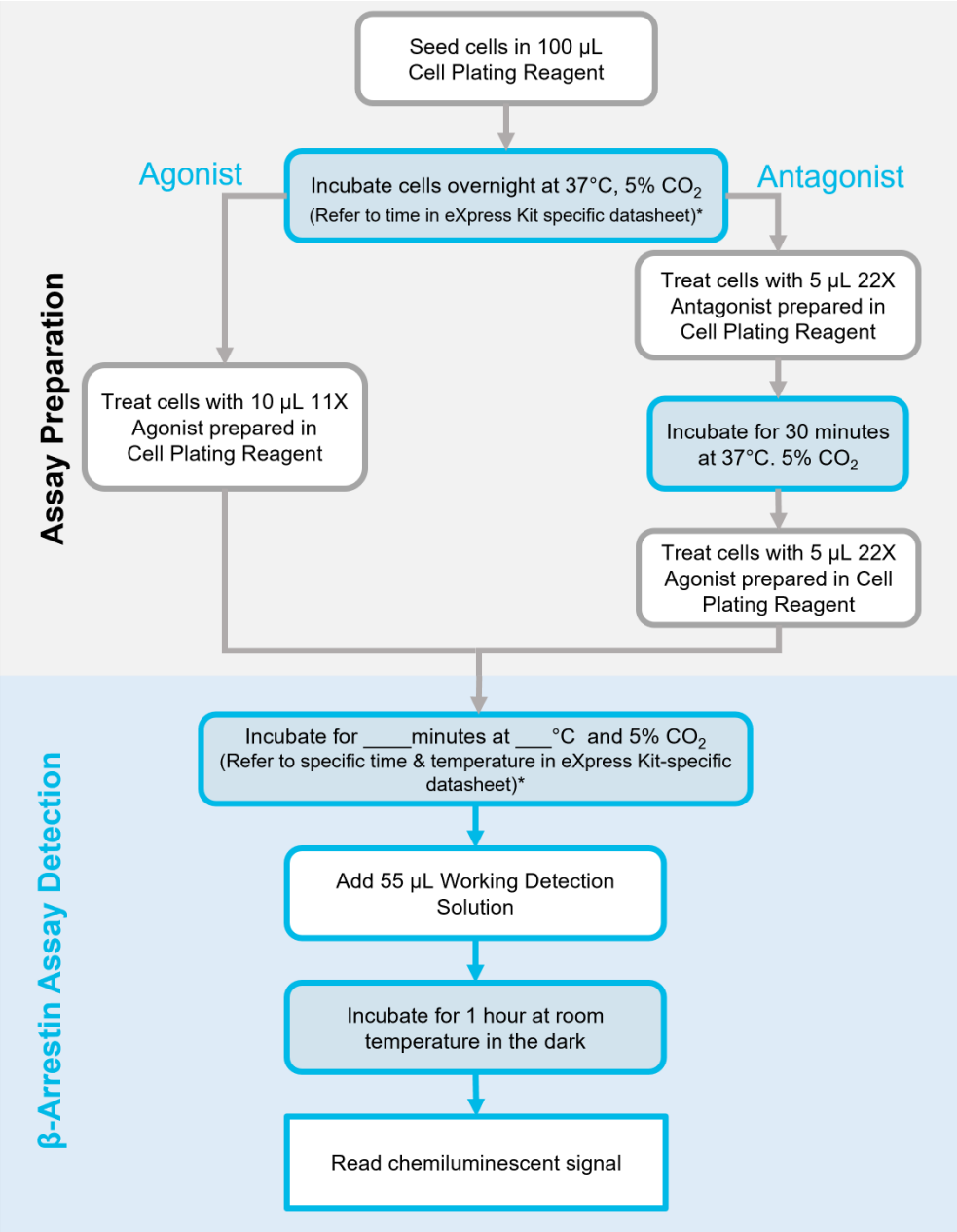
Protocol Schematic

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.



*For orphan receptors, refer to the Supplemental Information section in this user manual.

Figure 4. Quick-start Procedure

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
Compound (e.g., Agonist) (µL)	10
Working Detection Solution (µL)	55
Total Assay Volume	**165**

Table 3. Assay reagent volumes per well for 96-well plates (Agonist)

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 clean 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 cryovial from -80°C or liquid nitrogen vapor storage and immediately place the vial in dry ice prior to thawing.

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. 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. Do not touch the bottom of the vials at any time to avoid 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.

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

6. Transfer 0.6 mL of the thawed cell suspension to the 15 mL tube containing the remaining 11.5 mL of pre-warmed AssayComplete Cell Plating Reagent. Gently mix the cells, then transfer them to a sterile disposable reagent reservoir.

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.

   Note: For orphan receptors, refer to the Supplemental Information section in this user manual.

9. Proceed to compound preparation and addition.

Section II: Compound Preparation

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

1. Reconstitute the agonist compound in the vehicle specified in the ligand datasheet (e.g., DMSO, ethanol, PBS or other) to the recommended stock concentration.

2. Prepare compound (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 compound in Cell Plating Reagent as shown in the workflow below (Figure 5).

3. The concentration of each dilution should be prepared at 11X of the final screening concentration.

    a. For each compound, label wells of a dilution plate (or vials) numbers 1 through number 12.

    b. Add 40 µL of Cell Plating Reagent to well numbers 2 through number 12. This is enough volume required for duplicate rows of wells for each concentration in a 96-well plate. The volume may be adjusted according to the number of wells desired.

    c. Prepare the highest concentration of compound in Cell Plating Reagent. We recommend preparing a final screening concentration that is **250X** the expected EC_{50} value for the compound. Therefore, prepare a working concentration that is **2750X** the expected EC_{50} per well to get a **11X** working compound concentration. Example: For an expected EC_{50} of 1 nM, prepare the highest working concentration at 2750 nM. This is 11X the screening or final top concentration of 250 nM; the expected EC_{50} will lie near the center of the dose-response curve.

    d. Add 60 µL of the highest concentration of compound to well number 1 (Figure 5).

    e. Remove 20 µL from well number 1 and add it to well number 2. Mix gently.

    f. With a clean pipette tip, remove 20 µL of diluted compound from well number 2, and add it to well number 3. Mix gently.

    g. Repeat this process until well number 11 is reached. **DO NOT add agonist compound to well number 12.** This sample serves as the no agonist control and completes the dose curve.

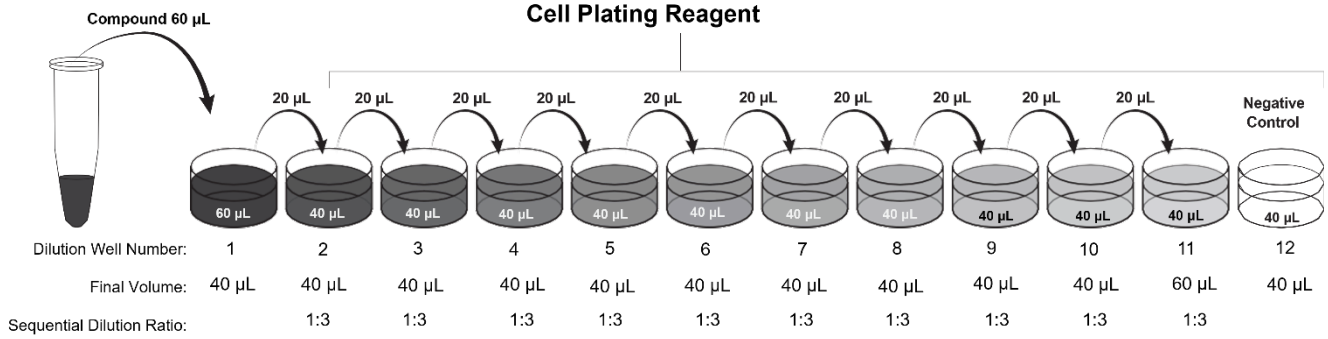


Figure 5. Compound serial dilutions - Agonist

h. Set up serial dilutions for any additional compounds in a similar manner.

i. Set compounds aside until they are ready to be added.

Section III: Compound Addition

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

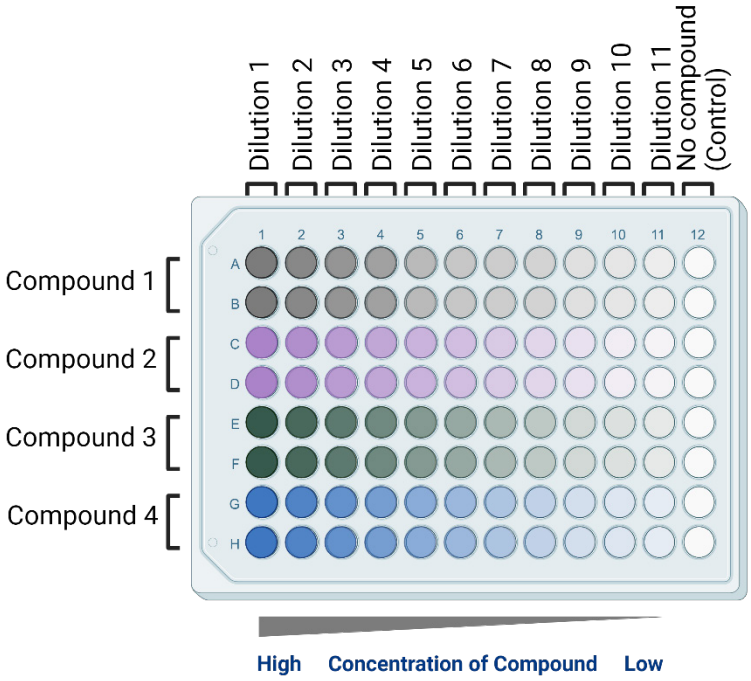


Figure 6. Compound Assay Plate Map - Agonist

Note: Create 11-point dilutions with four different compounds in duplicate.

1. Remove assay plate containing PathHunter® cells from the incubator (previously plated as described in Section I: Cell Preparation and Plating).

2. Using a 12-channel pipette, add 10 µL of each 11X agonist serial dilution in duplicate to the designated compound rows (e.g., Compound 1 in rows A and B; Compound 2 in rows C and D. Repeat for the remaining compounds as indicated on the Assay Plate Map - Agonist (Figure 6).

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

   Note: For orphan receptors, refer to the Supplemental Information section in this user manual.

Section IV: Detection Reagent Addition and Plate Reading

At this point, the agonist stimulation step has been completed. The following section contains procedures for preparing and adding the PathHunter® Detection Reagent, then 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 effect on assay performance.

Working Detection Solution for 96 Well-Plates		
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 Volume		**6.25**

Table 4. PathHunter Working Detection Solution Preparation – Agonist

Volume per Plate (mL) in the table above is recommended for a single plate run. For multi-plate runs, use volume ratios stated in the table.

1. Prepare a stock of Working Detection Solution a 15 mL polypropylene tube or reagent reservoir by mixing 19-parts of Cell Assay Buffer, 5-parts Substrate Reagent 1 and 1-part Substrate Reagent 2, as shown in Table 4.

   Use the Working Detection Solution stock within 24 hours.

2. Add 55 µL of Working Detection Solution to all wells of assay plate.

   Do not agitate or vortex plates after adding detection reagents.

3. Incubate the assay plate for 1 hour at room temperature in the dark.

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

4. Read samples on a standard luminescence plate reader at 0.1 to 1 second/ well for photomultiplier tube readers or 5 to 10 seconds for imagers. The actual signal characteristics are affected over time by lab conditions such as temperature. The user should establish an optimal read time accordingly. Luminescence readers collect signal from all wavelengths. Some instrument manufacturers may include a cut-off filter at higher wavelengths, but usually no wavelength setting is needed for luminescence readout.

   The assay plate should be read within 2 hours after adding the Working Detection Solution.

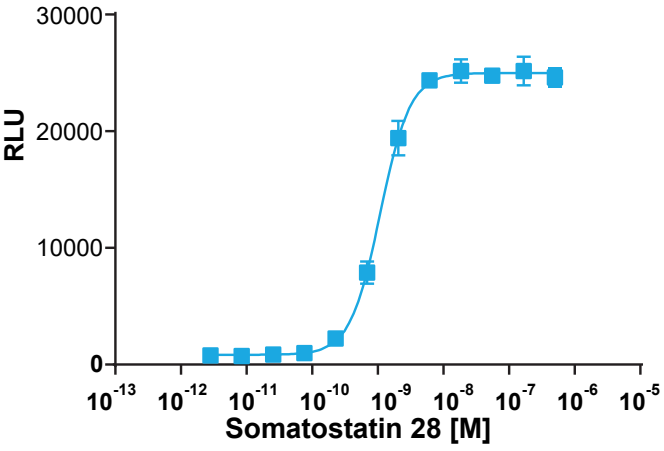
5. Data analysis can be performed using your choice of statistical analysis software (e.g., GraphPad Prism, Molecular Devices Softmax Pro, Biotek Instruments Gen5, Microsoft Excel, etc.).

Representative Data and Data Analysis

Representative data are shown below with typical results for the PathHunter® eXpress β-Arrestin GPCR Assay using the PathHunter eXpress CHO-K1 Somatostatin Receptor 2 (SSTR2) cells (A) and Glucagon-like Peptide Receptor 1 (GLP1R) cells (B).

A

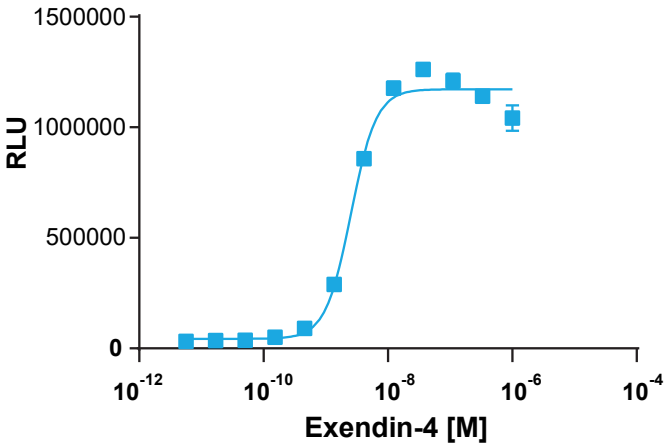
PathHunter eXpress SSTR2 CHO-K1 β-Arrestin GPCR Assay, Catalog Number 93-0181E2CP0L



EC_{50}	S/B
1.09 nM	29.7

B

PathHunter eXpress GLP1R CHO-K1 β-Arrestin GPCR Assay, Catalog Number 93-0300E2CP0L



EC_{50}	S/B
2.54 nM	37.3

Figure 7. Agonist Dose Response Curve

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
Total Assay Volume	**165**

Table 5. Assay reagent volumes per well for 96-well plates (Antagonist)

Section I: Antagonist Preparation

The following is a procedure for setting up a dilution series for an antagonist dose-response curve.

1. Reconstitute the antagonist compound in the vehicle specified in the ligand datasheet (e.g., DMSO, ethanol, PBS or other) to the recommended stock concentration.

2. Prepare an 11-point series of 3-fold antagonist (compound) serial dilutions in Cell Plating Reagent, in a separate dilution plate or vials as shown in the workflow below (Figure 8).

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

    a. For each compound, label wells of a dilution plate (or vials) numbers 1 through number 12.

    b. Add 40 µL of Cell Plating Reagent to well numbers 2 through number 12. This volume exceeds what is required for duplicate 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 compound in Cell Plating Reagent. We recommend preparing a final screening concentration that is **250X** the expected IC_{50} value for the compound. Therefore, prepare a working concentration that is **5500X** the expected IC_{50} per well to get a **22X** the working compound 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 top concentration of 250 nM and the expected IC_{50} will lie near the center of the dose-response curve.

    d. Add 60 µL of the highest concentration of antagonist compound to well number 1 (Figure 8).

e. Remove 20 µL from well number 1 and add it to well number 2. Mix gently.

f. With a clean pipette tip, remove 20 µL from well number 2, and add it to well number 3. Mix gently.

g. Repeat this process until well number 11 is reached. **DO NOT add antagonist compound to well number 12,** since this is the negative control well.

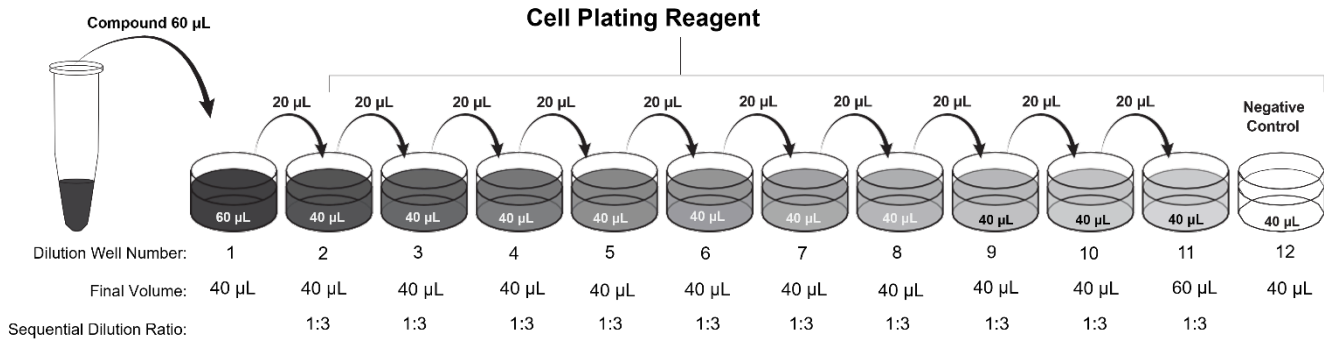


Figure 8. Compound serial dilutions - Antagonist

h. Set up serial dilutions for any additional compounds in a similar manner.

i. Set compounds aside until they are ready to be added.

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. Determine the agonist EC_{80} either by visually estimating it from the reference curve provided on the specific eXpress kit datasheet (orphan GPCR datasheets will not contain a reference curve) or experimentally following the steps below:

   a. Follow the steps in Section II of the Detailed Assay Protocol (Agonist) to generate an agonist reference curve.

   To establish a reference curve to calculate EC_{80}, run an agonist dose-response curve first.

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

   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 dosage.

3. Add equal volume aliquots of 22X agonist EC_{80} to well numbers 1 through number 12 of an empty row in a compound dilution plate.

Section III: Antagonist and Agonist EC_{80} Additions

The following is a procedure for adding the antagonist serial dilutions to generate a dose-response curve, followed by addition of agonist EC_{80} dose.

1. Remove assay plate containing PathHunter® cells from the incubator, previously plated as described in Section I: Cell Preparation and Plating under Detailed Assay Protocol (Agonist, 96-well).

2. Using a 12-channel pipette, add 5 µL of each 22X antagonist compound serial dilution in duplicate to the designated compound rows (e.g., Compound 1 in rows A and B; Compound 2 in rows C and D). Repeat for the remaining compounds as indicated on the compound assay plate map (Figure 9).

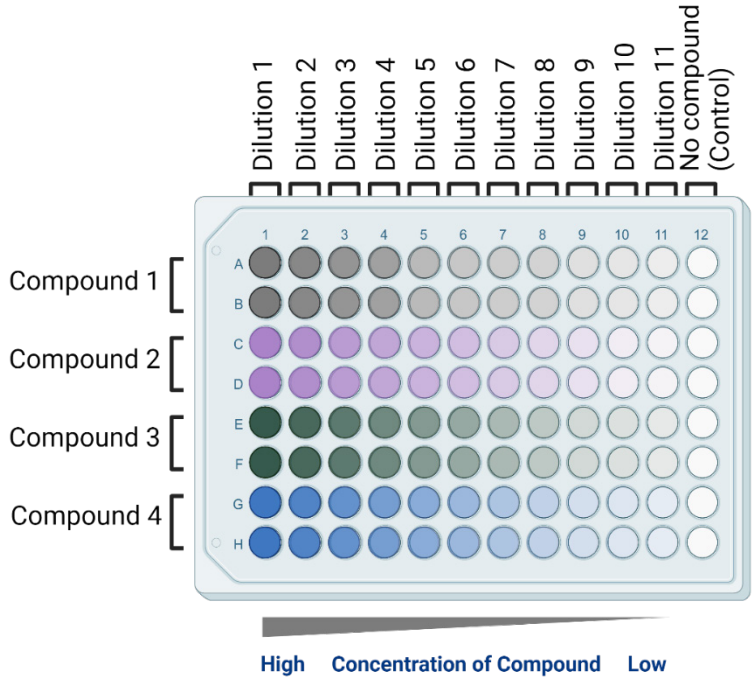


Figure 9. Compound Assay Plate Map – Antagonist

Note: Create 11-point dilution curves for four different compounds in duplicate.

2. Incubate the assay plate for 30 minutes at 37°C and 5% CO_2.

3. Add 5 µL of the 22X EC_{80} agonist dilution to each assay well.

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

   Note: For orphan receptors, refer to the Supplemental Information section in this user manual.

Section IV: Assay Detection and Plate Reading

Follow the instructions in Section IV: Detection Reagent Addition and Plate Reading under Detailed Assay Protocol (Agonist, 96-well).

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 bound by the anti-ligand antibody will be unable to bind to and activate the receptor.

The following procedure is for determining the dose-dependent inhibition of an agonist by an anti-ligand antibody in a 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	**165**

Table 6. Assay reagent volumes per well for 96-well-plates (Anti-Ligand Antibody)

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 the anti-ligand antibody serial dilution in row A of a separate dilution plate or vials (not provided in the kit) using Cell Plating Reagent, following the workflow below (Figure 10).

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

    a. For each antibody, label wells in Row A of a dilution plate (or vials) numbers 1 through number 12.

    b. Add 40 µL of Cell Plating Reagent to well numbers 2 through number 12. This volume exceeds what is required for duplicate rows of wells for each concentration in a 96-well plate. The dilution volume may be adjusted according to the number of wells desired.

    c. Prepare the highest concentration of the anti-ligand antibody 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 60 µL of the highest concentration of antibody to well number 1 (Figure 10).

    e. Remove 20 µL from well number 1 and add it to well number 2. Mix gently.

f. With a clean tip, remove 20 µL from well number 2, and add it to well number 3. Mix gently.

g. Repeat this process until well number 11 is reached. **DO NOT add antibody to well number 12**, as this is the negative control well.

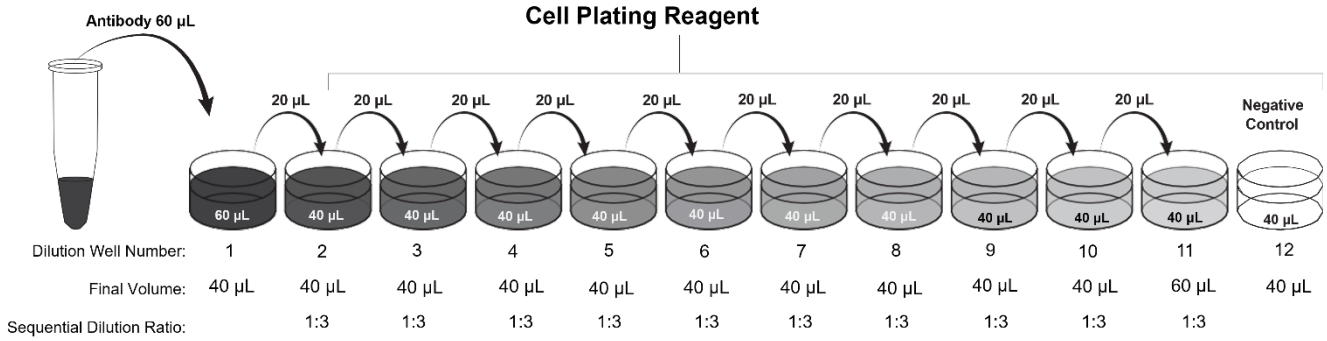


Figure 10. Compound serial dilutions – Anti-Ligand Antibody

h. Set up serial dilutions for any additional antibodies in a similar manner.

i. Set antibodies aside until they are ready to be added.

Section II: Agonist EC_{80} Challenge Preparation

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

1. First, determine the agonist EC_{80} either by visually estimating it from the reference curve provided on the specific eXpress kit datasheet (orphan GPCR datasheets will not contain a reference curve) or experimentally following the steps below:

   a. Follow the steps in Section II of the Detailed Assay Protocol (Agonist) to generate an agonist reference curve.

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

   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 dosage.

3. In Row B of the antibody dilution plate, aliquot 20 µL of the 22X agonist EC_{80} dilution into well numbers 1 through number 12.

Section III: 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.

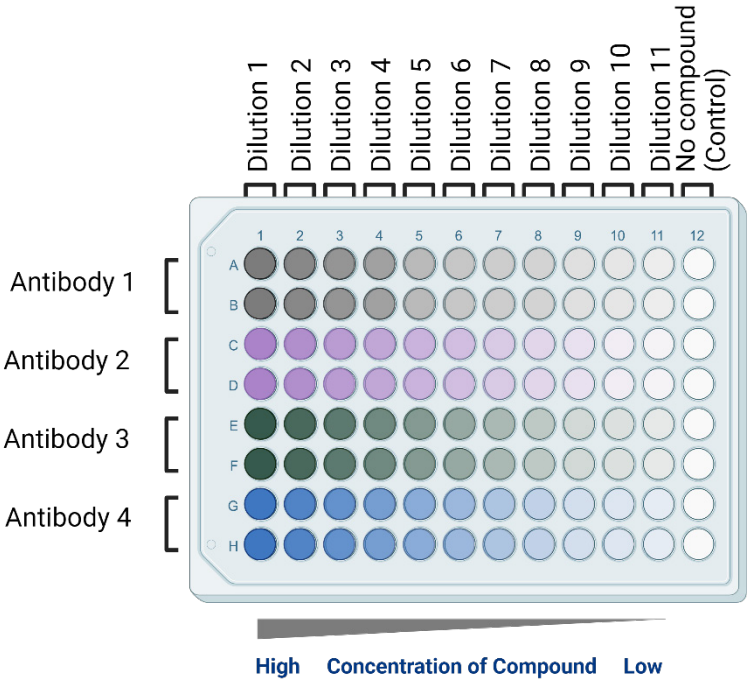


Figure 11. Assay Plate Map - Anti-Ligand Antibody

Note: Create 11-point dilution curves for four different anti-ligand antibodies in duplicate.

1. Using a 12-channel pipette, transfer 20 µL of 22X anti-ligand antibody dilution that is in well numbers 1 through number 12 of Row A to the 20 µL of agonist EC_{80} that is in well numbers 1 through number 12 of Row B 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 antibody and agonist mix for at least 15 minutes. The optimal pre-incubation time and temperature should be determined empirically.

3. Remove assay plate containing PathHunter® cells from the incubator, previously plated as described in Section I: Cell Preparation and Plating under Detailed Assay Protocol (Agonist, 96-well).

4. Using a 12-channel pipette, add 10 µL of each 11X 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 Figure 11.

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

   Note: For orphan receptors, refer to the Supplemental Information section in this user manual.

Section IV: Assay Detection and Plate Reading

Follow the instructions in Section IV: Detection Reagent Addition and Plate Reading under Detailed Assay Protocol (Agonist, 96-well).

Supplemental Information

Allosteric Modulators

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

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

Crude Biologic Samples

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

After sample treatment, it may be necessary to remove the biologic test sample from the cells and replace it with 25 µL fresh Cell Plating Reagent (or 110 µL for 96-well format), before adding 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.

Orphan GPCRs

Without knowing the reference agonist, optimal assay conditions for orphan GPCR assays are unknown. However, we suggest the following as a starting point to use until an agonist is discovered that could later be used to optimize assay conditions. Therefore, for eXpress assay cells (for orphan GPCRs), we recommend that cells be allowed to recover for 48 hours after being thawed and plated. If an agonist is discovered, then it would be possible to test if a 24-hour cell recovery is sufficient. Ligand incubation periods should start at 90 minutes at 37°C. Agonist incubation periods should generally not be less than 90 minutes. If an agonist is discovered, then incubations longer than 90 minutes and/or room temperature incubations can be tested.

Running Assay in 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
Compound (e.g., Agonist) (µL)	5
Working Detection Solution (µL)	12.5
Total Assay Volume	**37.5**

Table 7. Assay reagent volumes per well for 384-well plates (Agonist)

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	**37.5**

Table 8. Assay reagent volumes per well for 384-well plates (Antagonist)

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	**37.5**

Table 9. Assay reagent volumes per well for 384-well plates (Anti-Ligand Antibodies)

Frequently Asked Questions

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

- Determine the EC_{50} value and the Hill Slope from the agonist reference curve.
- Use an online EC_{80} calculator like QuickCalc by GraphPad (https://www.graphpad.com/quickcalcs/ECanything1/), or
- Use the formula below where F is the percent response and H is the Hill Slope from the agonist reference curve:

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

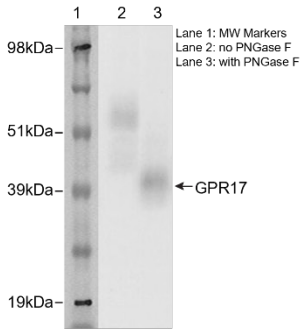
- An example of EC_{80} calculation:

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

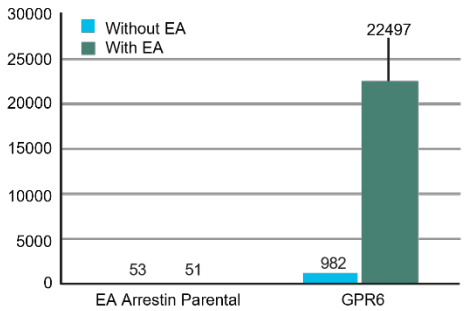
How do you characterize the orphan GPCR cell lines?

PathHunter® 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 AssayComplete™ Cell Plating Reagent



Cell lysates prepared from PathHunter ß-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 (2) resolves a band of appropriate size corresponding to GPCR-PK fusion protein and the PNGase F-treated lane 3 resolves a deglycosylated band indicative of proper expression and folding of GPCR protein.



PathHunter ß-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.

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What if there is no or low signal?

- Visually inspect the cells before and after compound incubation 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 that the cell line-specific detection reagents were used, were stored, and prepared as indicated in the datasheet.
- 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 solvents are not too high and 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 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 temperature indicated in the specific eXpress Assay datasheet.
- 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 avoid carryover.

Can the cells 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.

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 compound response?

- The β-Arrestin system is a stoichiometric system; receptor expression levels do not distort the response to ligands.

How do I use suspension cells?

- Harvest and resuspend suspension cells in Cell Plating Reagent at the optimal cell density. Typical suspension cell density is approximately 20,000 cells per well in 100 µL in a standard 96-well plate with cell viability >90%.

Can I use bacterial lysate samples?

- Yes, our assays tolerate bacterial lysates. We have tested up to 17% lysate concentrations with no change in assay performance. It is best to use E. coli strain with little or 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 I use my ligand, 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.

Do I need to use clear-bottom plates?

- We recommend using clear-bottom plates to visualize cells after plating. However, other plate formats can be used with some assay optimization.

Can these assays be run in 96-, 384-, and 1536-well formats?

- Yes. These assays can be used in high-throughput format.

What is the general liquid dispensing speed that you would recommend when using robotic dispensing instruments?

- This will depend on the particular instrument and has to be determined empirically, but in general, we would recommend a speed of 15 to 20 µL/second.

For additional information or Technical Support, see contact information at the bottom of this page.

Document Revision History

Revision Number	Date Released	Revision Details
5	June 2024	Document Revision [Eurofins DiscoverX format, minor spelling/grammar corrections] This document replaces the following User Manual: PathHunter® eXpress β-Arrestin GPCR Assay (70-255 Rev. 4)

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