



PathHunter[®] β -Arrestin Orphan GPCR Assays

For Chemiluminescent Detection of Activated GPCRs

User Manual

Simple Solutions for Complex Biology

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For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

INTENDED USE

PathHunter® β -Arrestin Orphan GPCR Assays are whole cell, functional assays that directly measure GPCR activity by detecting the interaction of β -Arrestin with the activated GPCR. Because Arrestin recruitment occurs independent of G-protein coupling, PathHunter β -Arrestin assays offer a powerful and universal screening platform that can be used for virtually any GPCR without knowing the coupling status of the receptor. The PathHunter system combines engineered clonal cell lines stably expressing the ProLink™ (PK)-tagged GPCR of interest and the Enzyme acceptor (EA)-tagged β -Arrestin fusion protein with optimized PathHunter® Detection Reagents (Cat. #93-0001, 93-0001L and 93-0001XL). By combining a simple, one-step addition protocol and standard chemiluminescent detection, these assays are ideally suited for surrogate ligand discovery, de-orphanization and compound profiling.

TECHNOLOGY PRINCIPLE

PathHunter β -Arrestin cell lines monitor GPCR activity by detecting the interaction of β -Arrestin with the activated GPCR using β -galactosidase (β -gal) enzyme fragment complementation (EFC, Figure 1). In this system, the GPCR of interest is fused in frame with the small, 42 amino acid fragment of β -gal called ProLink™ and co-expressed in cells stably expressing a fusion protein of β -Arrestin and the larger, N-terminal deletion mutant of β -gal (called enzyme acceptor or EA). Activation of the GPCR stimulates binding of β -Arrestin/EA to the ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β -gal enzyme. This action leads to an increase in enzyme activity that can be measured using chemiluminescent PathHunter Detection Reagents. Because Arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation.

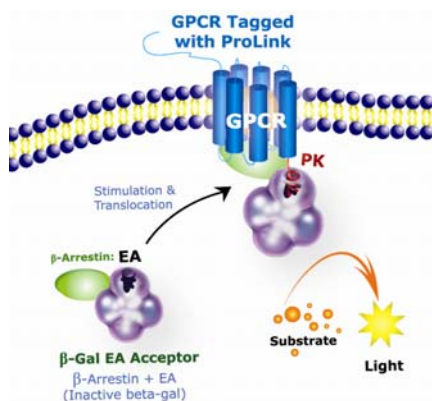


Figure 1. PathHunter® β -Arrestin Assay Principle. Activation of the ProLink-tagged GPCR results in β -Arrestin recruitment and formation of a functional enzyme capable of hydrolyzing substrate and generating a chemiluminescent signal.

CHARACTERIZATION OF ORPHAN GPCR CELL LINES

PathHunter eXpress β -Arrestin Orphan GPCR cell lines are validated using the following criteria: a) Confirmation of proper oGPCR expression at the predicted molecular weight (Figure 2) and; b) *In-vitro* complementation studies to measure basal activity and GPCR-PK expression (Figure 3).

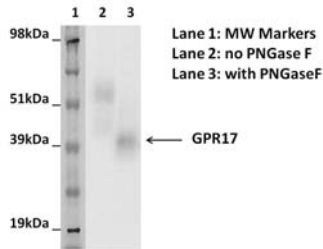


Figure 2. Cell lysates prepared from PathHunter® β -Arrestin Orphan GPCR cell lines were treated with PNGase F (Glyko; Cat. #GKE-5003), run on a SDS-PAGE gel and analyzed using the PathHunter® Eastern Blot Kit (DRx: 93-0053). Untreated lane resolves a band of appropriate size corresponding to GPCR-PK fusion protein and the PNGase F treated lane resolves a deglycosylated band indicative of proper expression and folding of GPCR protein.

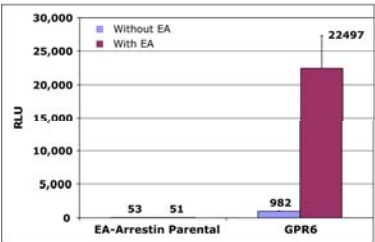


Figure 3. PathHunter® β -Arrestin Orphan GPCR cells were analyzed for basal activity as well as GPCR-ProLink™ expression by comparing the ratio of signal between untreated cells and cells treated with saturating amounts of exogenous EA, using ProLink™ Detection Kit (DRx: 92-0006). Signal from complementation of ProLink™ and EA fragments correlates to the amount of GPCR-PK expression in the cell line.

ASSAY OVERVIEW

Please read the entire protocol completely before running the assay. Successful results depend on understanding and performing these steps correctly. The **Assay Procedure** sections and **Quick Start Guides** in this booklet contain detailed information about how to run the assays. Refer to the cell-line specific datasheet for additional information on the optimized Cell Plating Reagent and reference ligand recommended for the assay.

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.

The following steps are required to monitor GPCR activity using a PathHunter[®] β -Arrestin Orphan GPCR cell line (Figure 4).

1. Plate cells (p.11).
2. Dilute and add compounds.
3. Perform functional assay in agonist mode (p.11).

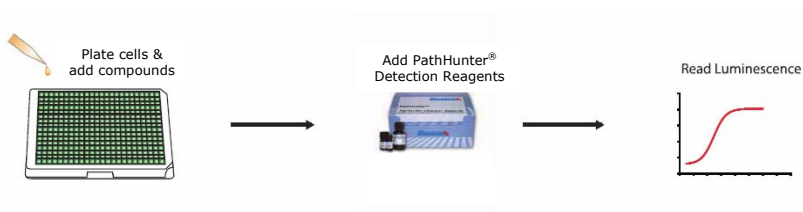


Figure 4. Simple chemiluminescent assay protocol for monitoring oGPCR activity in response to compound challenge.

MATERIALS PROVIDED

Description	Contents	Storage
PathHunter [®] β -Arrestin Orphan GPCR Cells*	2 vials	Liquid N ₂ (vapor phase)

*Please refer to the cell line specific datasheet for detailed information on the PathHunter[®] β -Arrestin Orphan GPCR cell line you are testing.

ADDITIONAL MATERIALS REQUIRED

The following additional materials are required to perform PathHunter β -Arrestin Orphan GPCR Assays:

Equipment	Materials
<ul style="list-style-type: none"> Green V-Bottom PP Ligand Dilution Plates, 10 plates/pack (DiscoverX, Cat. #92-0011) 96-well Clear Bottom TC treated, Sterile WCB, FB w/lid, 10 plates/pack (DiscoverX, Cat. #92-0014) 384-well Clear Bottom TC treated, Sterile WCB, FB w/lid, 10 plates/pack (DiscoverX, Cat. #92-0013) 384-well White Bottom TC treated, Sterile w/lid, 10 plates/pack (DiscoverX, Cat. #92-0015) Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar) Hemocytometer Cryogenic Freezing Container (Nalgene, Cat. #5100-0001 or similar) Cryogenic Freezer Vials (Fisher Scientific, Cat. #375418 or similar) Multimode or luminescence plate reader* Single and multi-channel pipettors and pipette tips Tissue culture disposables and plasticware (T25 and T75 flasks, etc.) 	<ul style="list-style-type: none"> PathHunter[®] Detection Kit (DiscoverX, Cat. #93-0001, #93-0001L or #93-0001XL) Revive[™] Media (DiscoverX, Cat. #92-0016RM Series) PathHunter[®] <i>select</i> Cell Culture Kits (DiscoverX, Cat. #92-0018G Series) Preserve[™] Freezing Reagent (DiscoverX, Cat. #92-0017FR Series) Cell Detachment Reagent (DiscoverX, Cat. #92-0009) PathHunter[®] Cell Plating (CP) Reagent (DiscoverX, Cat. #93-0563R Series) Phosphate buffered saline (PBS) GPCR test ligands

*For 96-well analysis, we recommend the LumiLITE[™] Microplate Reader (DiscoverX, Cat. #75-0001).

±Please refer to the cell line specific datasheet to determine catalog numbers for the media and reagent requirements for the PathHunter[®] β -Arrestin Orphan GPCR cell line you are testing.

FROZEN CELL HANDLING PROCEDURE

To ensure maximum cell viability, thaw the vial and initiate the culture as soon as possible upon receipt. If continued storage of the frozen vials is necessary, store vials in the vapor phase of liquid nitrogen (N₂). **DO NOT** store at -80°C as this could result in significant loss in cell viability.

CELL PLATING REAGENT REQUIREMENTS

Each PathHunter® β -Arrestin Orphan GPCR cell line has been validated for optimal assay performance using the recommended Cell Plating (CP) Reagent as indicated in the cell line specific datasheet. **For optimal performance using this PathHunter Certified System, always use the CP Reagent recommended for the cell line and DO NOT substitute at any time.**

SOLVENTS AND PREPARATION OF COMPOUND DILUTIONS

PathHunter β -Arrestin Orphan GPCR assays are routinely carried out in the presence of $\leq 1\%$ solvent (i.e. DMSO, ethanol, PBS or other). As solvents can affect assay performance, optimize the assay conditions accordingly if other solvents or solvent concentrations are required.

For preparation of test compounds, we recommend preparing the dilutions using the CP Reagent recommended for the cell line (containing the appropriate solvent). For antibodies or other compounds that may be sensitive to serum and/or other assay components, dilutions can be prepared in either Hanks Buffered Salt Solution (HBSS) + 10 mM HEPES + 0.1% Bovine Serum Albumin (BSA) or OptiMEM® + 0.1% BSA without affecting assay performance.

USE OF PLASMA OR SERUM CONTAINING SAMPLES

PathHunter β -Arrestin Orphan GPCR Assays can be run in the presence of high levels of serum or plasma without negatively impacting assay performance. Ligands can be prepared in neat, heparinized plasma and added directly to the cells (without further dilution, i.e. 100% plasma in the well). After ligand stimulation, the samples should be removed and replaced with fresh CP Reagent before the addition of the PathHunter Detection Reagents.

NOTE:

EDTA anti-coagulated plasma samples do not give a positive response in the assay. Therefore, the choice of anti-coagulant treatment is very important.

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Cells are shipped in 2 vials on dry ice and contain approximately 1×10^6 cells per vial in 1 mL of freezing medium. The following procedures are for safely storing and removing cryovials from liquid nitrogen storage.

1. PathHunter® cells must arrive in a frozen state on dry ice. If cells arrive thawed, do not proceed, contact technical support.
2. Frozen cells must be immediately transferred to liquid N₂ storage or thawed and put in culture immediately upon arrival.

3. When removing cryovials from liquid N₂ storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid N₂ inside the vial to evaporate.
4. Proceed with the thawing protocol in the following section.

SAFETY WARNING: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Some cryovials can leak when submerged in liquid N₂. Upon thawing, the liquid N₂ present in the cryovial converts back to its gas phase which can result in the vessel exploding.

CELL THAWING AND PROPAGATION

The following procedures are for thawing, seeding and expanding the cells, and for maintaining the cultures once the cells have been expanded. Cells are free of contamination prior to shipment and care should be taken in their handling to avoid contamination.

NOTE:

Face shield, gloves and a lab coat should be worn during the thawing procedure.

1. Pre-warm 15 mL Revive™ Media in a 37°C water bath.
2. Place the frozen cell vials **briefly** (10 seconds to 1 min) in a 37°C water bath under sterile conditions until only small ice crystals remain and the cell pellet is almost completely thawed. Caution: Longer incubation may result in cell death.
3. To remove DMSO from the media, carefully transfer the thawed cells to a sterile 15 mL tube and then fill tube with 10 ml pre-warmed Revive™ Media. Centrifuge at 300 x g for 4 minutes to pellet cells.
4. Remove media without disturbing cell pellet and resuspend in 5 mL of prewarmed Revive™ Media. Transfer cells to a T25 flask and incubate for 24 hours at 37°C, 5% CO₂.

NOTE:

Cell recovery is greatly improved when selection antibiotics are omitted for the first 24 hours.

5. After 24 hours, gently remove Revive™ Media (being careful not to disturb the cell monolayer) and replace with 5 mL of pre-warmed complete PathHunter®*select* Cell Culture Media.
6. Once the cells become >70% confluent in the T25 flask, aspirate media and wash cells with 5 mL PBS. Aspirate PBS and dissociate cells with 0.5 mL Cell Detachment Reagent and resuspend in 5 mL of complete PathHunter®*select* Cell Culture Media. Transfer the entire cell suspension to a T75 flask containing 15 mL of PathHunter®*select* Cell Culture Media for continued growth.

7. Passage the cells every 2-3 days, based on the doubling time of the cell line, using cell Detachment Reagent. For routine passaging, prepare a 1:3 dilution of cells in a total volume of 15 mL PathHunter^{®select} Cell Culture Media. Transfer 5 mL of the diluted cells to each new T75 flask.

NOTE:

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

8. Each PathHunter β -Arrestin Orphan GPCR Cell Line has been found to be stable for at least 10 passages with no significant drop in expression level.

CELL FREEZING PROTOCOL

The following procedures are for freezing cells from confluent T225 flasks. If smaller flasks are used, adjust the volumes accordingly. Care should be taken in handling to avoid contamination.

1. Remove T225 flasks from incubator and place in the tissue culture hood. Aspirate the media from the flasks.
2. Add 10 mL PBS into each T225 flask and swirl to rinse the cells. Aspirate PBS from flask.
3. Add 5 mL of Cell Detachment Reagent to the flask. Rock the flask back and forth gently to ensure the surface of the flask is covered. Incubate at 37°C, 5% CO₂ for 2–5 minutes or until the cells have detached.
4. 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.
5. Add 8–10 mL of Revive™ Media to each T225 flask. Rinse the cells from the surface of the flask using the added media. Remove the cells from the flask and transfer to a 50 mL conical tube. (If necessary, add an additional 5 mL
6. of media to the flask and rinse to collect the remaining cells and transfer the additional volume to the 50 mL conical tube). Remove 0.5 mL of the resuspended cells and count the cells using a hemocytometer.
7. Centrifuge the collected cells at 300 x g for 4 minutes.
8. After centrifugation, discard the supernatant. Resuspend the cell pellet in Preserve™ Freezing Reagent. Based on the cell number obtained from Step 5, dilute the resuspended cells to a concentration of 1.2×10^6 cells/mL using Preserve™ Freezing Reagent.
9. Transfer 1 mL cells to each 2 mL cryogenic tube. (Keep cells on ice during this process and transfer to a cryogenic container pre-chilled at 4°C).
10. Transfer tubes to –80°C and store overnight. Transfer tubes into the vapor phase of a liquid N₂ tank for long-term storage.

PREPARATION OF ASSAY PLATES

Each PathHunter β -Arrestin Orphan GPCR Assay has been validated for optimal assay performance using the specific PathHunter Cell Plating Reagent. **Always use the CP Reagent recommended for the cell line and DO NOT substitute at any time.**

- Harvest the cells as follows from a confluent T25 or T75 flask using Cell Detachment Reagent. **Do not use Trypsin.**
 - Remove PathHunter[®]*select* Cell Culture Media.
 - Gently wash cells with 5 mL PBS and aspirate.
 - Add 0.5 mL Cell Detachment Reagent to each T25 flask, or 1 mL to each T75 flask.
 - Place the flask in the incubator for 5 minutes or until cells have detached.
 - Add 3 mL of CP Reagent and transfer to a 15 mL conical tube.
- Determine the cell density using a hemocytometer. Centrifuge the cells at 300 x g for 4 minutes to pellet cells. Remove supernatant.
- Resuspend cells in CP Reagent at a concentration of 250,000 cells/mL (5,000 cells/20 μ L). Transfer 20 μ L of the cell suspension to each well of a 384-well microplate. Please refer to Appendix A for cell numbers and volumes for alternate formats.
- Incubate the plate overnight at 37°C, 5% CO₂.

ASSAY PROCEDURE — AGONIST DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing GPCR agonist assays using the PathHunter β -Arrestin Orphan GPCR Cell Lines and PathHunter Detection Reagents in a 384-well format. Refer to Appendix A for cell numbers and volumes for alternate formats. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least *duplicate* wells for each dilution.

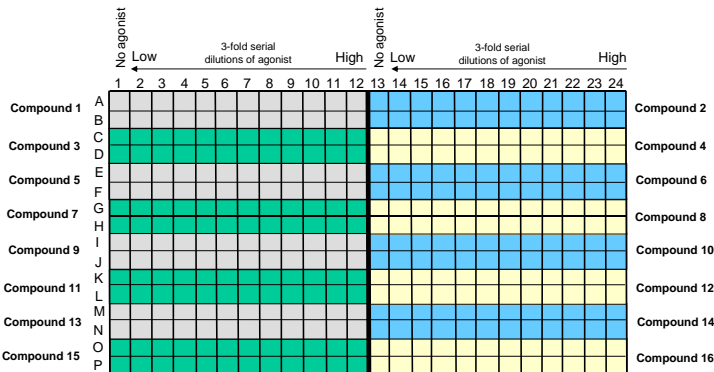


Figure 5. This plate map shows 12-point dose curves with 2 data points at each concentration. Plate map allows 16 compounds to be tested in duplicate per 384-well plate.

DAY 1: PREPARATION OF ASSAY PLATES

Plate PathHunter cells in the appropriate number of wells in a 384-well plate as described in the "Preparation of Assay Plates" section on p.11. Allow cells to incubate overnight.

DAY 2: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve agonist compound in the vehicle of choice (DMSO, ethanol, PBS or other) at the desired stock concentration.
2. Prepare a series of twelve 3-fold serial dilutions of agonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other) as described below. The concentration of each dilution should be prepared at 5X of the final screening concentration (i.e. 5 μ L compound + 20 μ L of cells). For each dilution, the final concentration of solvent should remain constant.
 - a) For each compound tested, label the wells of a 384-well dilution plate #1 through #12.
 - b) Add 20 μ L of CP Reagent containing appropriate solvent to wells #1-11.
 - c) Prepare a working concentration of agonist compound in the appropriate CP Reagent.
 - d) Add 30 μ L of the working concentration of agonist compound to well #12.
 - e) Remove 10 μ L of compound from well #12, add it to well #11 and mix gently by pipetting up and down. Discard pipet tip.
 - f) With a clean pipet tip, remove 10 μ L of diluted compound from well #11, add it to well #10 and mix gently by pipetting up and down. Discard the pipet tip.
 - g) Repeat this process 8 more times in succession to prepare serial dilutions for the remaining wells, from right to left across the plate.
DO NOT add agonist compound to well #1. This sample serves as the no agonist control and completes the dose curve.
 - h) Repeat this process for each additional agonist compound to be tested.
 - i) Set compounds aside until agonist compounds are ready to be added.
3. Remove PathHunter cells from the incubator (previously plated on day 1).
4. Transfer 5 μ L from wells #1-12 to duplicate wells according to the plate map shown on p.11.
5. Incubate for 90 minutes @ 37°C.

SUBSTRATE PREPARATION AND ADDITION

1. Prepare PathHunter Detection Reagent by combining 1 part **Substrate Reagent 2** with 5 parts **Substrate Reagent 1**, and 19 parts of **PathHunter® Cell Assay Buffer**.

Component	Entire Plate (384 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 1	1.25 mL
Substrate Reagent 2	0.25 mL

NOTE:

The working solution is stable for up to 8 hours at room temperature.

2. Add 12 μ L of prepared detection reagent to the appropriate wells. **DO NOT pipet up and down in the well to mix or vortex/shake plates.**
3. Incubate for 60 minutes at room temperature (23°C).
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response. See the example shown in Figure 6.

REPRESENTATIVE DATA AND DATA ANALYSIS

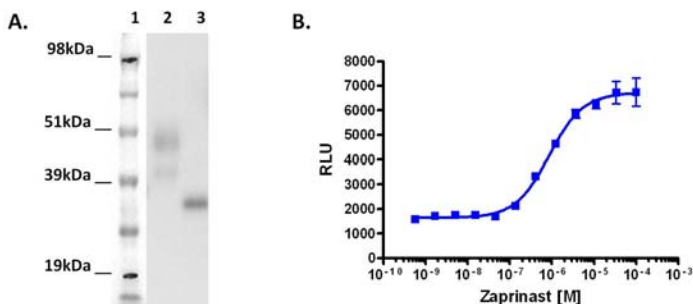
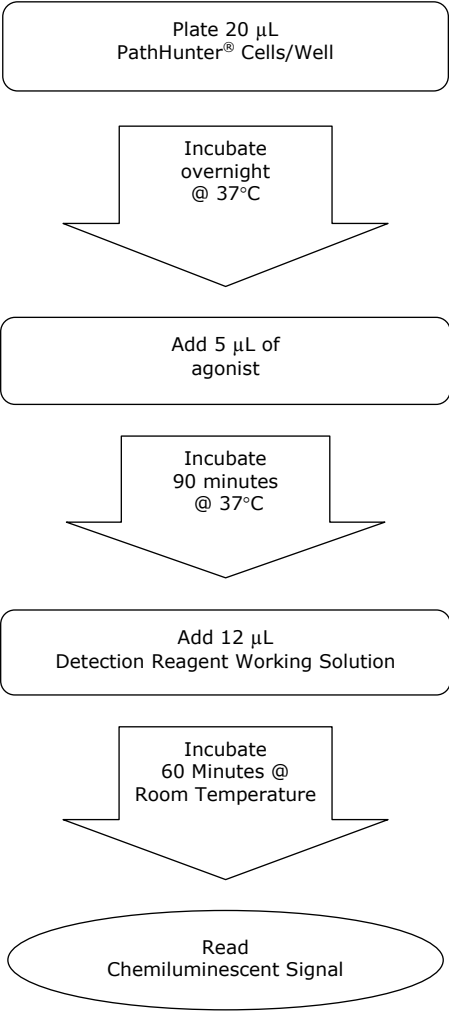


Figure 6. PathHunter® CHO-K1 GPR35 β -Arrestin Cells (93-0355C2).

(A) Expression of the GPR35 receptor was analyzed by Eastern blot, as shown by the presence of a 34 kD band (lane 3). **(B)** Cells were plated in a 384-well plate at 5,000 cells/well and stimulated with Zaprinast (DiscoverX, Cat. #92-1048) for 90 minutes. Signal was detected using the PathHunter Detection Kit (93-0001) according to the recommended protocol.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No Response	Improper cell growth conditions	See Product Insert for cell culture conditions
	High DMSO/solvent concentration	Maintain DMSO/solvent at <1% in serial dilutions of compounds.
	Improper ligand used or improper ligand incubation time	See Product Insert for recommended ligand and assay conditions
	Improper preparation of ligand	Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased Response	Higher passages give reduced performance	PathHunter cells are stable up to 10 passages. Use low passage cells whenever possible
	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscopy
Low or No Signal	Improper preparation of detection reagents	Detection reagents should be prepared just prior to use and are sensitive to light.
	Problem with cell growth, cell viability, cell adherence or cell density	See Product Insert for cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 sec/well.
Cells growing slowly	U2OS grow slower than CHO-K1 or HEK 293	Average doubling time is 3 days, so please observe cells under microscope and monitor cell health
	Slow growing clones	Use of DiscoverX functionally validated and optimized media and reagents improves assay performance

For additional information or technical support, please call 1.866.448.4864 (US) +44.121.260.6142 (Europe) or email info@discoverx.com

APPENDIX A: ASSAY FORMATS

PathHunter® Certified Assay Format				
Plate Format	96-well	FV 384-well	LV 384-well	1536-well
Total Volume	150 µL	40 µL	20 µL	8 µL
Cell Numbers	10,000	5,000	2,500	1,250
Cell Plating Reagents*	90 µL	20 µL	10 µL	4 µL
Ligand	10 µL	5 µL	2.5 µL	1 µL
Detection Reagents	50 µL	12 µL	6 µL	3 µL

*Cell Plating Reagent volume used to resuspend cells for assay plates

APPENDIX B: RELATED PRODUCTS

Description	Ordering Information
Control Ligands	www.discoverx.com/pathway_assays/control_ligands.php
PathHunter® Cell Plating Reagents	www.discoverx.com/certified/cell_plating_reagents.php
PathHunter® Certified Cell Culture Reagents <ul style="list-style-type: none"> • PathHunter® <i>select</i> Cell Culture kit • Revive™ Media • Preserve™ Freezing Reagent 	www.discoverx.com/certified/PH_cell-culture_reagents.php
PathHunter® Detection Reagents	www.discoverx.com/certified/PH_detection_reagents.php
Microplates	www.discoverx.com/certified/microplates.php
PathHunter® eXpress β-Arrestin GPCR Assays	www.discoverx.com/gpcrs/express_arrestin.php
PathHunter® eXpress β-Arrestin Orphan GPCR Assays	www.discoverx.com/gpcrs/express_orphan.php
PathHunter® eXpress β-Arrestin Ortholog GPCR Assays	www.discoverx.com/gpcrs/express_ortholog.php

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Contact Information

DRX UM PH ARRESTIN ORPHAN 0812V1

DiscoverX Corporation

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

t | 510.979.1415
f | 510.979.1650
toll-free | 866.448.4864

KINOMEScan

A division of DiscoverX

11180 Roselle Street, Suite D
San Diego, CA 92121
United States

t | 800.644.5687
f | 858.630.4600

DiscoverX Corporation Ltd.

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

t | +44.121.260.6142
f | +44.121.260.6143

www.discoverx.com

