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User Manual

PathHunter® β-Arrestin Assay for GPCR Cell Lines

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

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

w discoverx.com usa 1.866.448.4864 eu +44.121.260.6142 e CustomerServiceDRX@eurofins.com

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.

Technology 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.

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

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

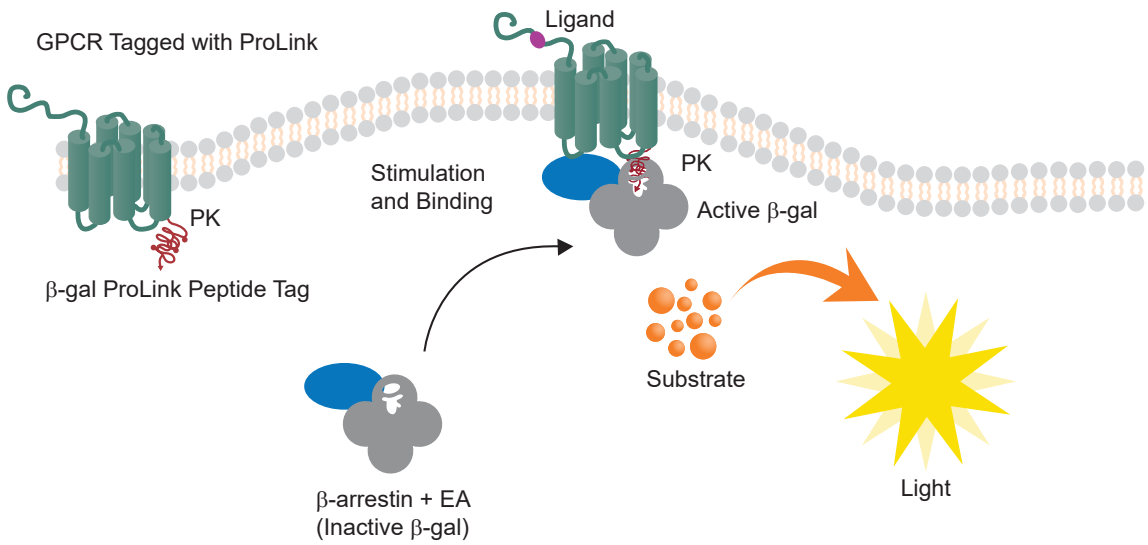


Figure 1. PathHunter β-Arrestin GPCR Assay

Materials Provided

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

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

Additional Materials Required

Refer to the cell line datasheet to determine catalog numbers for the media and reagent requirements for the specific PathHunter β-Arrestin cell line you are testing. Each PathHunter 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
AssayComplete Thawing Reagents	Refer to cell line-specific datasheet
AssayComplete Cell Plating Reagent	Refer to cell line-specific datasheet
AssayComplete Cell Culture Kits	Refer to cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Freezing Reagents	Refer to cell line-specific datasheet
Selection Antibiotics	Refer to cell line-specific datasheet
Compound Dilution Buffer (Protein Dilution Buffer: 92-0023 Series)	
Trypsin-EDTA, 0.25%	
Phosphate-Buffered Saline (PBS)	
Serial Dilution and Assay Plates	discoverx.com/microplates
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Tissue culture disposables pipettes (1 mL – 25 mL) and plasticware (T25 and T75 flasks, etc.)	
15 mL polypropylene tubes and 1.5 mL microtubes	
Single and multichannel micro-pipettors and pipette tips (10 µL – 100 µL)	
Cryogenic vials for freezing cells	
Hemocytometer	
Multimode or Luminescence reader	discoverx.com/instrument-compatibility
Humidified tissue culture incubator (37°C and 5% CO_2)	

Recommended Materials	Ordering Information
PathHunter Anti-PL/PK Antibody*	92-0010
Ligands	Refer to cell line-specific datasheet

*Recommended for PathHunter β-Arrestin Orphan GPCR cells.

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 provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for safe storage and removal of cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.
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.
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.
4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line-specific datasheet for appropriate AssayComplete products mentioned in the protocol below.

Contact technical support immediately, if cells are thawed upon arrival.

A face shield, gloves, and a lab coat should be worn during these procedures.

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

Cell Culture Protocol

The following procedures are for thawing 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 while handling cells 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 below. DO NOT add selection antibiotics to the thawing reagent.
3. Remove the cell cryovials from -80°C or liquid nitrogen 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.
5. Decontaminate the vial by spraying and wiping 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 the surface of the flask is completely covered with trypsin.
8. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm that the cells have detached by viewing them 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 the flask to the incubator for an additional 1 to 2 minutes, and repeat this step until all 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 added media.
12. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK 293	1:3
U2OS	1:2

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.

13. Fill a new T75 or T225 flask with AssayComplete Thawing Reagent until the total volume equals 12 mL for T75 flasks (or 45 mL for T225 flasks), and add cell suspension (added volume determined at Step 12) to the media in the flask. Transfer flask to a tissue culture incubator and incubate 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.
14. After 24 hours, examine the cells under a microscope. If the cells appear to be healthy (adhering to the surface of the flask with only a small number of cells remaining in suspension) exchange the AssayComplete™ Thawing Reagent with 12 mL for T75 flasks (or 45 mL for T225 flasks) cell culture media supplemented with the recommended concentration of selection antibiotic. Refer to cell line-specific datasheet to determine the correct Cell Culture Kit and the recommended antibiotic and antibiotic concentration for your cell line.

The cell culture media comprises 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.

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

16. 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 cell culture media containing 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

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 x 10^6 per vial).

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

Care should be taken in 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.

Keep cells on ice during this process and transfer to a cryogenic container.

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 cell culture media containing appropriate selection antibiotic, 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. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional cell culture media containing appropriate selection antibiotic, to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.

12. For the purpose of determining 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 x 10^6 to 1 x 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent.

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

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

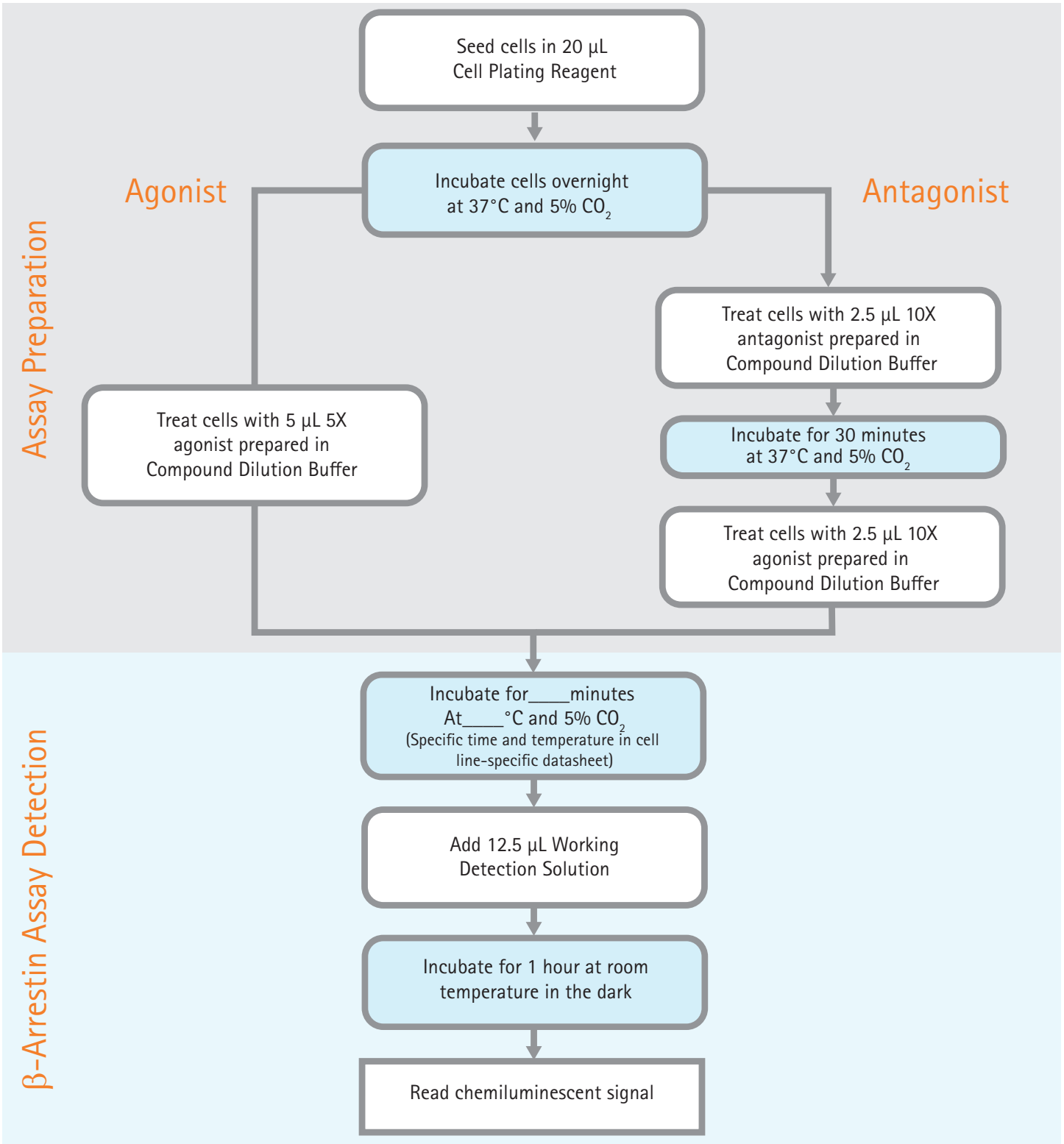
Protocol Schematic

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

Assay Name: _____ Date: _____

Product Details: _____

Quick-Start Procedure: In a 384-well tissue culture treated plate, perform the following steps.



Detailed Assay Protocol (Agonist)

The following procedure is for determining the dose-dependent agonist response 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 cell line-specific datasheet*	Refer to cell line-specific datasheet
AssayComplete™ Cell Plating Reagent (µL)	100	20
Compound (µL)	10	5
Working Detection Solution (µL)	55	12.5
Total Assay Volume (µL)	**165**	**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.

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 been cultured in their specified cell culture media. The cell culture media comprises 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.

1. Aspirate the media from the flask.
2. 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.

3. Gently aspirate AssayComplete Cell Detachment Reagent from flask.
4. Add 1 mL fresh AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL to the T225 flasks).
5. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with AssayComplete Cell Detachment Reagent .
6. Incubate at 37°C and 5% CO_2 for 2 to 5 minutes or until the cells have detached.
7. 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.
8. Add 4 mL AssayComplete Cell Plating Reagent to T75 flasks (or 7 mL to T225 flasks). Note: Refer to cell line-specific datasheet to determine the correct Cell Plating Reagent for that cell line.
9. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
10. Remove the entire amount of cells from the flask and transfer to a 15 mL conical centrifuge tube.

11. For the purpose of determining 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 other 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.

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

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

13. After centrifugation, discard the supernatant, and re-suspend the cell pellet in AssayComplete™ Cell Plating Reagent. Based on the cell number obtained in Step 11 above, dilute the resuspended cells to the desired concentration (e.g. 250,000 cells/mL or 5,000 cells/20 µL).

14. Transfer 20 µL/well of the cell suspension to a 384-well (or 100 µL/well to a 96-well) assay plate.

15. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line-specific datasheet for recommended cell incubation time.

16. Proceed to the compound preparation and addition steps.

Section II: Compound Preparation

The following is a procedure for setting up 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. Prepare an 11-point series of 3-fold agonist (compound) serial dilutions in Compound Dilution Buffer, in a separate dilution plate or vials.

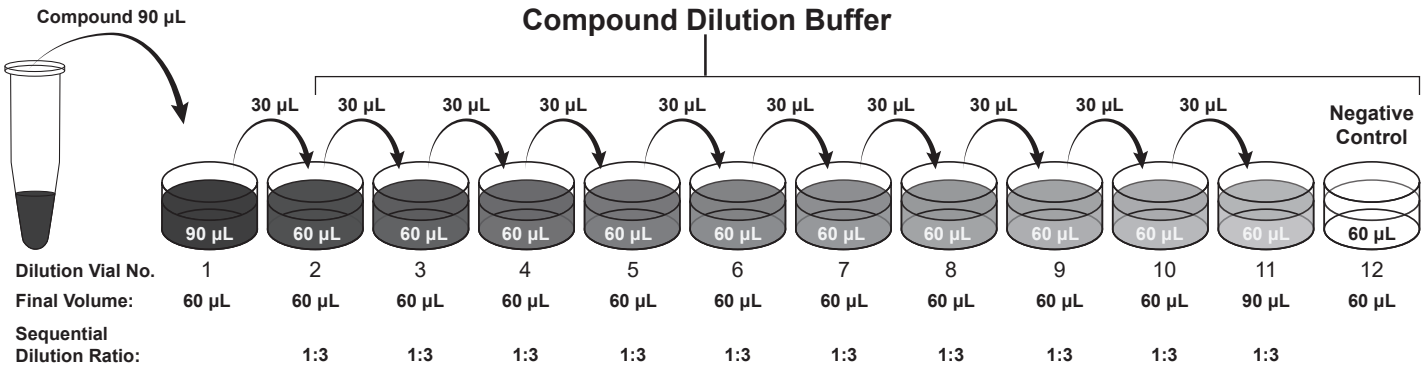
2. The concentration of each dilution should be prepared at 5X of the final screening concentration.

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

    b. Add 60 µL of Compound Dilution Buffer to dilution wells No. 2 through No. 12. This is enough volume required for four rows of wells for each concentration. 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} of the compound. Therefore, prepare a working concentration that is 1250X the expected EC_{50} per well to get a 5X working compound concentration. For example, for an expected EC_{50} of 1 nM, prepare the highest working concentration at 1250 nM. This is 5X the screening or final top concentration of 250 nM, and the expected EC_{50} will lie near the center of the dose-response curve.

d. Add 90 µL of the highest concentration of compound to well No. 1 (see figure: Compound Serial Dilutions).



Compound Serial Dilutions: Make eleven 3-fold serial dilutions of the compound in a dilution plate or vials.

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

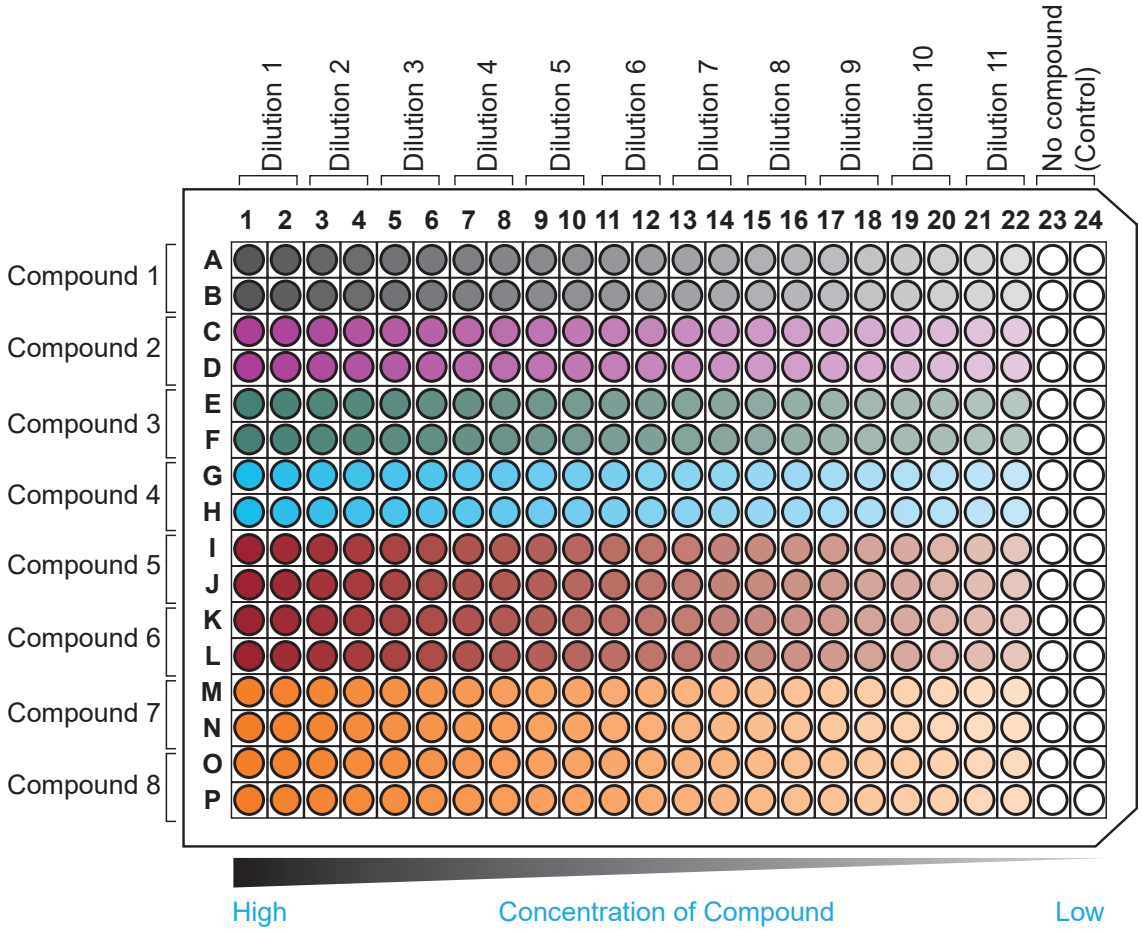
f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

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

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

Section III: Compound Addition

The following is a procedure for adding the agonist dilution series to the assay plate, to generate dose-response curves.



Agonist Assay Plate Map: Create 11-point dilution curves for eight different compounds in quadruplicate.

1. Add 5 µL of each 5X compound serial dilution in quadruplicate to the designated compound rows (e.g. compound 1 in Rows A and B, Columns 1 and 2; compound 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated in the figure: Agonist Assay Plate Map.

2. 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.

Section IV: Detection Reagent Addition and Plate Reading

At this point, the agonist stimulation step has been completed. The following section contains procedures for adding the PathHunter Detection Reagent (Cat. No. 93-0001 Series) and reading the assay plate on a luminometer. The working detection solution is unique to specific cell lines.
Please refer to the PathHunter β-Arrestin Cell Line-specific datasheet to determine the correct detection kit to use before proceeding to the next step.

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

Detection reagents must be prepared as a working solution prior to use. Once thawed, the working 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**

1. Prepare a stock of Working Detection Solution in a 15 mL polypropylene tube or reagent reservoir by mixing 19-parts of cell assay buffer, 5-parts of Substrate Reagent 1, and 1-part of Substrate Reagent 2.

Use the working stock solution within 24 hours.

2. Add 12.5 µL (for 384-well format) [equivalent to 50% of the assay volume] of Working Detection Solution to all wells of the 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.

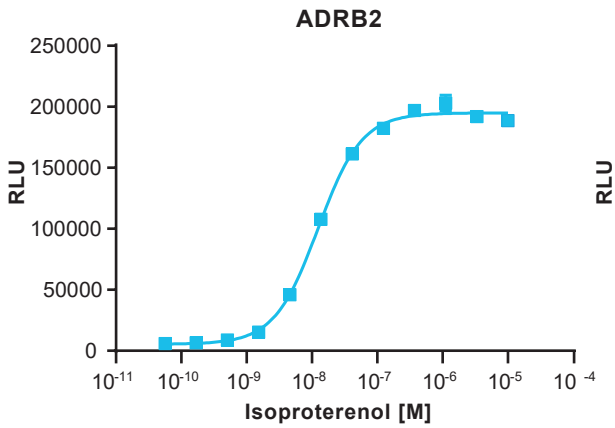
4. Read samples on a standard luminescence plate reader at 0.1 to 1 second/ well for photomultiplier tube readers or 5 to 10 seconds for imagers. The actual signal characteristics are affected over time by lab conditions such as temperature. The user should establish an optimal read time accordingly. 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 detection reagent solution.

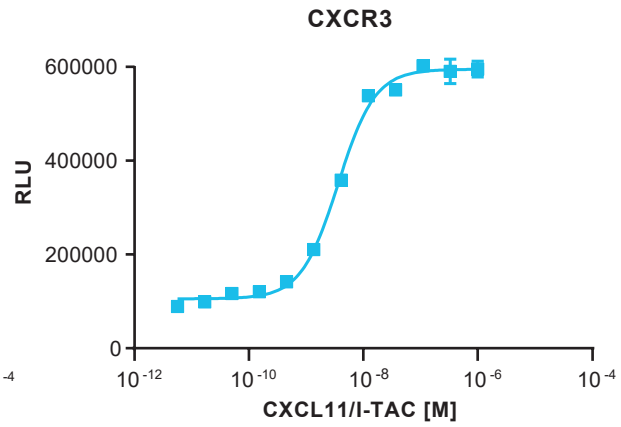
5. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, Biotek Instruments Gen5, Microsoft Excel, etc.).

Typical Results

Shown below are typical results for the PathHunter CHO-K1 ADRB2 β-Arrestin Cell Line with the isoproterenol agonist and the PathHunter CHO-K1 CXCR3 β-Arestin Cell Line with the CXCL11 agonist.



EC_{50}	S/B
12 nM	32.3



EC_{50}	S/B
38 nM	6.7

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.

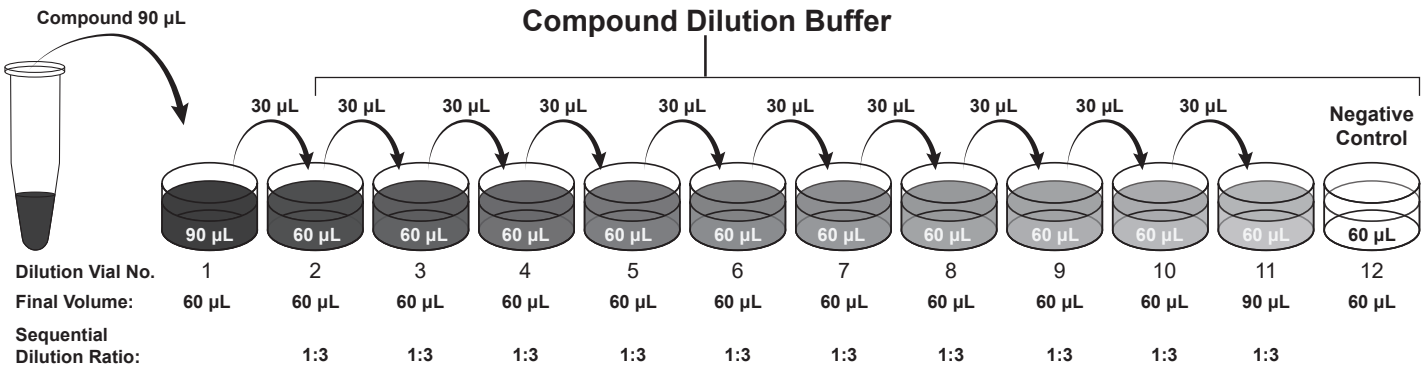
The following procedure is for determining the dose-dependent antagonist inhibition in a 384-well assay plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes. For running antagonist experiments in a 96-well plate, the agonist EC_{80} dose should be 22X the final screening concentration. Refer to the table below for specific reagent volumes to run the assays in 96-well or a 384-well plate.

Assay Reagent	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)	100	20
Antagonist (µL)	5	2.5
Agonist EC_{80} (µL)	5	2.5
Working Detection Solution (µL)	55	12.5
Total Assay Volume (µL)	**165**	**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.

Section I: Antagonist Preparation

1. Prepare an 11-point series of 3-fold antagonist (compound) serial dilutions in Compound Dilution Buffer, in a separate dilution plate or vials.
2. The concentration of each dilution should be prepared at 10X of the final screening concentration.
    a. For each compound, label the wells of a dilution plate (or polypropylene vials) No. 1 through No. 12.
    b. Add 60 µL of Compound Dilution Buffer to dilution wells No. 2 through No. 12. This volume exceeds what is required for four 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 Compound Dilution Buffer. We recommend preparing a final screening concentration that is 250X the expected IC_{50} of the compound. Therefore, prepare a working concentration that is 2500X the expected IC_{50} per well to get a 10X working compound concentration. For example, for an expected IC_{50} of 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 90 µL of the highest concentration of compound to well No. 1 (see figure: Compound Serial Dilutions).



Compound Serial Dilutions: Make eleven 3-fold serial dilutions of the compound in a dilution plate or vials.

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

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

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

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

Section II: Agonist EC_{80} Challenge Preparation

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

1. 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.

   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).

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

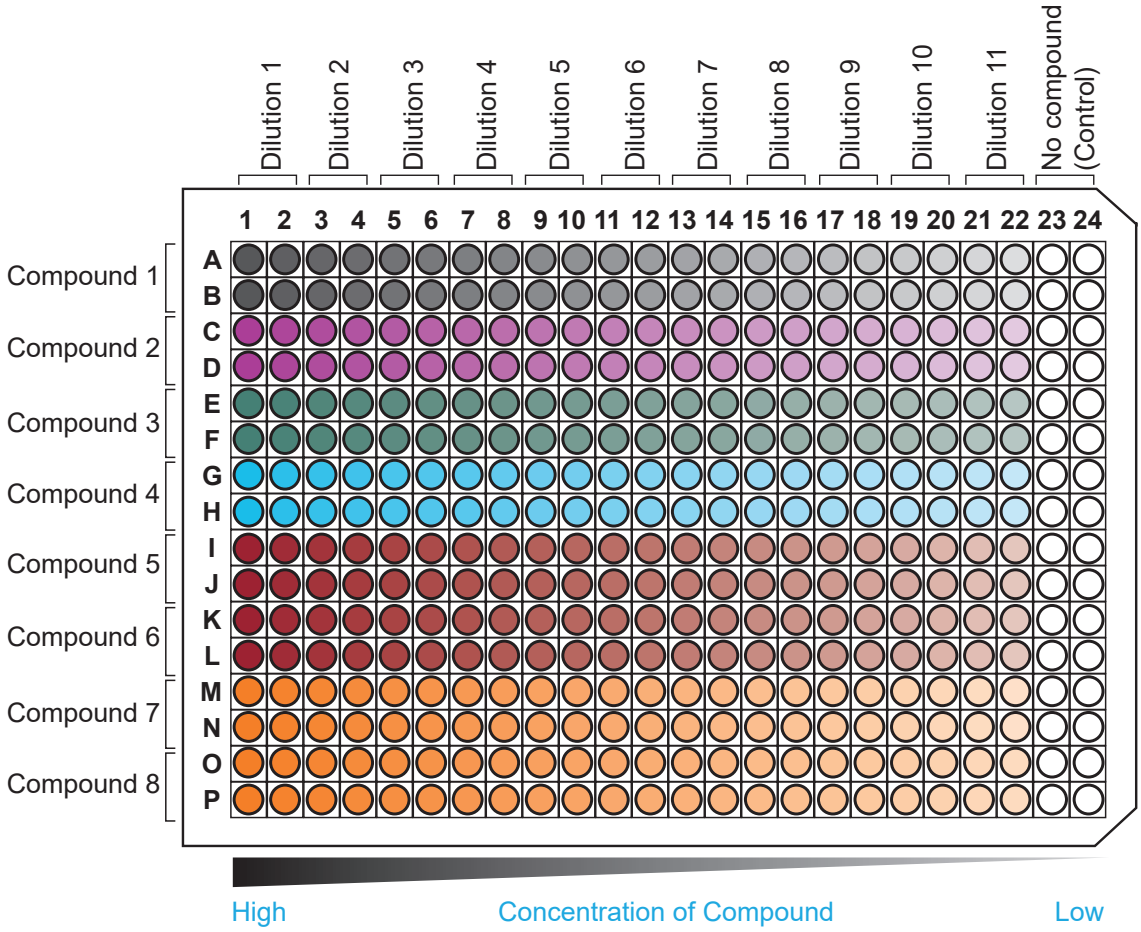
2. Prepare an agonist EC_{80} dilution in Compound Dilution Buffer in a separate tube to a concentration that is 10X the final desired agonist dosage.

3. Add equal volume aliquots of 10X agonist EC_{80} to wells No. 1 through No. 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 dose-response curve, followed by addition of the agonist EC_{80} dose.

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



Antagonist Assay Plate Map: Create 11-point dilution curves for eight different compounds in quadruplicate.

2. Add 2.5 µL of each 10X antagonist serial dilution in quadruplicate to the designated antagonist rows (e.g. antagonist 1 in Rows A and B, Columns 1 and 2; antagonist 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated in the figure: Antagonist Assay Plate Map.

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

4. Add 2.5 µL of the 10X agonist EC_{80} dilution to each assay well.

5. 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 90 minutes at 37°C and 5% CO_2. For best results, optimal incubation time should be empirically determined.

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 occupied 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 Reagent (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)	100	20
Antibody and Agonist mix (µL)	10	5
Working Detection Solution (µL)	55	12.5
Total Assay Volume	**165**	**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.

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.

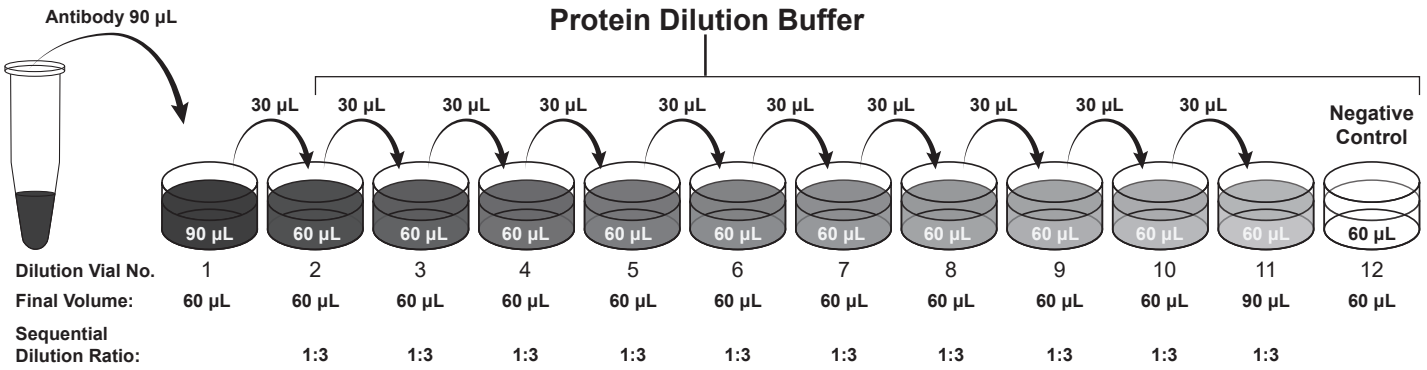
2. The concentration of each dilution should be prepared at 10X of the final screening concentration.

   a. For each antibody, label the wells in Row A of a dilution plate (or polypropylene vials) No. 1 through No. 12.

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

   c. Prepare the antibody 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 final 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 90 µL of the highest concentration of antibody to well No. 1 (see figure: Antibody Serial Dilutions).



Antibody Serial Dilutions: Make eleven 3-fold serial dilutions of the antibody in a dilution plate or vials.

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

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

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

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

Section II: Agonist EC_{80} Challenge Preparation

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

1. Determine the agonist EC_{80} 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 the Section II of 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 compound dilution plate, aliquot 30 µL of the 10X agonist EC_{80} dilution into wells No. 1 through No. 12.

Section III: Antibody/Agonist Pre-Incubation and Addition

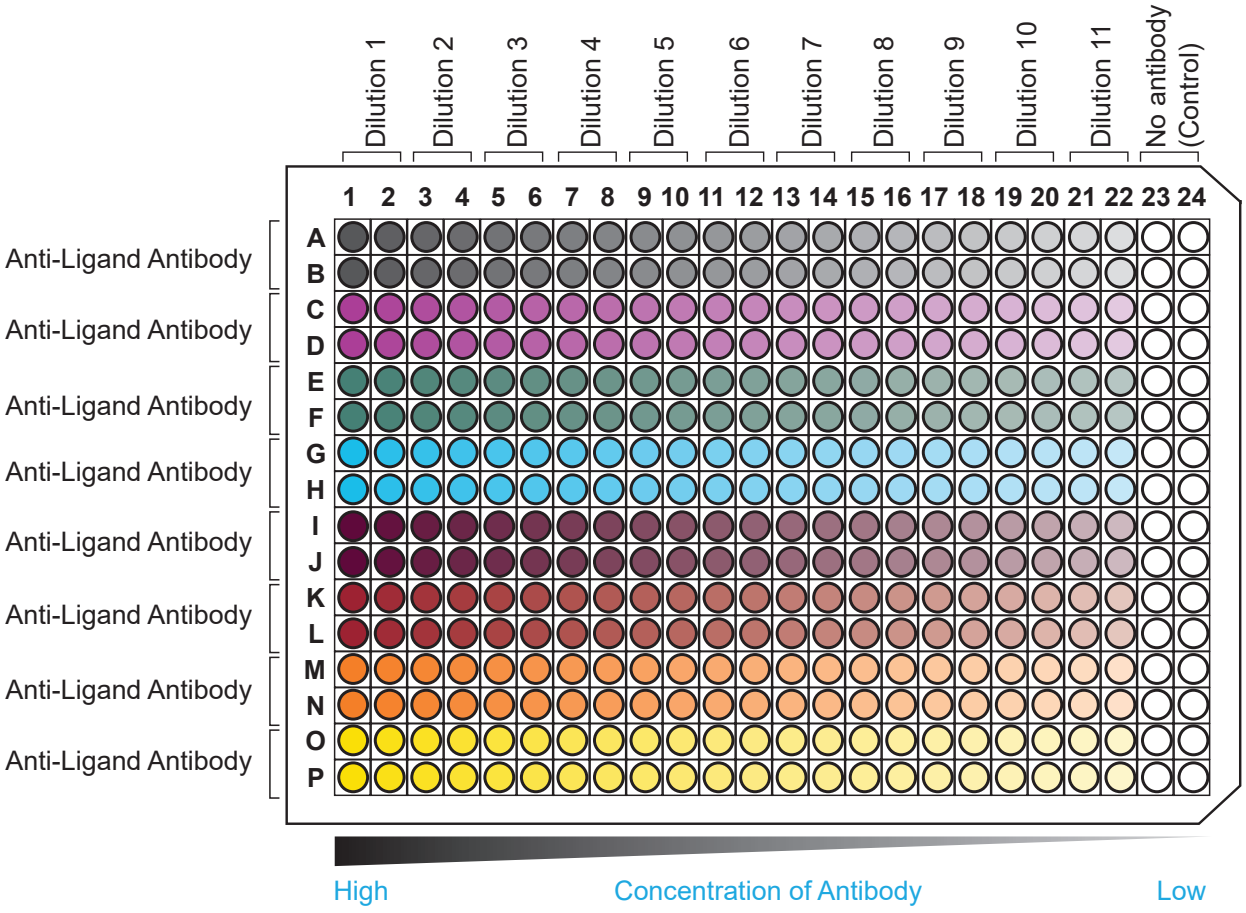
The following is a procedure for the mixing and pre-incubation of the anti-ligand antibody and agonist EC_{80} dose.

1. Using a multi-channel pipet, transfer 30 µL of 10X antibody dilution that is in wells No. 1 through No. 12 of Row A to the 30 µL of agonist EC_{80} that is in wells No. 1 through No. 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}.

2. Pre-incubate the anti-ligand and agonist mix for at least 15 minutes. The optimal pre-incubation time and temperature should be determined empirically.

3. Add 5 µL of each 5X antibody/agonist mix in quadruplicate to the designated antibody rows (e.g. antibody/agonist mix 1 in Rows A and B, Columns 1 and 2; antibody/agonist mix 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining antibody/agonist mix as indicated in the figure: Anti-Ligand Antibody Assay Plate Map.

4. 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 90 minutes at 37°C and 5% CO_2.
   For best results, the optimal incubation time should be determined empirically.



Anti-Ligand Antibody Assay Plate Map: Create 11-point dilution curves for eight different anti-ligand antibodies in quadruplicate.

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 ultimately 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.

Frequently Asked Questions

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

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

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

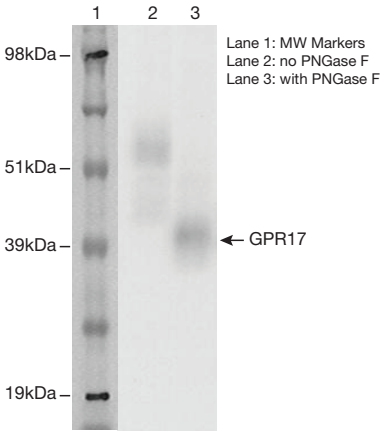
An example of EC_{80} calculation:

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

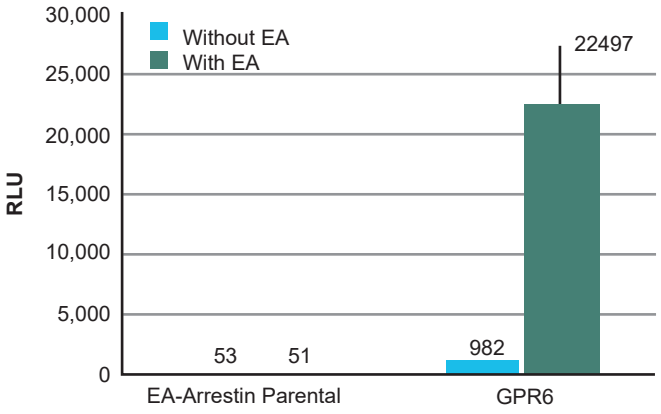
How do you characterize the orphan GPCR cell lines?

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 β-Arrestin Orphan GPCR cells were treated with PNGase F (Glyko; Cat. No. GKE-5003), run on a SDS-PAGE gel and analyzed. Alternatively, lysates can be analyzed by running a western blot using PathHunter Anti-PK/PL Antibody (DiscoverX Cat. No. 92-0010) and a commercially available secondary antibody. Untreated lane (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 β-Arrestin Orphan GPCR cells were analyzed for basal activity as well as GPCR-ProLink expression by comparing the ratio of signal between untreated cells and cells treated with saturating amounts of exogenous EA, using ProLink Detection Kit (DiscoverX Cat. No. 92-0006). Signal from complementation of ProLink and EA fragments correlates to the amount of GPCR-PK expression in the cell line.

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

- The cells are shipped at 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 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 plates 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, and 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 correctly, and ensure it is completely dissolved before use.
- Make sure DMSO and other solvent concentrations are not too high (not more than 1% final concentration).
- When testing ligands at high concentrations, make sure the vehicle is compatible.
- Make sure ligand is incubated for the specified time and at the specific 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 a cell viability >90%.

Can I use bacterial lysate samples?

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

Can 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.

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