PathHunter™ eXpress β-Arrestin Assays

Product Booklet: 93-0446E1 (Contains OCC medium, P/N 30-402)
93-0446E2 (Contains OCC2 medium, P/N 30-409)
93-0446E3 (Contains OCC3 medium, P/N 30-412)
93-0446E4 (Contains OCC4 medium, P/N 30-413)
93-0446E5 (Contains OCC5 medium, P/N 30-414)

Simple Solutions for Complex Biology

CONTENTS

ATTENTION:

Read the entire product insert prior to beginning the assay. Refer to the data sheets for additional information on cell-line specific media requirements.

For additional information or Technical Support, contact DiscoveRx or visit www.discoverx.com.

LEGAL SECTION

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For additional info or further questions regarding the rights conferred with purchase of the Materials, please contact:

Licensing Department
DiscoveRx Corporation
42501 Albrae Street
Fremont, CA 94538
t | 510.979.1415 x 104
e | info@discoverx.com

INTENDED USE

PathHunter™ eXpress β-Arrestin kits provide a robust, highly sensitive and easy-to-use method for monitoring G-protein Coupled Receptor (GPCR) activation through arrestin recruitment in live cells. The eXpress kits contain everything needed for a complete GPCR experiment including cells, detection reagents, media and plates*. The pre-validated, frozen eXpress cells have been manufactured for short term use and are provided in a convenient, ready-to-screen format that saves time and adds convenience. Assays have been designed for 96-well plate analyses.

*Test compounds are not included and must be provided by the researcher.

TECHNOLOGY PRINCIPLE

PathHunter β-Arrestin products monitor GPCR activity by detecting the interaction of arrestin with the activated GPCR using enzyme fragment complementation. In this system, the β-galactosidase (β-gal) enzyme is split into two inactive fragments (Fig. 1). The larger portion of β-gal, termed EA for enzyme acceptor, is fused to the C-terminus of β-Arrestin. The smaller, 4 kDa complementing fragment of β-gal, the ProLink™ tag, is expressed as a fusion to the C-terminus of the GPCR of interest. Upon activation, the interaction of β-Arrestin and the GPCR forces the interaction of ProLink and EA, resulting in complementation of the two fragments of β-gal and the formation of a functional enzyme capable of hydrolyzing substrate and generating a chemiluminescent signal.

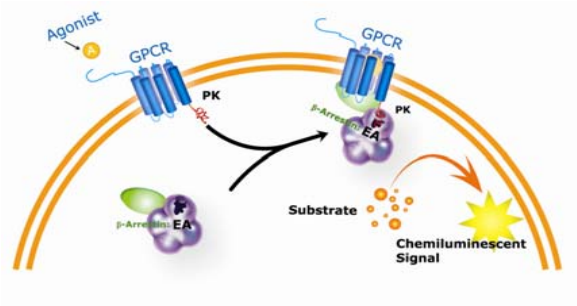


Figure 1. PathHunter β-Arrestin Assay Principle.

STORAGE CONDITIONS

PATHHUNTER EXPRESS COMPONENTS REQUIRE MULTIPLE STORAGE TEMPERATURES. OPEN BOXES IMMEDIATELY AND STORE CONTENTS AS INSTRUCTED.

BOX 1: PATHHUNTER EXPRESS CELLS: Store at -80°C*

NOTE:
PathHunter eXpress cells arrive frozen on dry ice. Cells are delivered in 2 vials each containing $1x10^6$ cells in 100 µL of freezing medium. Each vial contains sufficient cell numbers to generate (1) 96-well microplate prepared at the seeding density described.

*For short term storage (2 weeks or less), store vials at -80°C immediately upon arrival. For storage longer than 2 weeks, place vials in the vapor phase of liquid nitrogen (N_2). **Do not touch the bottom of the tubes at any time to avoid inadvertent thawing of the cells. If cells are not frozen upon arrival, do not proceed. Contact technical support.**

When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N_2 inside the vial to evaporate and proceed with the thawing protocol (p.6).

BOX 2: PATHHUNTER DETECTION REAGENT AND OCC MEDIA: Store at -20°C

NOTE:
Reagents can be frozen and thawed at least 2 times without affecting performance.

BOX 3: 96-WELL TISSUE CULTURE TREATED PLATES: Store at Room Temperature

MATERIALS PROVIDED

Each Shipment Contains:

Description	Contents	Storage
Box 1: PathHunter™ eXpress β-Arrestin Cells	2 vials $1x10^6$ cells each	-80°C (short term) Liquid N_2 (long term)
Box 2: PathHunter Detection Reagents - Cell Assay Buffer - Substrate Reagent 1 - Substrate Reagent 2* Optimized Cell Culture Media±	 200 data points 9.5 mL 2.5 mL 0.5 mL 2 X 15 mL	 -20°C
Box 3: 96-well Tissue Culture Treated Plates	2 plates	Room Temperature

***Centrifuge vial before opening to maximize recovery.**
±Refer to cell-line specific data sheets for optimized cell culture media included with each kit.

MATERIALS NOT PROVIDED

The following additional materials are required but not provided:

• Tissue culture disposables. • Disposable Reagent Reservoir (such as Thermo Scientific, Cat. #8094).	• GPCR control agonist. • GPCR test compound(s). • Multi-mode or luminescence plate reader.

MEDIA REQUIREMENTS

Each PathHunter eXpress β-Arrestin Assay has been validated for optimal assay performance using the specific OCC media included in the kit. **Always use the media included in the kit and DO NOT substitute media from an alternate kit at any time.**

For PathHunter eXpress β-Arrestin Assay catalog numbers ending in:
C1: use OCC Medium (P/N 30-402).
C2: use OCC2 Medium (P/N 30-409).
C3: use OCC3 Medium (P/N 30-412).
C4: use OCC4 Medium (P/N 30-413).
C5: use OCC5 Medium (P/N 30-414).

ASSAY PROCEDURE - AGONIST DOSE RESPONSE

The following steps outline the procedure for performing an Agonist assay using the PathHunter eXpress β-Arrestin cells and PathHunter Detection Reagents. Although plate layouts and experimental designs will vary, we recommend performing an 11-point dose curve using at least duplicate wells for each dilution. Refer to the plate map on p.14 for more details.

NOTE:
Solvents can affect assay performance. PathHunter eXpress assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). If you use other solvents or solvent concentrations, optimize the assay conditions accordingly.

DAY 1: THAWING AND PLATING FROZEN CELLS

The following are procedures for thawing and plating frozen PathHunter eXpress cells from freezer vials:

1. Pre-warm OCC medium in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor plate storage and place immediately on dry ice prior to thawing. **DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.**

   NOTE:
   When removing cryovials from liquid N_2, place immediately on dry ice in a covered container. Wait at least one minute before opening for any liquid N_2 inside the vial to evaporate.

3. Place the cell vial(s) ***briefly*** (10 seconds to 1 min) in a 37°C water bath until only small ice crystals remain and the cell pellet(s) is almost completely thawed.
4. Add 0.5 mL of pre-warmed OCC medium to the cell vial. Pipette up and down gently several times to ensure that the cells are evenly distributed.
5. Immediately transfer the cells to 11.5 mL of pre-warmed OCC medium and pour into a disposable reagent reservoir.
6. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
7. After seeding the cells into the microplate, place it into a 37°C, 5% CO_2 in a humidified incubator for 48 hours prior to testing.

DAY 3: COMPOUND ADDITION

1. Dissolve agonist compound in the vehicle of choice (DMSO, Ethanol, water or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of agonist compound in OCC medium containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **11X** of the final screening concentration (i.e. 10 µL compound + 100 µL of cells). For each dilution, the final concentration of solvent should remain constant.

   Guidelines for preparation of 11-point dose curve serial dilutions:
   - Label tubes 1 through 12.
   - Prepare a working concentration of agonist compound in appropriate OCC medium.

     NOTE:
     We recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (**550X** the final screening concentration).
   - Add 90 µl of the working concentration of agonist compound to tube #1.
   - Add 60 µL of OCC medium to subsequent tubes.
   - Remove 30 µL of diluted compound from tube #1, add it to the second tube and mix. Label this as tube #2.
   - Remove 30 µL of diluted compound from tube #2, add it to the third tube and mix. Label this as tube #3.
   - Repeat this process 8 more times.
   - **DO NOT** add agonist compound to tube #12. Add only appropriate OCC medium containing solvent. This sample serves as the no agonist control and completes the dose curve.
   - Repeat process when testing additional compounds.
3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 10 µL from tubes 1-12 to each well according to the plate map on p.14.
5. Incubate for 90 minutes @ 37°C.

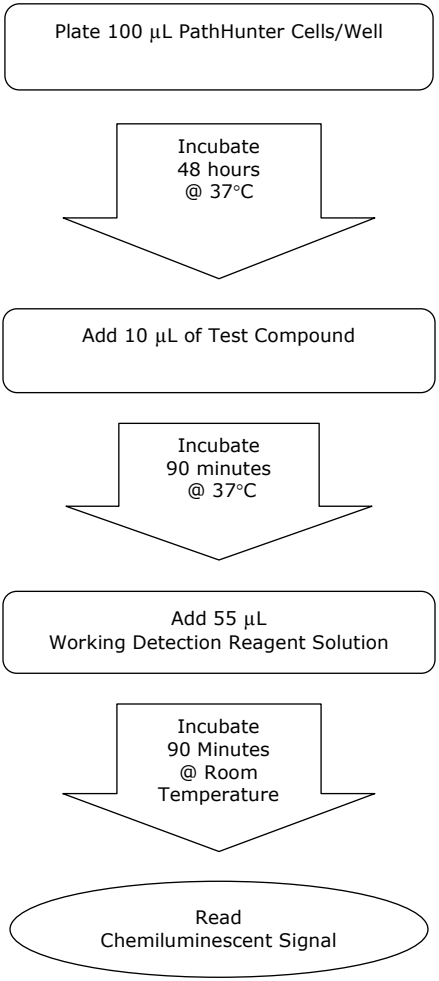
SUBSTRATE PREPARATION AND ADDITION

6. During the incubation period, prepare a working solution of the detection reagents for each plate by mixing the following reagents:
   - Cell Assay Buffer 4.75 mL (19 parts)
   - Substrate Reagent 1 1.25 mL (5 parts)
   - Substrate Reagent 2 0.25 mL (1 part)

   NOTE:
   The working solution is stable for up to 8 hours at Room Temperature.

7. Add 55 µL of prepared detection reagent per well and incubate for 90 minutes at room temperature (23°C).
   DO NOT pipette up and down in the well to mix or vortex/shake plates.
8. Read samples on any standard luminescence plate reader.
9. Use GraphPad Prism® or other comparable program to plot your agonist dose response.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



ASSAY PROCEDURE — ANTAGONIST DOSE RESPONSE

The following steps outline the procedure for performing an Antagonist assay using the PathHunter eXpress β-Arrestin cells and PathHunter Detection Reagents. Although plate layouts and experimental designs will vary, we recommend performing a 10-point dose curve using at least duplicate wells for each dilution. Refer to the plate map on p.14 for more details.

NOTE:
Solvents can affect assay performance. PathHunter eXpress assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). If you use other solvents or solvent concentrations, optimize the assay conditions accordingly.

DAY 1: THAWING AND PLATING FROZEN CELLS

The following are procedures for thawing and plating frozen PathHunter eXpress cells from freezer vials:

1. Pre-warm appropriate OCC medium in a 37°C water bath.
2. Remove cell vial(s) from -80°C or liquid N_2 vapor plate storage and place immediately on dry ice prior to thawing.
   DO NOT EXPOSE VIALS TO ROOM TEMPERATURE.
   NOTE:
   When removing cryovials from liquid N_2, place immediately on dry ice in a covered container. Wait least one minute before opening for any liquid N_2 inside the vial to evaporate.
3. Add 0.5 mL of pre-warmed OCC medium to the frozen cell pellet. Pipette up and down gently several times to ensure that cells are evenly distributed.
4. Immediately transfer the cells to 11.5 mL of OCC medium and pour into a disposable reagent reservoir.
5. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.
6. After seeding the cells into the microplate, place it into a 37°C, 5% CO_2 in a humidified incubator for 48 hours before testing.

DAY 3: COMPOUND ADDITION

1. Dissolve antagonist compound in the vehicle of choice (DMSO, Ethanol, water or other) at the desired concentration.
2. Prepare 3-fold serial dilutions of antagonist compound in appropriate OCC medium containing the appropriate solvent (DMSO, ethanol, PBS or other). The concentration of each dilution should be prepared at **22X** of the final screening concentration (i.e. 5 µL antagonist compound will be used in a final volume of 110 µL). For each dilution, the final concentration of solvent should remain constant.

   Guidelines for preparation of 10-point dose curve serial dilutions:
   - Label tubes 1 through 11.
   - Prepare a working concentration of antagonist compound in OCC medium.
     NOTE:
     We recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (**1100X** the final screening concentration).

- Add 90 µl of the working concentration of antagonist compound to tube #1.
- Add 60 µL of OCC medium to subsequent tubes.
- Remove 30 µL of diluted compound from tube #1, add it to the second tube and mix. Label this as tube #2.
- Remove 30 µL of diluted compound from tube #2, add it to the third tube and mix. Label this as tube #3.
- Repeat this process 7 more times.
- **DO NOT** add antagonist compound to tube #11. Add only appropriate OCC media containing solvent. This sample serves as the no antagonist control and completes the dose curve.
- Repeat process when testing additional compounds.

3. Remove PathHunter eXpress cells (previously plated on day 1) from the incubator.
4. Transfer 5 µL from tubes 1-11 to each well according to the plate map on p.14. In addition, add 5 µL of OCC medium to the wells in column 1.
5. Incubate for 60 minutes @ 37°C.
6. Determine the EC_{80} concentration of the agonist from the agonist dose response curve (described on p. 6).
7. Prepare the agonist compound in OCC medium containing the appropriate solvent (DMSO, ethanol, PBS or other) at **22X** of the final screening concentration (i.e. 5 µL agonist compound will be used in a final volume of 110 µL).
8. Add 5 µL of agonist compound to each well. Add 5 µL of OCC medium to the no antagonist/no agonist wells (columns 1).
9. Incubate for 90 minutes @ 37°C.

SUBSTRATE PREPARATION AND ADDITION

10. During the incubation period, prepare a working solution of the detection reagents for each plate by mixing the following reagents:
    - Cell Assay Buffer 4.75 mL (19 parts)
    - Substrate Reagent 1 1.25 mL (5 parts)
    - Substrate Reagent 2 0.25 mL (1 part)

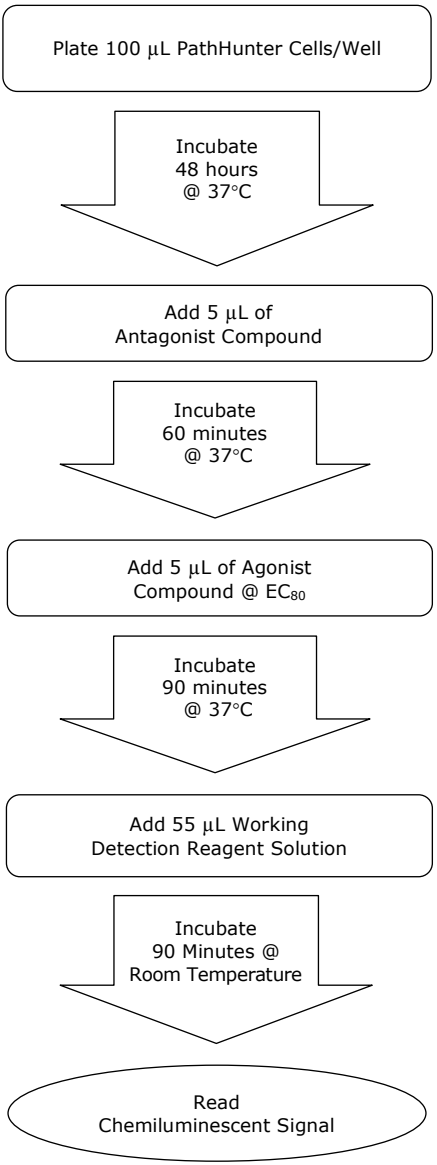
    NOTE:
    The working solution is stable for up to 8 hours at Room Temperature.

11. Add 55 µL of prepared detection reagent per well and incubate for 90 minutes at room temperature (23°C) in the dark.
    DO NOT pipette up and down in the well to mix or vortex/shake plates.

12. Read samples on any standard luminescence plate reader.

13. Use GraphPad Prism® or other comparable program to plot your antagonist dose response.

QUICK-START PROCEDURE : ANTAGONIST DOSE RESPONSE



FREQUENTLY ASK QUESTIONS

Q: I did not see a signal with my control agonist.
A: There may be differences in agonist purchased from different vendors. Confirm that the control agonist used is the same ligand used in the dose response shown in the provided cell-specific data sheet.

Q: I did not see a response with my compound.
A1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the agonist/antagonist diluent at ≤ 1%.
A2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
A3: Confirm that the cell line responds to the control agonist.
A4: Repeat the experiment using a new lot of control agonist.

Q: My cells arrived thawed. Can I use them?
A: No. Call technical support for a replacement.

Q: How long is the prepared detection reagent good for?
A: The working detection reagent solution must be used within 8 hours of mixing.

Q: What instruments can I use to read the plates?
A: Any bench top luminometer will work with the PathHunter™ eXpress β-Arrestin Assays.

Q: How long is the signal stable for?
A: The signal is stable for 5 hours after addition of detection reagent.

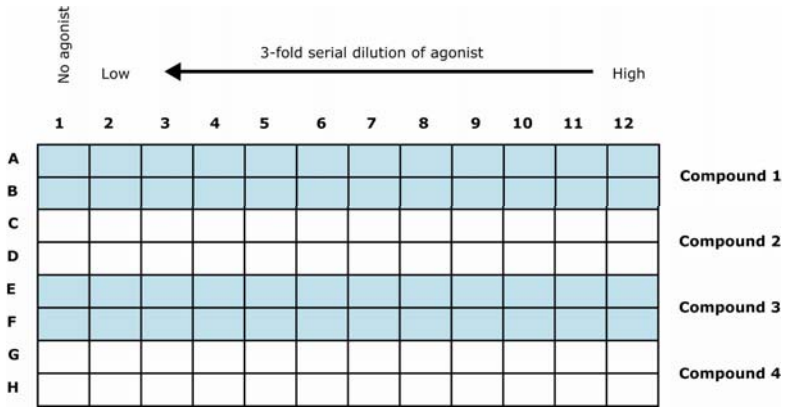
Q: My cells are floating after the 48 hours incubation.
A: The cells are not viable, contact technical support for a replacement.

Q: Can I switch plates or should I use the plate provided?
A: You can use any clear bottom white or opaque walled plate.

Q: How do I generate a dose curve for my agonist compound?
A: Prepare eleven serial 3-fold dilutions of the agonist compound in the desired vehicle of choice. Prepare agonist dilutions such that the concentration is **11X** of the final screening concentration (10 µl agonist + 100 µl cells). Pipette all compound dilutions following the plate map on page 14.

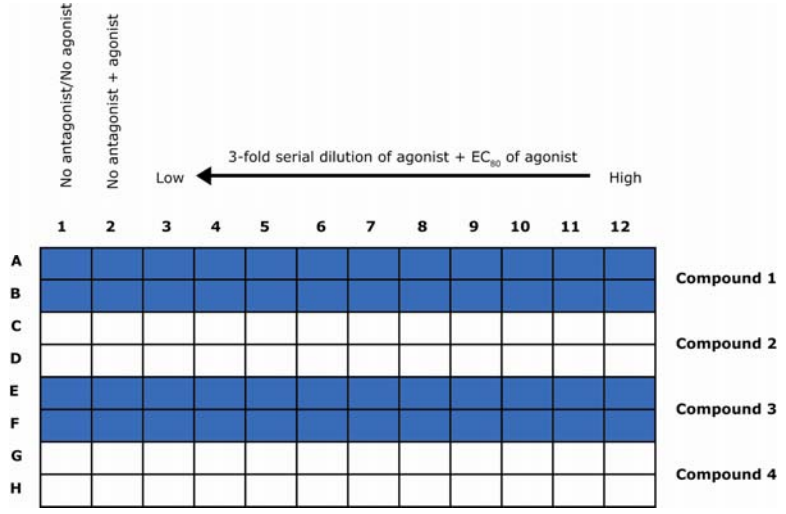
Q: How do I generate a dose curve for my antagonist compound?
A: For antagonist assays, first prepare ten serial 3-fold dilutions of the antagonist compound in the desired vehicle of choice. Prepare antagonist dilutions such that the concentration is **22X** of the final screening concentration (5 µl antagonist in a final volume of 110 µl). Then prepare a working stock of agonist com pound at **22X** of the EC_{80} concentration. Pipette all compound dilutions per well following the plate map on page 14.

A. Agonist Dose Response (96-well plate layout):



This illustration shows an 11-point dose curve with 2 data points each 4 compounds per plate for a total of 8 compounds per eXpress kit.

B. Antagonist Dose Response (96-well plate layout):



This illustration shows a 10-point dose curve with 2 data points each 4 compounds per plate for a total of 8 compounds per eXpress kit.

NOTES:

Contact Information

DiscoveRx Corporation
(World Wide Headquarters)
42501 Albrae Street
Fremont, CA 94538
United States

t | 510.979.1415
f | 510.979.1650
toll-free | 866.484.4464

DiscoveRx Corporation Ltd.
(Europe Headquarters)
Faraday Wharf, Holt Street
Aston Science Park
Birmingham, B7 4BB
United Kingdom

t | +44.121.260.6142
f | +44.121.260.6143

www.discoverx.com