PathHunter® Exendin-4 Bioassay Kit

For chemiluminescent detection of exendin-4 activity

Simple Solutions for Complex Biology

DiscoveRx

Table of Contents

Read the entire product insert before beginning the assay
For additional information or Technical Support, contact support@discoverx.com or visit www.discoverx.com.

Overview

Intended Use

PathHunter® Exendin-4 Bioassay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study exendin-4 potency and neutralizing antibodies. The Bioassay kits contain all the reagents needed for a complete assay including cells, detection reagents, cell plating reagent, positive control agonist, and assay plates. The pre-validated, frozen cells have been manufactured for short term use and are provided in a ready-to-assay format that saves time and adds convenience. Assays have been designed for 96-well and 384-well plate formats.

Technology Principle: PathHunter® Exendin-4 Bioassay

The PathHunter Exendin-4 Bioassay monitors GPCR activity by detecting the interaction of β-Arrestin with the activated GPCR using β-galactosidase (β-gal) enzyme fragment complementation. 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 to the ProLink-tagged GPCR and forces complementation of the two enzyme fragments of β-galactosidase, 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 Bioassay Detection Reagents. Because Arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation, thus providing an ideal tool to characterize functional biologics.

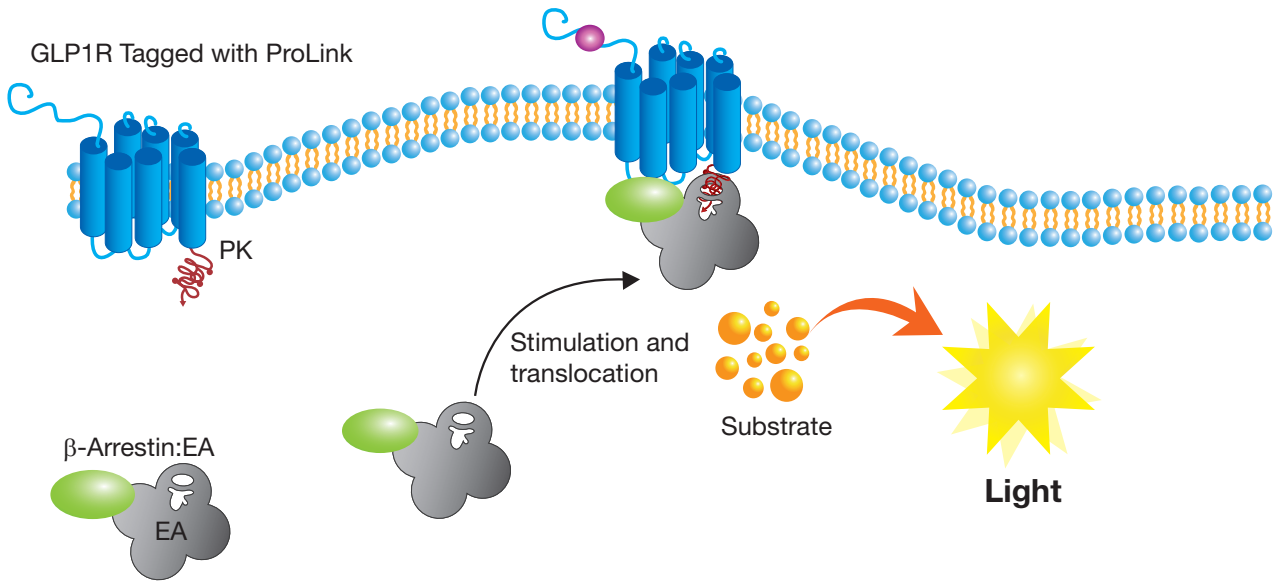


Figure 1. PathHunter Exendin-4 Bioassay Principle

Materials Provided

List of Components	(93-0300Y2-00029)	(93-0300Y2-00030)
Description Kit Size	**Contents**	**Contents**
Box 1: PathHunter CHO-K1 GLP1R Bioassay Cells	2 vials	10 vials
Box 2A: PathHunter Bioassay Detection Kit	200 dp	1,000 dp
• Detection Reagent 1	2 mL	10 mL
• Detection Reagent 2	8 mL	40 mL
Box 2B: AssayComplete Cell Plating Reagent 0	1 x 100 mL	3 x 100 mL
Box 2C: Protein Dilution Buffer	1 x 50 mL	2 x 50 mL
Box 3: Control Agonist (Exendin-4)	1 vial	1 vial
Box 4: 96-well Tissue Culture Treated Plates	2 plates	10 plates

Storage Conditions

Shipping Conditions	Dry Ice
Storage Conditions	**Box 1:** Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vials as soon as possible upon receipt. If continued storage of the frozen vials is necessary, store as follows: • **Short term** (2 weeks or less): Store vials at -80°C immediately upon arrival. (DO NOT store at –80°C for more than 2 weeks). • **Long term** (greater than 2 weeks): Vials should ONLY be stored in the **vapor phase** of liquid nitrogen (LN_2).*
	Box 2A, 2B, and 2C: PathHunter Bioassay Detection Reagents, Cell Plating Reagent 0 (CP0), and Protein Dilution Buffer: • **Store at -20°C** **Note:** Once thawed, the CP0 and Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. PathHunter Bioassay Detection Reagents can be kept at 4°C for up to 4 days. Reagents can be aliquoted and kept at -20°C until needed to avoid excessive freeze/thaw cycles.
	Box 3: Store at -20°C until ready to use. Equilibrate the vial to room temperature and **c**entrifuge the vial prior to opening to maximize recovery. Reconstitute in the provided buffer as described on the ligand data sheet. **Box 4:** 96-well tissue culture treated plates: Store at room temperature.

***Safety Warning:** A face shield, gloves and lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid N_2. Upon thawing, if liquid N_2 is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the liquid nitrogen tank.

Additional Materials Required

The following equipment and additional materials are required to perform these assays:

Equipment
Single and multichannel micro-pipettors and pipette tips
Multimode or luminescence plate reader
V-Bottom 96-well compound dilution plates (DiscoveRx 92-0011 or similar)
Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar)

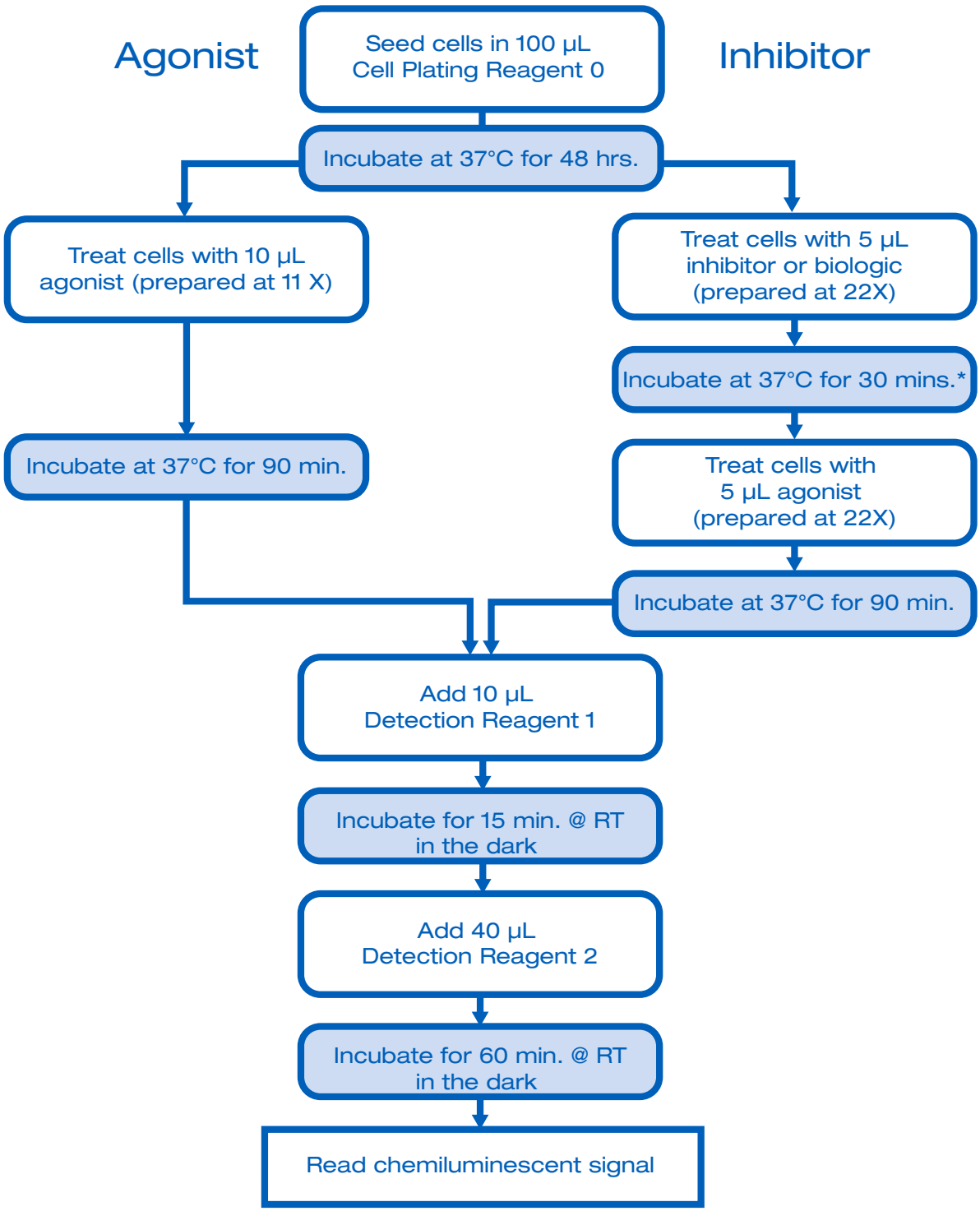
Protocol Schematic

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

Assay Name:_____

Product Number: _____

Quick-Start Procedure: In a 96-well tissue culture treated plate perform the following:



* For an anti-ligand approach, pre-incubate ligand with antibody for 30 minutes or more.

Detailed Protocols

The following protocols are for thawing and plating frozen PathHunter GLP1R β-Arrestin Bioassay cells from cryovials.

Cell Thawing Method

1. Pre-warm AssayComplete Cell Plating Reagent 0 in a 37˚C water bath for fifteen minutes to equilibrate temperature.
2. Remove cryovials from liquid N_2 storage and place immediately on dry ice.

Caution: 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. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

3. Decontaminate the vial by wiping with 70% ethanol.
4. Add 0.5 mL of pre-warmed AssayComplete Cell Plating Reagent 0 to the cell vial to thaw the cells. Pipette up and down gently to ensure that the cells are evenly distributed.

Caution: **Do not thaw the vials in 37˚C water bath.** Do not centrifuge to remove DMSO.

5. Immediately tranfer the cells to 11.5 mL of pre-warmed AssayComplete Cell Plating Reagent 0. Rinse the cryovial to ensure complete cell recovery. Mix and pour into a disposable reagent reservoir.
6. Plate 100 µL of cells into each well of the provided 96-well tissue culture plate.

Assay Protocol

Purified Biologics

These assays are optimized for 96- and 384-well plates. For volumes associated with 384-well format, please refer to page 9.

96-Well Assay Protocol for Purified Biologics

	Agonist Assay Protocol	Inhibitor Assay Protocol
Step 1: Plate Cells	Seed cells in 100 µL AssayComplete Cell Plating Reagent 0.	
Step 2: Pre-treat with Inhibitor and Incubate	NA	Treat cells with 5 µL inhibitor (22X)* in dose curve. Incubate for 30 min. at 37°C
Step 3: Treat Cells and Incubate	Induce cells with 10 µL agonist (11X)* in dose curve. Incubate at 37°C for 90 minutes.	Induce cells with 5 µL agonist challenge (22X)* at EC_{90}.** Incubate at 37°C for 90 minutes.
Step 4: Add Bioassay Detection Reagent 1	Add 10 µL of Detection Reagent 1 to each well. Incubate plate at room temperature for 15 minutes in the dark.	
Step 5: Add Bioassay Detection Reagent 2	Add 40 µL Detection Reagent 2 to each well. Incubate plate at room temperature for 1 hour in the dark.	
Step 6: Read Samples	Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for PMT readers or 5-10 seconds for imager.	

*** Note:** Agonists are prepared at 11X of final screening concentration for agonist assays. The inhibitor and agonist challenge are prepared at 22X of final screening concentration for inhibitor assays. For an anti-receptor antibody, antibody and agonist can be pre-incubated together before combined addition to the assay plate. The pre-incubation time should be optimized empirically. Pre-incubation times of 0-60 min. are typical.

** The specific concentration of agonist challenge to be used can vary from EC_{75} to EC_{100}, based on the target and assay conditions.

For more information on agonist/inhibitor preparation and reconstitution, please refer to the product datasheet for the appropriate DiscoveRx Synergy products.

For detailed instructions on setting up a dose response curve, please see Supplemental Protocols on page 7.

Assay Protocol

Crude Biologics

The PathHunter assays can be run in the presence of high levels of serum or plasma without significantly impacting assay performance. Therefore, standard curves of control 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 (MRD) of crude samples should be empirically determined. These assays are optimized for 96- and 384-well plates. For volumes associated with 384-well format, please refer to page 9.

96-Well Assay Protocol for Non-Purified Biologics

	Agonist Assay Protocol	Biologic Sample Assay Protocol
Step 1: Plate Cells	Seed cells in 100 µL AssayComplete Cell Plating Reagent 0.	
Step 2: Aspirate Media	NA	Gently remove the Cell Plating Reagent from the cells.
Step 3: Pre-treat with Crude Biologic and Incubate	NA	Treat cells with 100 µL crude biologic samples (1X)* in dose curve, diluted in Protein Dilution Buffer as necessary. Incubate for optimized amount of time at 37°C
Step 4: Treat Cells with Agonist and Incubate	Induce cells with 10 µL agonist (11X)* in dose curve. Incubate at 37°C for 90 minutes.	Induce cells with 10 µL agonist challenge (11X)* at EC_{90}.** Incubate at 37°C for 90 minutes.
Step 5: Aspirate Incubation Mixture	Gently remove the incubation mixture from the cells. Immediately add 100 µL of fresh Cell Plating reagent to each well.	
Step 6: Add Bioassay Detection Reagent 1	Add 10 µL of Detection Reagent 1 to each well. Incubate plate at room temperature for 15 minutes in the dark.	
Step 7: Add Bioassay Detection Reagent 2	Add 40 µL Detection Reagent 2 to each well. Incubate plate at room temperature for 1 hour in the dark.	
Step 8: Read Samples	Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for PMT readers or 5-10 seconds for imager.	

*** Note:** Agonists are prepared at 11X of final screening concentration. Crude biologic sample is prepared at 1X final screening concentration. These concentrations will result in 1x concentration when added to the assay plate. For a detailed protocol, refer to the ligand datasheet and Supplemental Information on the following page.

** The specific concentration of agonist challenge to be used can vary from EC_{75} to EC_{100}, based on the target and assay conditions.

For more information on agonist/inhibitor preparation and reconstitution, please refer to the product datasheet for the appropriate DiscoveRx Synergy products.

For detailed instructions on setting up a dose response curve, please see Supplemental Protocols on page 7.

Supplemental Information

Appropriate Methods to Set Up Dose Curves and Dilutions

1. Dissolve test sample in the appropriate solvent (see specific information on data sheet).
2. Prepare a series of 12 three-fold dilutions of test sample in Protein Dilution Buffer or AssayComplete Cell Plating Reagent 0. The concentration of each dilution should be prepared at 11X or 22X of the final screening concentration for agonist or inhibitor assays, respectively (e.g. 10 µL of 11X agonist + 100 µL of cells will give 1X final screening concentration).

    a. Label the wells of the dilution plate.

    b. Add 30 µL of vehicle to dilution wells #1 to #11. (The dilution volume needs to be adjusted according to the number of duplicate wells)

    c. Prepare the highest concentration of test sample in appropriate solvent. [We recommend targeting a working concentration 500 times the expected EC_{50}. e.g. For an expected EC_{50} of 10 ng/mL, prepare the highest concentration in working dilution at 5,000 ng/mL. This is 11X the screening or final concentration of 454.5 ng/mL. This will result in an EC_{50} concentration near the center of the dose curve].

    d. Add 45 µL of highest concentration to well #12.

    e. Remove 15 µL from well #12 and add it to well #11 and mix gently.

    f. With a clean tip, remove 15 µL from well #11 and add it to well #10. Mix gently.

    g. Repeat process until well #2 is reached. DO NOT add test sample to wells #1 and #2.

    h. Set up additional dose curves for agonists and inhibitors. See figures 1 and 2 for sample plate maps with agonist and inhibitor dose curves.

Important Tips for Preparing Accurate Dilution Curves

- Peptides and proteins should be diluted in Protein Dilution Buffer.
- Test samples in organic solvents like DMSO or ethanol need special handling to ensure accuracy:
    - Prepare dilution curves at 110X the final screening concentration in organic solvent.
    - Dilute the 110X dilution series in organic solvent from the above step, 10-fold in Protein Dilution Buffer to get the working 11X concentration for entire dose curve.
    - Add 10µL of 11X working concentration to 100 µL of cells to get 1X screening concentration. The final solvent concentration will be 1% in each well.
    - Test samples that are soluble in organic solvents will precipitate out of aqueous solution at higher concentrations, decreasing accuracy in subsequent dilutions. This protocol ensures that insufficient solubility of test sample at high concentrations in aqueous solution will not affect subsequent lower dilution series.

Representative Plate Maps for Agonist/Inhibitor Curve

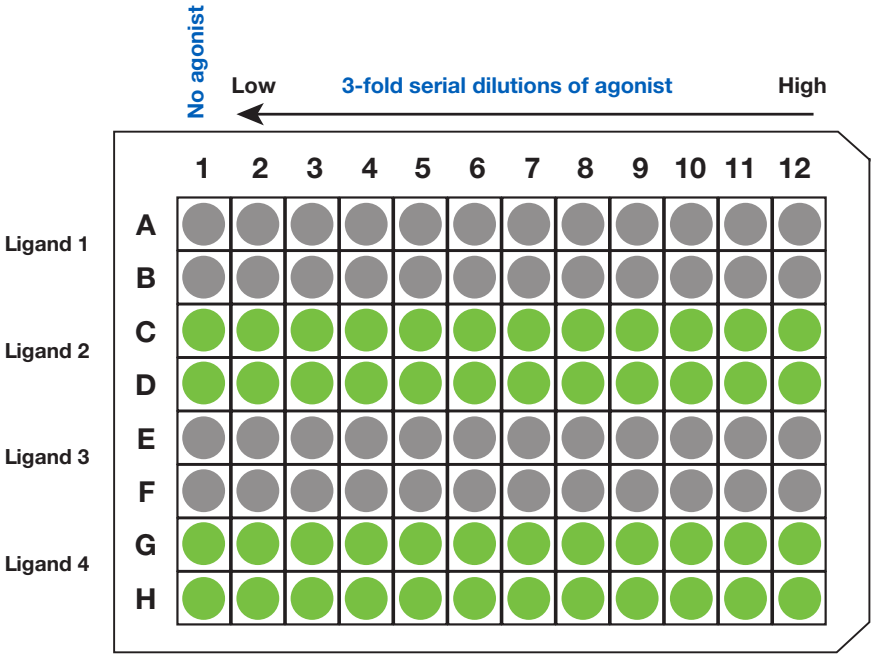


Figure 1. This agonist plate map shows 11-point dose curves with 2 data points at each concentration for 4 agonists per plate for a total of 88 data points per 96-well plate.

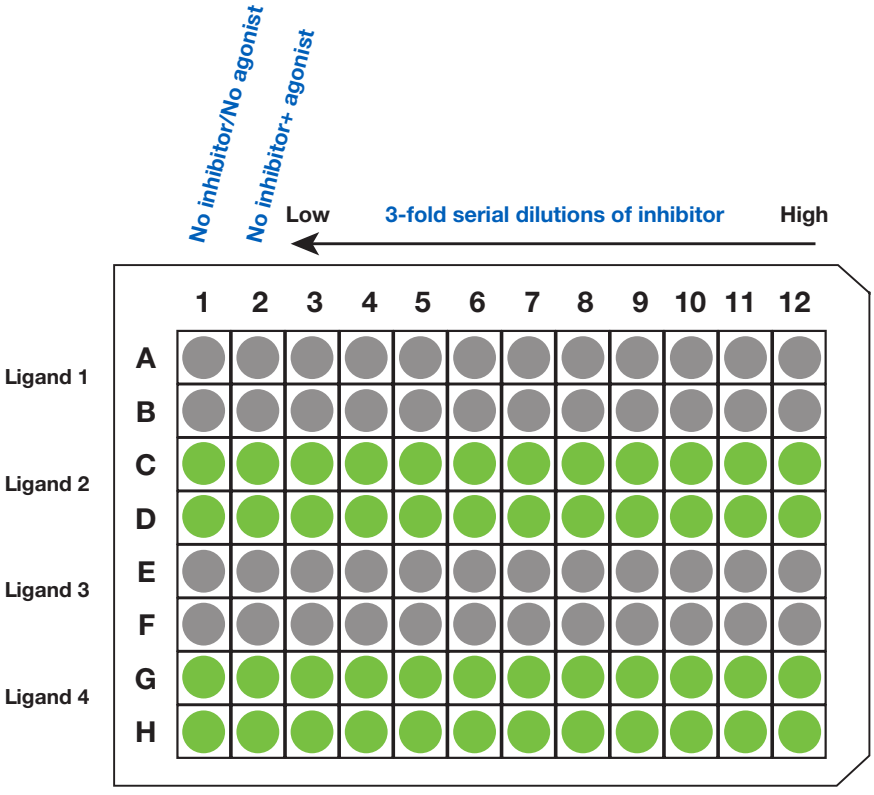


Figure 2. This inhibitor plate map shows a 10 point dose curve with 2 data points each, 4 inhibitors per plate for a total of 80 data points per 96-well plate.

Assay Formats Table

PathHunter Certified Assay Format		
Plate Format	96-well	384-well
Total Volume	160 µL	37.5 µL
Cell Numbers	10,000	5,000
AssayComplete Cell Plating Reagent	100 µL	20 µL
Test Sample	10 µL	5 µL
Bioassay Detection Reagents	50 µL	12.5 µL

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

Related Products	
Description	**Ordering Information**
PathHunter Bioassay Detection Reagents	www.discoverx.com/detectionreagents
Cell Culture Kits, Reagents & Consumables	www.discoverx.com/cell-culture-kits-reagents-consumables
AssayComplete Cell Plating Reagents	www.discoverx.com/cellplatingreagents
Control Ligands	www.discoverx.com/controlligands

Instrument Compatibility Chart		
Assay	**Instrument**	**Read-Out**
All PathHunter® assays HitHunter® cAMP HitHunter® cGMP	**COMPATIBLE WITH ANY LUMINOMETER** **BMG:** PheraStar, Cytostar, LumiStar **Perkin Elmer:** TopCount, Victor 2 or V, Fusion, LumiCount, Envision, Micro-beta (Trilux), Viewlux, Northstar **GE:** LEAD seeker, Farcyte **Molecular Devices:** FLIPR, SpectraMax M3/ M4/M5/M5e, FlexStation 3, SpectraMax L **Tecan:** Ultra, Evolution **Turner BioSystems:** Modulus Microplate **Caliper** LabChip 3000 & EZ Reader **Berthold Technologies:** Mithras LB940, CentroLIApc **Hamamatzu** FDS6000, FDSS/RayCatcher **Thermo Scientific:** Luminoskan Ascent **Biotek:** Synergy 2	Luminescence

*For other instruments not listed here, please contact technical support at support@discoverx.com to ensure compatibility.

Troubleshooting Guide

Problem	Cause	Solution
No Response	Improper thawing procedure	Refer to thawing instructions on page four of this user manual.
	Improper ligand used or improper ligand incubation time	See datasheet for recommended ligand and assay conditions
	Improper preparation of ligand (agonist or antagonist)	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	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope
Low or No Signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 sec/well.
Experimental S:B does not match datasheet value	Incorrect incubation temperature	Confirm assay conditions
		Check and repeat assay at correct incubation temperature as indicated on the assay datasheet
	Improper preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands
EC_{50} is right-shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in Protein Dilution Buffer
		Non-binding surface plates may be necessary for hydrophobic compounds

For additional information or technical support, please contact technical support at support@discoverx.com.

Limited Use License Agreement

A. This product and/or its use is covered by U.S. patents #6,342,345 B1, #7,135,325, B2, #8,101,373 B2 and/or related foreign patents and pending applications and trade secrets that are either owned by or licensed to DiscoveRx corporation. The cells and detection reagents (collectively "Materials") purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Cell-Based assay ("Assay") that employs genetically modified cells and vectors (collectively, the “Cells”), and related detection reagents (the “Reagents”) (collectively referred to as “Materials”). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

1. Purchaser will not further propagate the Cells included in the Assay kit.
2. Purchaser is permitted to use the Cells only for use in the Assay and in connection with Reagents purchased from DiscoveRx Corporation or its authorized distributor.
3. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.
4. The Reagents contain or are based upon the proprietary and valuable know-how developed by DiscoveRx, and the Reagents have been optimized by DiscoveRx to function more effectively with the Cells in performing the Assay. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser’s behalf.
5. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.
6. Purchaser will not use the Cells with any other reagents or substrates, other than the Reagents that are provided by or purchased from DiscoveRx or an authorized DiscoveRx distributor, in connection with the Materials.
7. The number of Assays performed will not exceed the authorized number for which Materials were purchased.

B. The use of this cell line may require third party licenses. Purchaser shall have the sole responsibility for obtaining any such licenses prior to use of the cell lines.

If the purchaser has any further questions regarding the rights conferred with purchase of the Materials, please contact:

Licensing Department
DiscoveRx Corporation
42501 Albrae Street
Fremont, CA 94538 USA
t | 1.866.448.4864
e | info@discoverx.com