User Manual

PathHunter® β-Arrestin Assay for GPCR Cell Lines

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

Document Number 70-247 Revision 6

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

Table of Contents

Table of Figures

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, in addition to being used to develop potency assays for the QC lot release testing of numerous biologic drugs. Since β-arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation.

These assays utilize stable cell lines and a simple, homogenous protocol and can easily be implemented in a high-throughput format.

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.

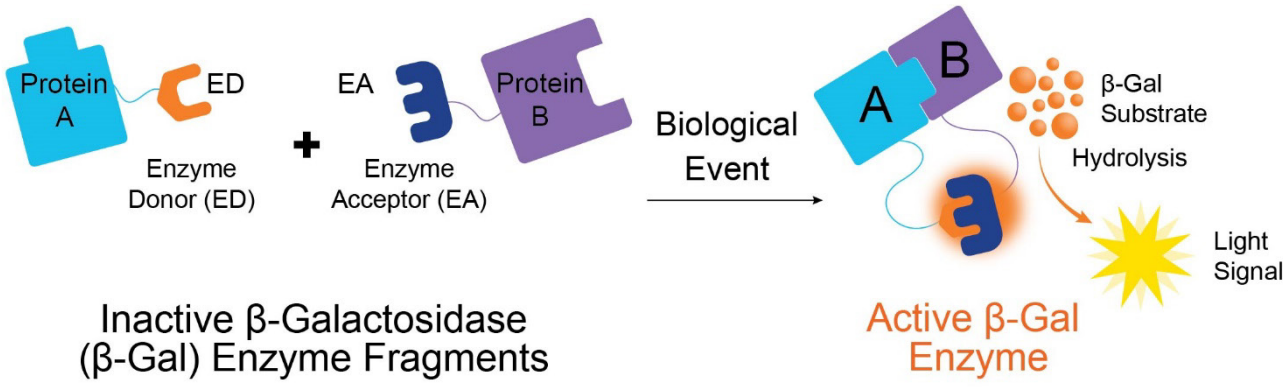


Figure 1. **Enzyme Fragment Complementation (EFC) technology**

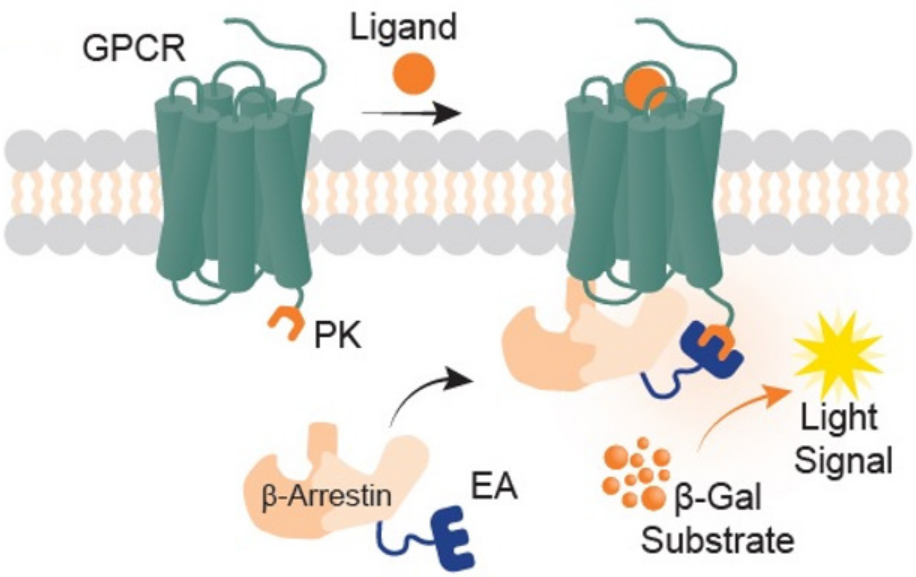


Figure 2. Assay Principle

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

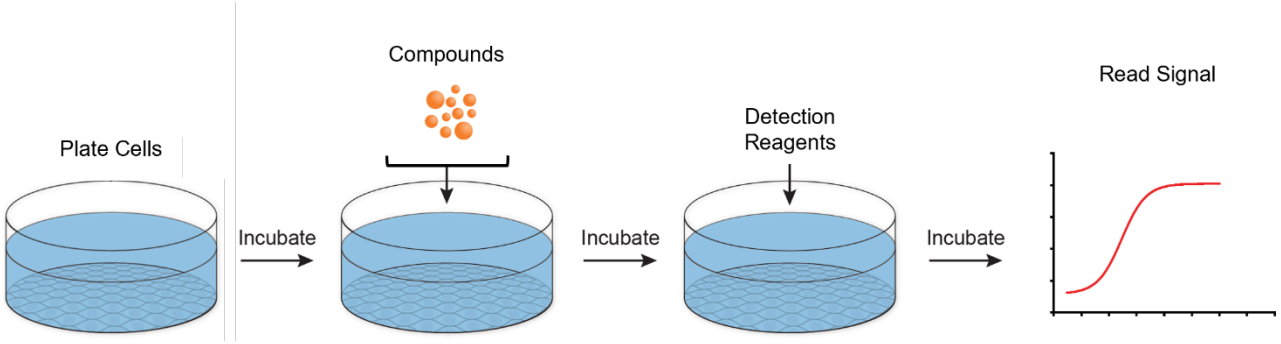


Figure 3. Assay Workflow

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 using a fresh split of low-passage cells that have not been allowed to reach confluency for more than 24 hours. 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 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

Number of Components	Configuration
2 vials	Refer to cell-line specific datasheet

Table 1. Materials Provided

Storage Conditions

Cells must arrive in a frozen state on dry ice and should be transferred to the vapor phase of liquid nitrogen storage immediately upon arrival.

If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For storage longer than 24 hours, store vials in the vapor phase of liquid nitrogen storage.

Additional Materials Required

Refer to the cell line-specific datasheet to determine the appropriate media and reagents required for the specific cell line used in the assay. Each PathHunter® β-Arrestin cell line has been validated for optimal assay performance using the recommended AssayComplete™ Cell Plating (CP) Reagent and its ligand, as indicated on the cell line datasheet.

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

Materials	Ordering Information
PathHunter® Detection Kit	**93-0001** series*
AssayComplete Cell Thawing Reagents	Refer to the cell line-specific datasheet
AssayComplete Cell Plating Reagent	Refer to the cell line-specific datasheet
AssayComplete Cell Culture Kits	Refer to the cell line-specific datasheet
AssayComplete Cell Detachment Reagent	**92-0009** (for adherent cells)
AssayComplete Cell Freezing Reagent	Refer to the cell line-specific datasheet
Selection Antibiotics	Refer to the cell line-specific datasheet
Compound Dilution Buffer (Protein Dilution Buffer)	**92-0023** series*
Trypsin EDTA, 0.25%	
Phosphate-Buffered Saline, (PBS)	
Green V-Bottom PP Ligand Dilution Plates	**92-0011**
96-well Clear bottom TC Treated, Sterile WCB, FB w/ LID	**92-0014**
384-well Clear bottom TC Treated, Sterile WCB, FB w/ LID	**92-0013**
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Cryogenic Vials for Freezing Cells	Fisher Scientific, Cat. No.375418 or similar
Multimode or Luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/tools-resources/instrument-compatibility/
Single and multi-channel micro-pipettes and pipette tips (10 µL-1,000 µL)	
50 mL and 15 mL polypropylene tubes and 1.5 mL microtubes	
Tissue Culture Disposables (Pipettes 1 mL to 25 mL) and Plastic-Ware (T25 and T75 Flasks, etc.)	
Hemocytometer	
Humidified tissue culture incubator (37° C and 5% CO_2)	

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

Table 2. Materials Required

Recommended Materials	Ordering Information
Ligands	Refer to the cell line-specific datasheet

Table 3. Recommended Materials

Unpacking Cell Cryovials

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells shipped in the vial). The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.

   Contact technical support immediately if cells are thawed upon arrival.

2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.

   Cryovials should be stored in the vapor phase. They are not rated for storage in the liquid phase of liquid nitrogen.

3. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid nitrogen inside the vial to evaporate.

   A face shield, gloves, and a lab coat should be worn during these procedures. Hold the vial by the cap. Do not touch the sides of the vial.

4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line-specific datasheet for the appropriate AssayComplete™ products indicated in the following protocols.

Cell Culture Protocol

The following procedures are for thawing adherent cells in cryovials, seeding, and expanding the cells, maintaining the cell cultures once the cells have been propagated, and preparing them for assay detection.

Care should be taken in cell handling to avoid contamination.

Cell Thawing

The following is a protocol for thawing cells in a T75 flask.

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.

2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask in a sterile tissue culture hood. Set aside for Step 6. DO NOT add selection antibiotics to the thawing reagent.

3. Remove the cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them on 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. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain, and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.

Do not leave the frozen cell vials in the water bath for longer than 1 minute. Prolonged thawing at 37ºC may result in cell death.

5. Decontaminate the external surface of the vial by spraying and wiping it with 70% ethanol and transfer it to a tissue culture hood.

6. With a pipette, gently transfer the thawed cells to the pre-filled T75 flask and incubate at 37°C and 5% CO_2.

7. Maintain the cells in culture until they are >70-80% confluent. Then proceed to Cell Propagation instructions. Do not split if cells are below this confluency or growth issues may occur.

Cell Propagation

The following is a protocol for propagating cells once they become >70-80% confluent in a T75 flask.

1. Pre-warm AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.

2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.

3. Gently aspirate media from the T75 flask.

4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire surface of the flask to rinse the cells.

5. Gently aspirate PBS from flask.

6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.

7. Gently rock the flask back and forth to ensure that the inner surface of the flask is uniformly covered with trypsin solution.

8. Incubate the flask at 37°C, 5% CO_2 for 2 to 3 minutes or until the cells have detached.

9. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the

cells have not detached, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

Prolonged treatment with Trypsin- EDTA may compromise cell viability.

10. Add 4 mL of AssayComplete™ Thawing Reagent to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the reagent.
12. Gently pipette the cell suspension up and down several times to generate a single cell suspension without any clumps.
13. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are included in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK293	1:3
U2OS	1:2

Table 4. Split Ratios for Selected Cell Backgrounds (1st Passage)

For example, for CHO-K1 cells, add 4 mL of AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5th of the total reagent in the flask) into each new tissue culture flask.

14. Add 5 mL of AssayComplete Thawing Reagent to a new T75 or T225 flask, followed by addition of the cell suspension (added volume determined in Step 13). Add an additional volume of the thawing reagent to reach a final volume of 15 mL for a T75 flask, or 45 mL for a T225 flask.
15. Transfer flask to a tissue culture incubator and incubate the cells for 24 hours at 37°C and 5% CO_2.

To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

16. After 24 hours, examine the cells under a microscope. If the cells appear healthy (adhering uniformly to the surface of the flask, with only a few cells remaining in suspension), exchange the AssayComplete Thawing Reagent with 15 mL of AssayComplete Cell Culture Reagent for a T75 flask (45 mL for T225 flasks), supplemented with the recommended concentration of selection antibiotic. Refer to the cell line-specific datasheet to determine the recommended/appropriate Cell Culture Kit, recommended selection antibiotics, and antibiotic concentration for the cell line.

Cell culture media is prepared by mixing the components provided in the cell line-specific AssayComplete™ Cell Culture Kit, and appropriate selection antibiotics. Refer to the Cell Culture Kit's datasheet for instructions on using its components.

17. Return the flask to a tissue culture incubator. If the cells do not appear to be healthy, or if confluency is <25%, incubate the flask for additional 24 to 48 hours to allow for additional cell recovery before splitting the cells.

18. Once the cells become ≥70% confluent in the flask, split the cells every 2 to 3 days, based on the doubling time of the cell line, using AssayComplete Cell Culture Reagent supplemented with the appropriate selection antibiotics. Typical split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:10
HEK 293	1:5
U2OS	1:3

Table 5. Split Ratios for Selected Cell Backgrounds (Propagation)

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g., 1-2 x 10^6 cells/vial).

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.

   Care should be taken in cell handling to avoid contamination.

2. Gently aspirate the media from the flasks.
3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).
6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.
7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
9. Add 5 mL AssayComplete™ Cell Culture Kit media to each T75 flask (or 15 mL to each T225 flask).
10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.

11. Using a pipette, transfer the cell suspension from the T75 flask into a 15 mL conical centrifuge tube. If using aT225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add an additional volume of the cell culture media to the flask (e.g., 3 mL for T75 flasks or 5 mL for T225 flasks). Rinse the flask to collect the remaining cells, then transfer it into the conical tube. Slowly pipette up and down several times to ensure that a single cell suspension is formed.

12. To determine the concentration of cells in the suspension:

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.

    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.

13. Centrifuge the collected cells at 300 X g for 4 minutes.

14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.

15. Based on the total cell number calculated in step 12 above, re-suspend cells to the desired concentration (e.g., 1-2 X 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent (as defined in the cell line-specific datasheet).

Keep cells on ice during this process to protect cell viability.

16. Make aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes. Tightly cap tubes.

17. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

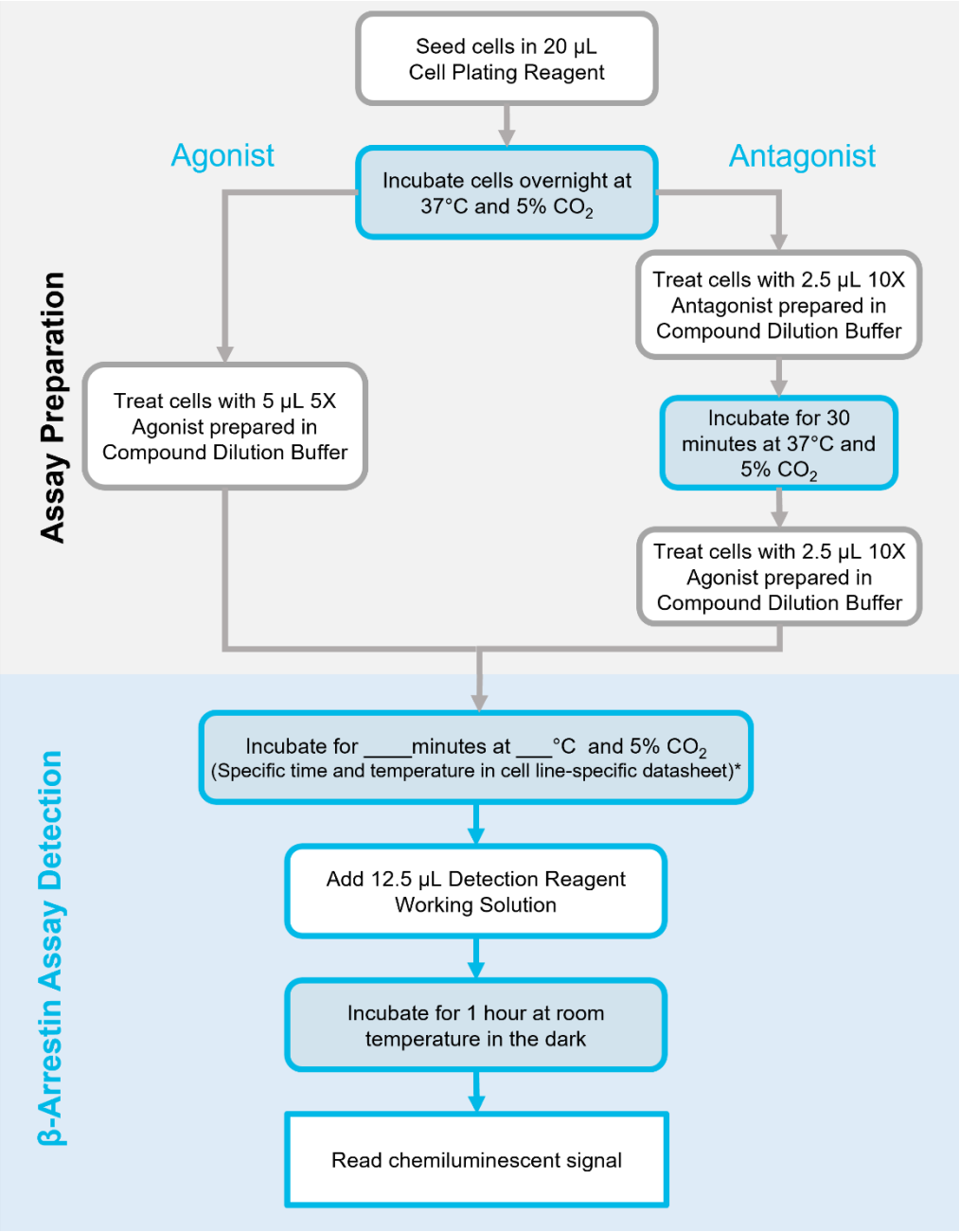
Protocol Schematic

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 flat bottomed, sterile, 384-well tissue culture treated plate, 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)

The following procedure is for determining the dose-dependent agonist response using the PathHunter® β-Arrestin Cell Lines and PathHunter Detection Reagents in a 384-well tissue culture plate. For assays to be run in a 96-well plate, refer to the table below for assay reagent volumes.

Assay Reagents (Volume per Well)	96-Well Plate	384-Well Plate
Number of Cells	Refer to the cell line-specific datasheet*	Refer to the cell line-specific datasheet
AssayComplete™ Cell Plating Reagent (µL)	90	20
Compound (e.g., Agonist) (µL)	10	5
Working Detection Solution (µL)	50	12.5
Total Assay Volume	**150**	**37.5**

* For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

Table 6. Assay reagent volumes per well for 96-well and 384-well plates (Agonist)

Section I: Cell Preparation and Plating

The following is a protocol for harvesting cells from a confluent T75 or T225 flask and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that the cells have reached a 70-80% confluency and have been cultured in the recommended cell culture media. The cell culture media is comprised of the cell line-specific AssayComplete™ Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic(s). For cell line-specific information on the appropriate AssayComplete Cell Plating Reagent, Cell Culture Kit, control ligand, selection antibiotic(s), incubation times and temperature, please refer to the cell line-specific datasheet.

1. Ensure that the cells are in the logarithmic growth phase prior to use in the assay.
2. Warm the AssayComplete Cell Plating Reagent and cell culture media in a clean 37°C water bath for 15 minutes. Refer to the cell line-specific datasheet for the recommended AssayComplete Cell Plating Reagent and cell culture media.
3. Aspirate the media from the flask.
4. Add 2 mL AssayComplete™ Cell Detachment Reagent into each T75 flask (or 3 mL to T225 flasks). Swirl to rinse the cells.

Do not use trypsin for this step; especially with assays interrogating membrane-anchored receptors (e.g., GPCRs). Use of trypsin at this step can negatively affect assay results. Additionally, it is important to rinse the cells with AssayComplete Cell Detachment Reagent. It is not recommended to use PBS to rinse cells as PBS may inhibit the detachment of cells from the plate.

5. Gently aspirate AssayComplete™ Cell Detachment Reagent from the flask.
6. Add 1 mL of fresh AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL to the T225 flasks).
7. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with AssayComplete Cell Detachment Reagent.
8. Incubate at 37°C and 5% CO_2 for 2 to 5 minutes or until the cells have detached.
9. Remove the flask from the incubator and view it under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
10. Add 4 mL AssayComplete Cell Plating Reagent to T75 flasks (or 7 mL to T225 flasks).

    Note: Refer to the datasheet to determine the correct Cell Plating Reagent for this cell line.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
12. Remove the entire amount of cells from the flask and transfer them to a 15 mL conical centrifuge tube. Pipette up/down to disperse any cell clumps.
13. To determine the concentration of cells in the suspension,

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    Keep suspended cells on ice to protect cell viability until ready for transfer to the assay plate.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or automated cell counting device.

    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.
14. Centrifuge the collected cells at 300 X g for 4 minutes.
15. After centrifugation, discard the supernatant, and re-suspend the cell pellet in AssayComplete™ Cell Plating Reagent. Based on the cell number obtained in Step 13 above, dilute the re-suspended cells to the desired concentration (e.g., 250,000 cells/mL or 5,000 cells/20 µL).
16. Transfer 20 µL/well of the cell suspension to a 384-well (or 90 µL/well to a 96-well) assay plate.
17. Incubate the assay plate overnight at 37°C and 5% CO_2.
18. Proceed to the compound preparation steps.

Section II: Compound Preparation

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

For testing antagonists or anti-ligand antibodies, see respective Detailed Assay Protocols later in this user manual.

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 an 11-point series of 3-fold agonist (compound) serial dilutions in Compound Dilution Buffer (this is equal to the 92-0023 series AssayComplete™ Protein Dilution Buffer), in a separate dilution plate or vials.

3. The concentration of each dilution should be prepared at 5X 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 Compound Dilution Buffer to well numbers 2 through number 12. This is enough volume required for four replicates for each concentration in a 384-well plate. The volume may be adjusted according to the number of wells desired.

    c. Prepare the highest concentration of compound in Compound Dilution Buffer. 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 **1250X** the expected EC_{50} per well to get a **5X** working compound concentration. Example: For an expected EC_{50} is 1 nM, prepare the highest working concentration at 1250 nM. This is 5X 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.

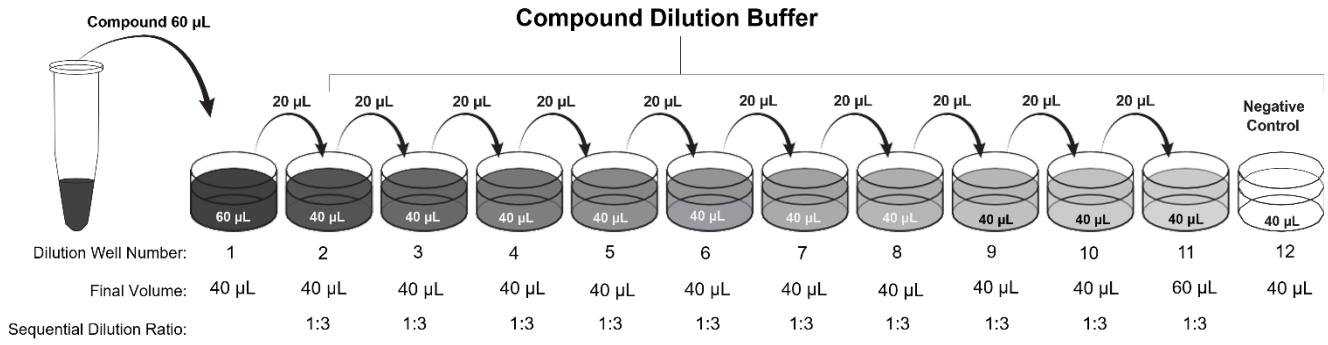


Figure 5. Compound serial dilutions - Agonist

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 dilution series to the assay plate, to generate dose-response curves.

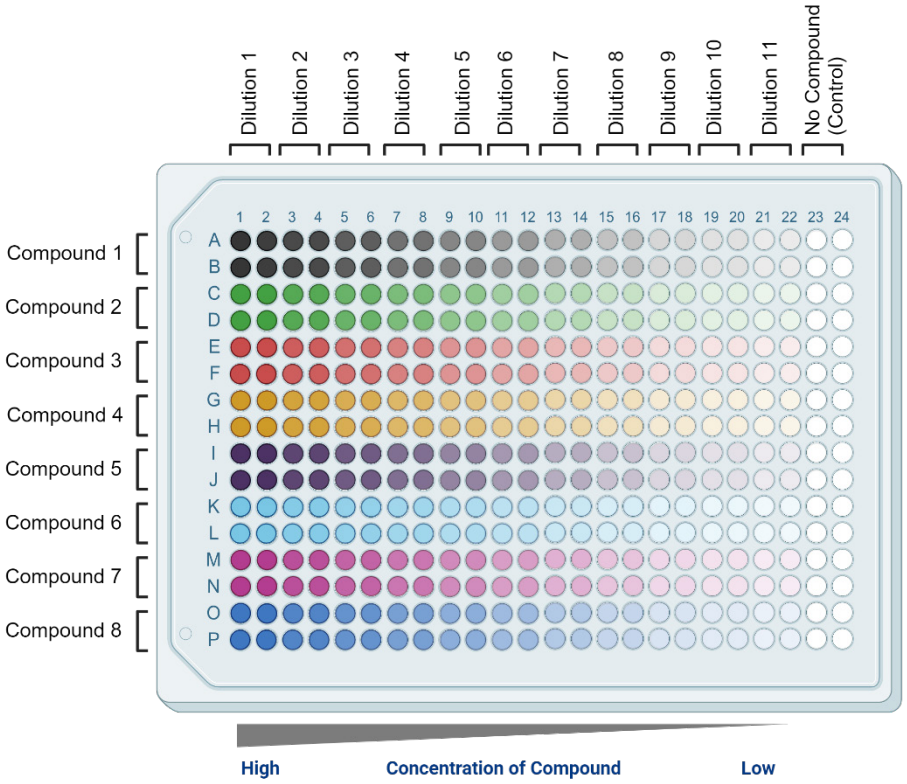


Figure 6. Compound Assay Plate Map - Agonist

Note: This plate map shows 11-point dose curves with 4 data points at each concentration. Plate map allows 8 compounds to be tested in quadruplicate per 384-well plate.

1. Remove PathHunter® β-Arrestin cells from the incubator (previously plated as described in Section I: Cell Preparation and Plating).

2. Using a 12-channel pipette, add 5 µL of each 5X compound serial dilution in quadruplicate into the designated compound rows of the assay plate (e.g., Compound 1, with Dilution 1 in rows A and B, Columns 1 and 2; Compound 2, with Dilution 1 in rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated in the Compound Assay Plate Map - Agonist (Figure 6).

3. Incubate assay plate at the indicated time and temperature for the specific cell line (refer to the specific cell line datasheet for conditions). For most cell lines, incubate 90 minutes at 37°C and 5% CO_2. For the best results, the optimal incubation time should be empirically determined.

   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 (Cat. No. 93-0001 Series), then reading the assay plate on a luminometer.

Please refer to the PathHunter β-Arrestin Cell Line-specific datasheet to determine the correct detection kit to use before proceeding with this step. If this is not the correct detection kit, then the instructions below will not apply.

Refer to specific PathHunter β-Arrestin Cell Line-specific datasheet for appropriate detection before proceeding.

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 384 Well-Plates		
Components	**Volume Ratio**	**Volume per Plate (mL)**
Cell Assay Buffer	**19**	5.7
Substrate Reagent 1	**5**	1.5
Substrate Reagent 2	**1**	0.3
Total Volume		**7.5**

Table 7. 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 (Table 7) in 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.

Use the working stock solution within 24 hours.

2. For a 384-well plate format, add 12.5 µL of Working Detection Solution (equivalent to 50% of assay volume) 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.

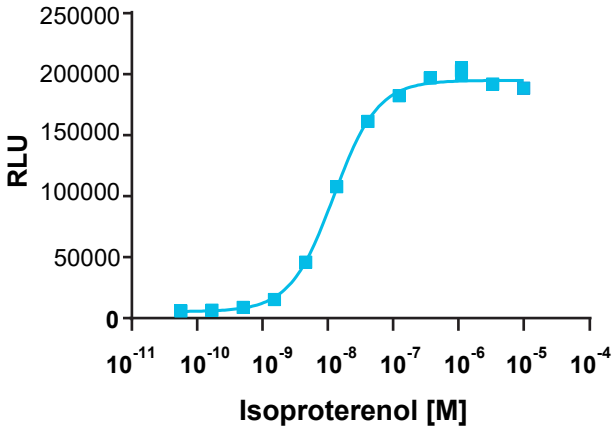
5. Data analysis can be performed using your choice of statistical analysis software (e.g., GraphPad Prism, Molecular Devices Softmax Pro, Biotek Instruments Gen5, Microsoft Excel, etc.).

Representative Data and Data Analysis

Representative data are shown below with typical results for PathHunter® β-Arrestin Assay for GPCR Cell Lines - Agonist Dose Response Curve (Figure 7) using: A) PathHunter CHO-K1 ADRB2 β-Arrestin Cell Line with the isoproterenol agonist and B) PathHunter CHO-K1 CXCR3 β-Arestin Cell Line with the CXCL11 agonist.

A

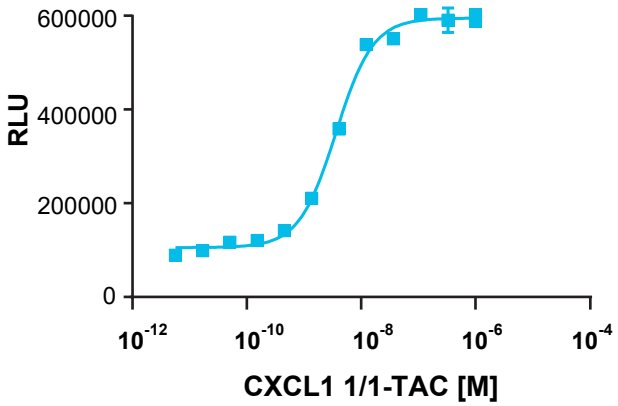
PathHunter CHO-K1 ADRB2 β-Arrestin Cell Line
Catalog Number 93-0182C2



EC_{50}	S/B
12 nM	32.3

B

PathHunter CHO-K1 CXCR3 β-Arrestin Cell Line
Catalog Number 93-0271C2



EC_{50}	S/B
38 nM	6.7

Figure 7. Agonist Dose Response Curve

Detailed Assay Protocol (Antagonist)

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 is for determining the dose-dependent antagonist inhibition in a 384-well assay plate. For running antagonist experiments in a 96-well plate, the agonist EC_{80} dose should be 20X the final screening concentration. Refer to Table 8 below for specific reagent volumes to run the assays in 96-well or a 384-well plate.

Assay Reagents (Volume per Well)	96-Well Plate	384-Well Plate
Number of Cells	Refer to cell-line specific datasheet*	Refer to cell-line specific datasheet
AssayComplete™ Cell Plating Reagent (µL)	90	20
Antagonist (µL)	5	2.5
Agonist EC_{80} (µL)	5	2.5
Working Detection Solution (µL)	50	12.5
Total Assay Volume	**150**	**37.5**

* For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

Table 8. Assay reagent volumes per well for 96-well and 384-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., DSMO, ethanol, PBS or other) to the recommended stock concentration.

2. Prepare an 11-point series of 3-fold antagonist (compound) serial dilutions in Compound Dilution Buffer, 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 10X 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 Compound Dilution Buffer to well numbers 2 through number 12. This is enough volume required for four replicates for each concentration in a 384-well plate. The dilution volume may be adjusted according to the number of wells desired.

    c. Prepare the highest concentration of compound in Compound Dilution Buffer. 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 **2500X** the expected IC_{50} per well to get a **10X** the working compound concentration. For example, for an expected IC_{50} is 1 nM, prepare the highest working concentration at 2500 nM. This is 10X 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.

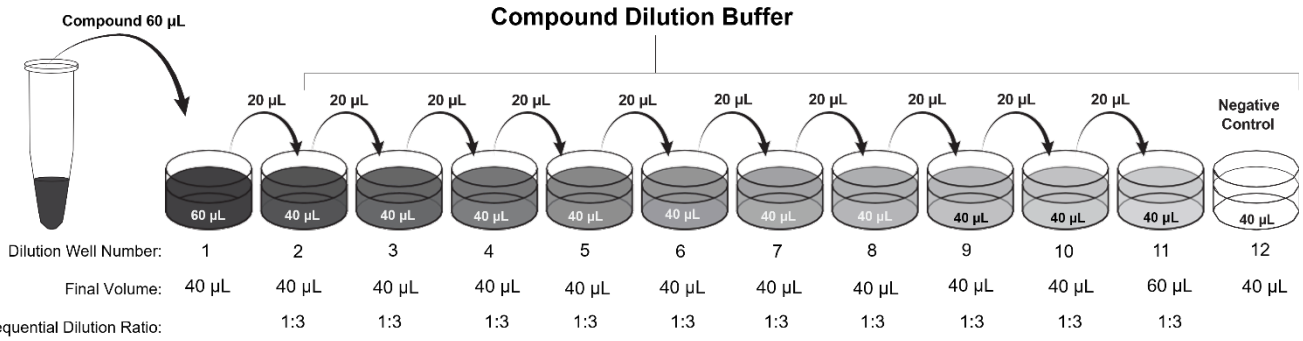


Figure 8. Compound serial dilutions - Antagonist

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} needed for the experiment.

   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 Compound Dilution Buffer in a separate tube to a concentration that is 10X the final desired agonist dosage.

3. Add equal volume aliquots of 10X agonist EC_{80} to well numbers 1 through number 12 of 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.

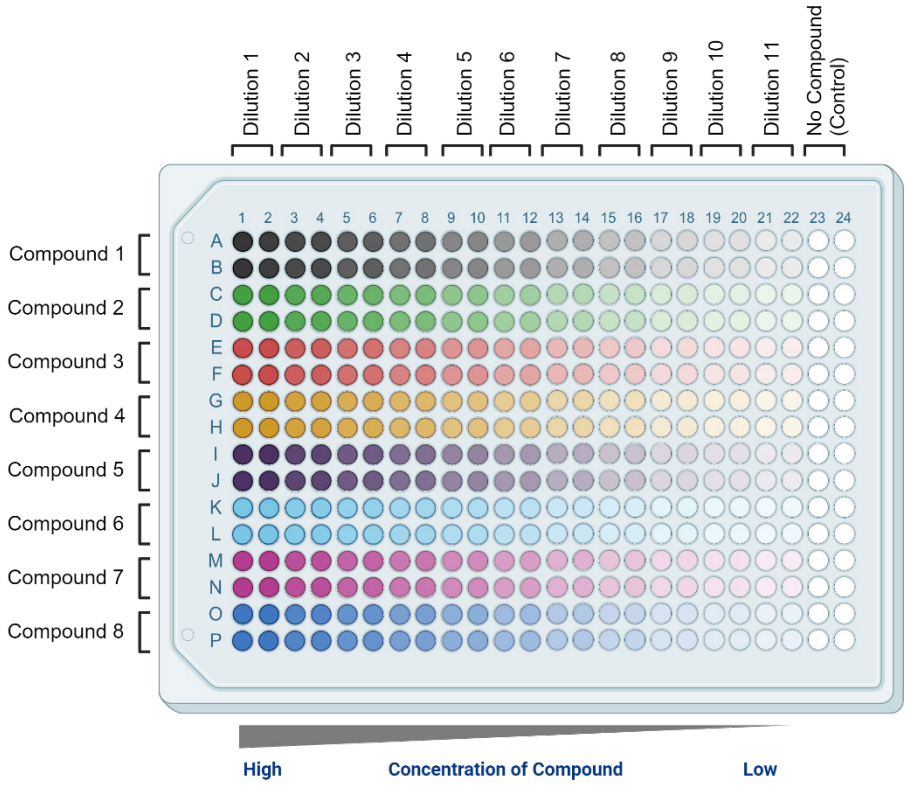


Figure 9. Compound Assay Plate Map – Antagonist

Note: Create 11-point dilution curves for eight different compounds in quadruplicate.

1. Perform Cell Preparation and Plating (Section I) in Detailed Assay Protocol (Agonist).

2. Remove assay plate containing PathHunter® cells from the incubator (previously plated as in Step 1, above).

3. Using a 12-channel pipette, add 2.5 µL of each 10X compound serial dilution in quadruplicate into the designated compound rows of the assay plate (e.g., Compound 1, with Dilution 1 in rows A and B, Columns 1 and 2; Compound 2, with Dilution 1 in rows C and D, Columns 1 and 2). Repeat

for the remaining compounds as indicated in the Compound Assay Plate Map – Antagonist (Figure 9).

4. Incubate the assay plate for 30 minutes at 37°C and 5% CO_2.
5. Add 2.5 µL of the 10X EC_{80} agonist dilution to each assay well.
6. Incubate assay plate at the indicated time and temperature (for agonist incubation) for the specific cell line (refer to the cell line- specific datasheet for incubation conditions). For most cell lines, incubate for 3 hours at 37°C and 5% CO_2. For best results, optimal incubation time should be empirically determined.

   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 Detection Reagent Addition and Plate Reading (Section IV) under Detailed Assay Protocol (Agonist).

Detailed Assay Protocol (Anti-Ligand Antibody)

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 384-well assay plate. For running the assay in a 96-well plate, refer to the table below for specific reagent volumes.

Assay Reagents (Volume per Well)	96-Well Plate	384-Well Plate
Number of Cells	Refer to cell line-specific datasheet*	Refer to cell line-specific datasheet
AssayComplete™ Cell Plating Reagent (µL)	90	20
Antibody and Agonist mix (µL)	10	5
Working Detection Solution (µL)	50	12.5
Total Assay Volume	**150**	**37.5**

*For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

Table 9. Assay reagent volumes per well for 96-well and 384-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 anti-ligand antibody serial dilutions in Protein Dilution Buffer (PDB) in a separate dilution plate or vials, following the workflow below (Figure 10).

2. The concentration of each dilution should be prepared at 10X 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 PDB to well numbers 2 through number 12. This is enough volume required for four replicates for each concentration in a 384-well plate. The dilution volume may be adjusted according to the number of wells desired.

    c. Prepare the antibody dilutions for the curve in PDB. We recommend a starting concentration of 10 µg/mL as the final top dose or approximately **250X** the antibody binding ED_{50}). Therefore, prepare a working concentration that is **10X** the final top dose in the well. For example, for an expected top dose of 10 µg/mL, prepare the highest working concentration at 100 µg/mL (or 2500X the antibody binding ED_{50}). This is 10X the screening or final top concentration of 10 µg/mL.

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 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 antibody to well number 12**, as this is the negative control well.

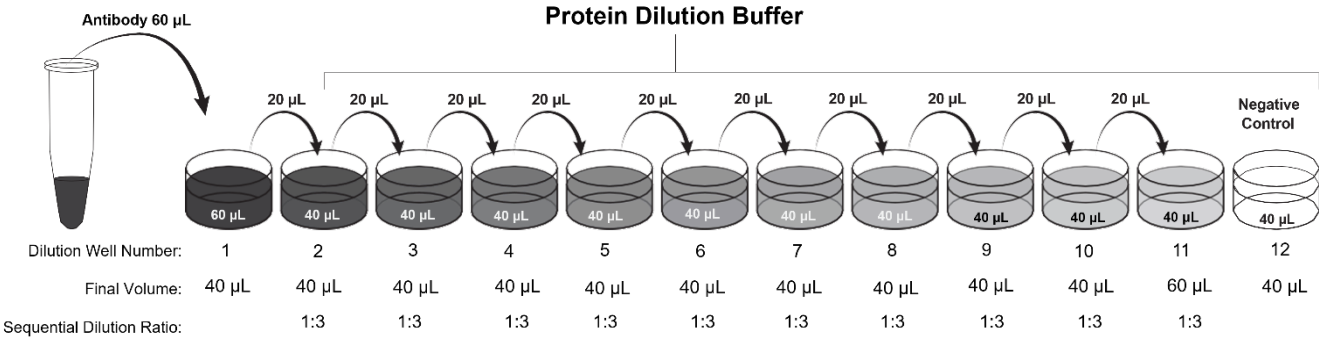


Figure 10. Compound serial dilutions – Anti-Ligand Antibody

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 dose-response curve.

1. Determine the EC_{80} concentration of the agonist needed for the experiment.

   a. To establish a reference curve to calculate EC_{80}, run an agonist dose-response curve first. Follow the steps in Section II of the Detailed Assay Protocol (Agonist) to generate an agonist reference curve. Plot the agonist response data using a variable slope sigmoidal curve.

   b. Determine EC_{50} and Hill Slope.

   c. 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 10X the final desired agonist dosage.

3. In Row B of the antibody dilution plate, aliquot 20 µL of the 10X 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 adding the mixing and pre-incubation of the anti-ligand antibody and agonist / EC_{80} dose.

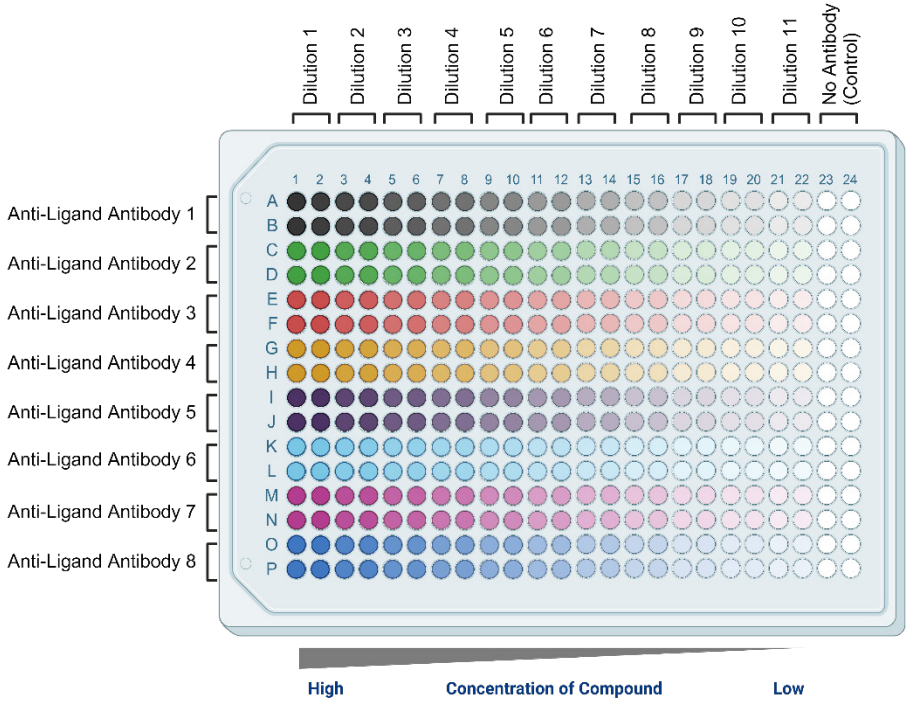


Figure 11. Assay Plate Map – Anti-Ligand Antibody

Note: Create 11-point dilution curves for eight different anti-ligand antibodies in quadruplicate.

1. Perform Cell Preparation and Plating (Section I) in Detailed Assay Protocol (Agonist).

2. Using a 12-channel pipette, transfer 20 µL of 10X 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 5X anti-ligand antibody and 5X agonist EC_{80}.

3. 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.

4. Remove assay plate containing PathHunter® cells from the incubator (previously plated as in Step 1, above).

5. Using a 12-channel pipette, add 5 µL of each 5X antibody/agonist serial dilution in quadruplicate into the designated compound rows of the assay plate (e.g., antibody/agonist 1, with Dilution 1 in rows A and B, Columns 1 and 2; antibody/agonist 2, with Dilution 1 in rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated in the Assay Plate Map – Anti-Ligand Antibody (Figure 11).

6. Incubate assay plate at the indicated time and temperature (for agonist incubation) for the specific cell line (refer to the specific cell line datasheet for conditions). For most cell lines, incubate at 90 minutes at 37°C and 5% CO_2. For best results, the optimal incubation time should be determined empirically.

   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 Detection Reagent Addition and Plate Reading (Section IV) under Detailed Assay Protocol (Agonist).

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 100 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 can suggest a starting point to use until an agonist is discovered that could later be used to optimize assay conditions. When working with a cell line, assays should be run the day after cells are plated. 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.

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:

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

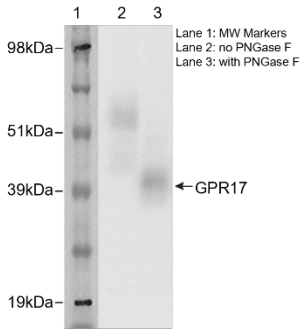
- An example of EC_{80} calculation:

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

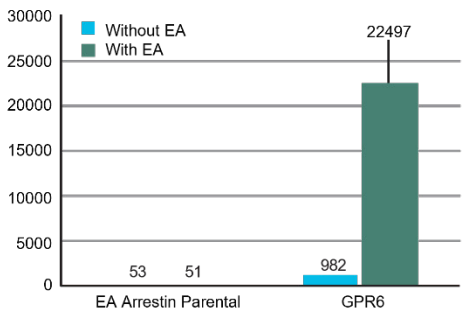
How do you characterize the orphan GPCR cell lines?

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.

What is the passage number of the frozen cells in the vials I receive when I purchase the cell line?

- The cells are shipped in passages 2 or 3, according to the cell line.

I understand that you generate your cell lines via retroviral infection as it is a very efficient system. Do the final cell lines produce any viable retroviral particles?

- No. We have confirmed the lack of retroviral particles by using a Marker Rescue Assay.

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 cell line-specific 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.

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.

Do you perform any quantitative expression analysis of your recombinant cell lines?

- No. Expression level is crucial to induce the correct response to its ligands. We select clones based on agonist response rather than the expression level.

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
6	June 2024	Document Revision [Eurofins DiscoverX format, minor spelling/grammar corrections] This document replaces the following User Manual: PathHunter® β-Arrestin Assay for GPCR Cell Lines (70-247 Rev. 5)

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