PathHunter® eXpress Dimerization Assay Kit

For chemiluminescent detection of protein dimerization

Simple Solutions for Complex Biology

DiscoveRx

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

DiscoveRx

Overview

Intended Use

PathHunter eXpress Dimerization Assay Kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study receptor-receptor interactions at the cell surface. The eXpress kits contain all the reagents needed for a complete assay including cells, flash detection reagents, cell plating reagents, and plates. The pre-validated, frozen eXpress 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® Dimerization Assay

These assays utilize Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two framents, ProLink (PK) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

The PathHunter eXpress Dimerization assay detects ligand induced dimerization of two subunits of a receptor-dimer pair. The cells have been engineered to co-express one receptor subunit (target protein 1) fused to Enzyme Donor (ED), and a second dimer partner (target protein 2) fused to Enzyme Acceptor (EA). Binding of an agonist to one receptor subunit induces it to interact with its dimer partner, forcing complementation of the two enzyme fragments. This results in the formation of a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.

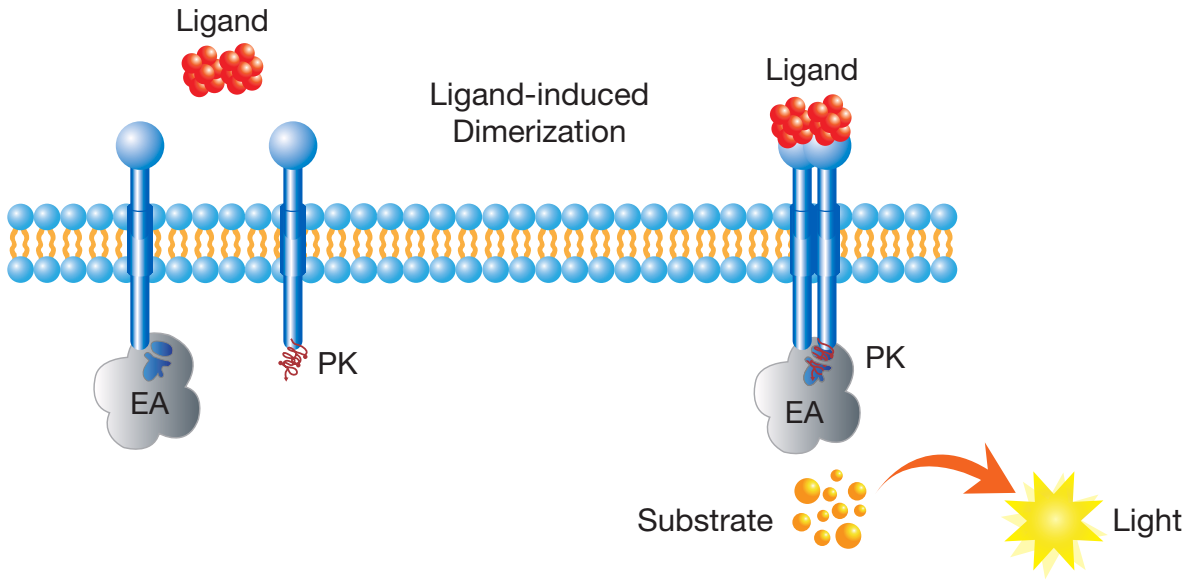


Figure 1: PathHunter eXpress Dimerization Assay Principle

Materials Provided

List of Components			
Description Kit Size	**2-Plate Kit**	**10-Plate Kit**	**Storage**
PathHunter eXpress Dimerization Cells	2 vials (1 x 10^6 cells each)	10 vials (1 x 10^6 cells each)	Short-term: -80°C Long-term: Liquid Nitrogen
PathHunter Flash Detection Kit*	200 dp	1000 dp	-20°C
• Flash Cell Assay Buffer	5 mL	25 mL	
• Flash Substrate	20 mL	100 mL	
AssayComplete™ Cell Plating Reagent**	1 x 100 mL	2 x 100 mL	
96-Well Tissue Culture-Treated Plates	2 plates	10 plates	Room Temperature

*Please discard the user manual provided in the Detection Reagent Kit box. Refer to the protocol outlined in this user manual for running the assay.

**Refer to cell-line specific data sheets for the optimized Cell Plating Reagent included with each kit.

Storage Conditions

Shipping Conditions	**Dry Ice**
Storage Conditions	Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vial 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).*
	PathHunter Flash Detection Reagents and Cell Plating (CP) Reagent: • **Store at -20°C** **Note:** Once thawed, store the PathHunter Flash Detection Reagents and CP Reagent at 4°C. Avoid multiple freeze/thaw cycles.
	96-well tissue culture treated plate: 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 (Not Provided)

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

Equipment
Single and multichannel micro-pipettors and pipette tips
Hemocytometer
Multimode or luminescence plate reader
AssayComplete™ Protein Dilution Buffer (DiscoveRx 92-0023M, 92-0023L)

Recommended Materials

The following products* are recommended:

Product Description	Catalog No.
CytoTracker™ LDH Quantification Kit	92-2002
CytoTracker™ Glutathione Quantification Kit	92-2003
CytoTracker™ DNA Damage Quantification Kit	92-2004M
Control Ligands	www.discoverx.com/controlligands

* Products not available in all countries. Please inquire.

Protocol Schematic

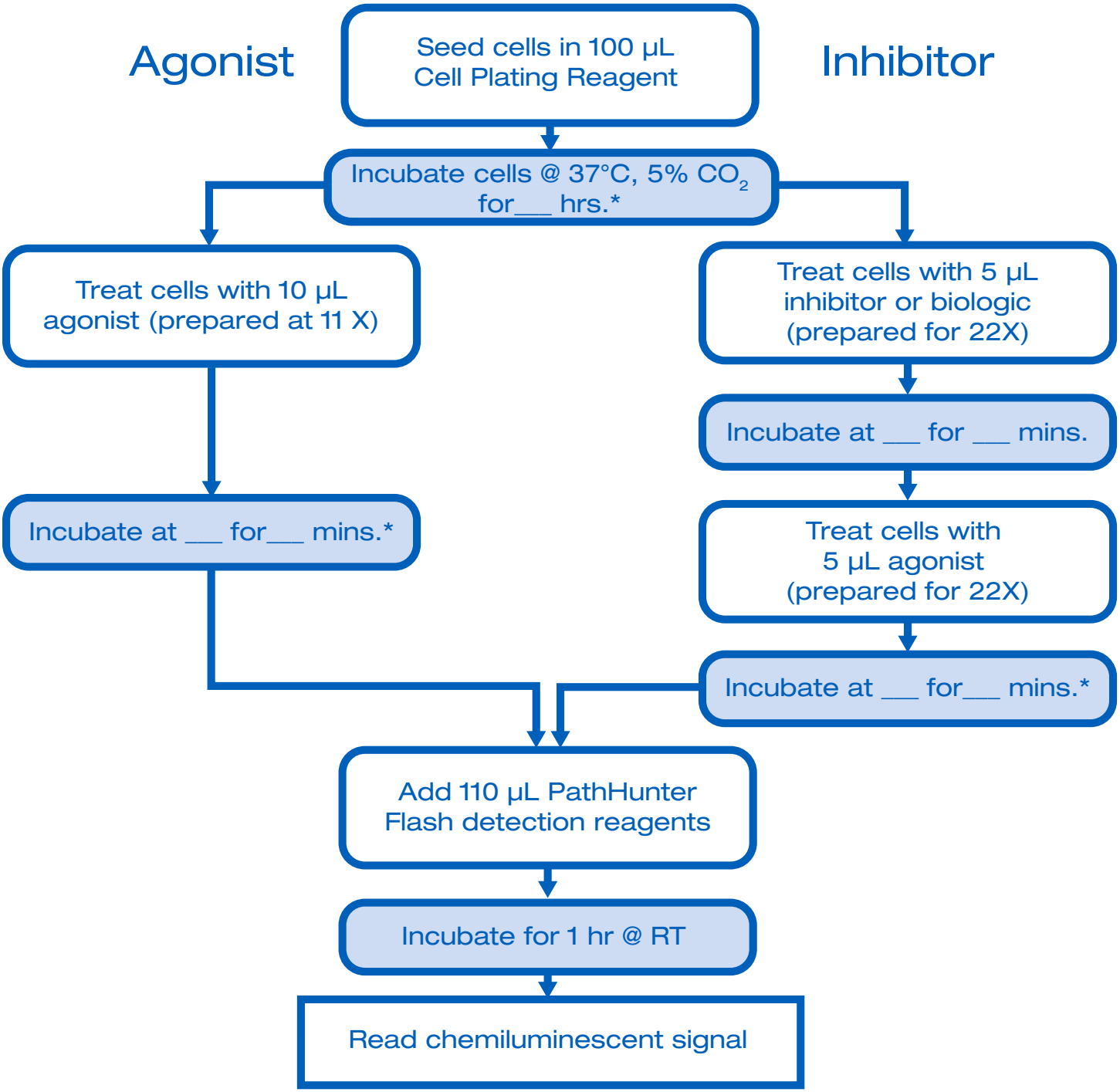
Tip: *Use this sheet to note your assay specific conditions. Post on your bench to use as a quick reference guide.*

Assay Name:_____

Product Number: _____

Quick-Start Procedure: Agonist Dose Response

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



* Assay specific time and temperature in datasheet

Detailed Protocols

The following protocols are for thawing and plating frozen PathHunter eXpress Dimerization cells from cryovials.

Cell Thawing Method

1. Pre-warm CP reagent 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 CP reagent 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 CP reagent. 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.
7. Place the seeded plate in a 37˚C 5% CO_2 humidified incubator for 24 or 48 hours prior to testing (refer to target-specific datasheet for recommended incubation time).

Assay Protocol

Small Molecules and Purified Biologics

The PathHunter assays are routinely carried out in the presence of <1% solvent (eg. DMSO, ethanol, PBS etc.). Solvents may affect the assay performance, so please optimize assay conditions when using other solvent(s) concentrations. These assays are optimized for 96- and 384-well plates. For volumes associated with 384-well format, please refer to page 9.

Assay Protocol For Small Molecules and Purified Biologics

	Agonist Assay Protocol	Inhibitor Assay Protocol
Step 1: Plate Cells and Incubate	Seed cells in 100 µL Cell Plating Reagent. Incubate for 24 or 48 hrs. at 37°C*	
Step 2: Pre-treat with Inhibitor and Incubate	NA	Treat cells with 5 µL inhibitor (22X)** in dose curve. Incubate for indicated amount of time at indicated temperature.*
Step 3: Treat Cells with Agonist and Incubate	Induce cells with 10 µL agonist (11X)** in dose curve. Incubate at appropriate temperature for indicated amount of time.	Induce cells with 5 µL agonist challenge (22X)** at EC_{90}.*** Incubate at appropriate temperature for indicated amount of time.
Step 4: Prepare Detection Reagents	Prepare a working solution of detection reagents for each plate by mixing the following reagents: Flash Cell Assay Buffer - 2.5 mL (1 part) Flash Substrate - 10 mL (4 parts) **Note:** The working solution is stable for up to 8 hrs @ RT in the dark. These reagents are light sensitive.	
Step 5: Add Detection Reagents	Add 110 µL PathHunter Flash Detection Reagents to each well. Incubate plate at room temperature for 1 hr in the dark.	
Step 6: Read Samples	Read sample on a standard luminescence plate reader at 0.1 to 1 sec/well for PMT readers or 5-10 secs for imager.	

*** Note:** For product-specific information on cell plating reagent, control agonist, control inhibitor (if available), incubation times and temperature, please see product data sheet.

** Agonists are prepared at 11X of final screening concentration for agonist assays. The inhibitor and agonist challenge is 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. 0-60 min. pre-incubation times 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 appropriate DiscoveRx Synergy products.

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

Assay Protocol

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

Assay Protocol For Non-Purified Biologics

	Agonist Assay Protocol	Biologic Sample Assay Protocol
Step 1: Plate Cells and Incubate	Seed cells in 100 µL AssayComplete™ Cell Plating Reagent. Incubate for 24 or 48 hrs. at 37°C*	
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 AssayComplete Protein Dilution Buffer as necessary. Incubate for indicated amount of time at indicated temperature.*
Step 4: Treat Cells with Agonist and Incubate	Induce cells with 10 µL agonist (11X)** in dose curve. Inbubate at appropriate temperature for indicated amount of time.	Induce cells with 10 µL agonist challenge (11X)** at EC_{90}.*** Incubate at appropriate temperature for indicated amount of time.
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: Prepare Detection Reagents	Prepare a working solution of detection reagents for each plate by mixing the following reagents: Flash Cell Assay Buffer - 2.5 mL (1 part) Flash Substrate - 10 mL (4 parts) **Note:** The working solution is stable for up to 8 hrs @ RT in the dark. These reagents are light sensitive.	
Step 7: Add Detection Reagents	Add 110 µL PathHunter Flash Detection Reagents to each well. Incubate plate at room temperature for 1 hour in the dark.	
Step 8: Read Samples	Read plate on a standard luminescence plate reader at 0.1 to 1 sec/well for PMT readers or 5-10 seconds for imager.	

*** Note:** For product-specific information on AssayComplete Cell Plating Reagent, control agonist, control inhibitor (if available), incubation times and temperature, please see product data sheet.

** Agonists are prepared at 11X 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 compound datasheet and Supplemental Information on the following page.

*** The specific concentration of agonist 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 article in the appropriate solvent (see specific information on data sheet).
2. Prepare a series of 12 three-fold dilutions of test article in Cell Plating Reagent or appropriate solvent. 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 article in Cell Plating Reagent. [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 5000 ng/mL. This is 11X the screening or final concentration of 454.5 ng/mL].
    d. Add 45µL 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 article to well #1.
    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 can be diluted in AssayComplete Protein Dilution Buffer.
- Test articles 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 AssayComplete 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 articles 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 article at high concentrations in aqueous solution will not affect subsequent lower dilution series.

Representative Plate Maps for Agonist/Inhibitor Curve

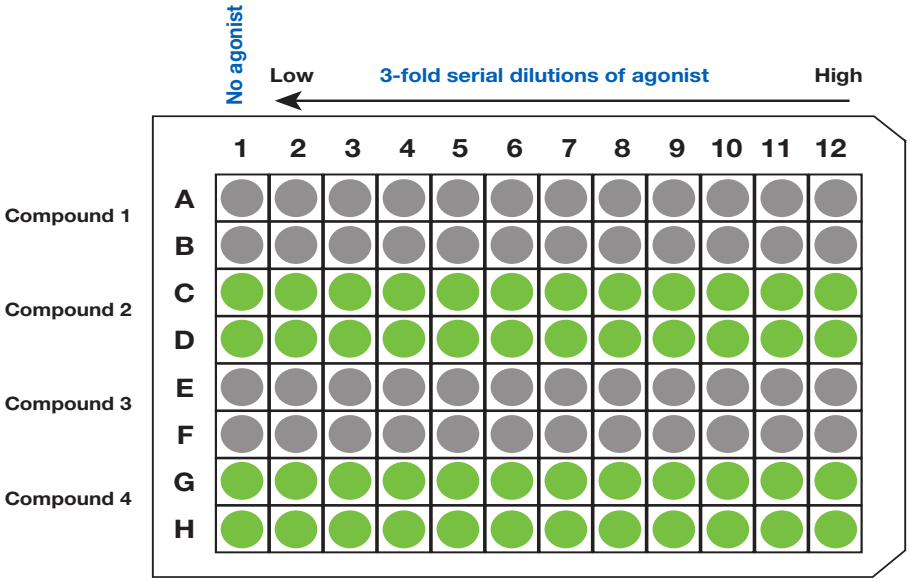


Figure 1. This agonist plate map shows 11-point dose curves with 2 data points at each concentration for 4 agonists per plate for a total of 88 data points per 96-well plate.

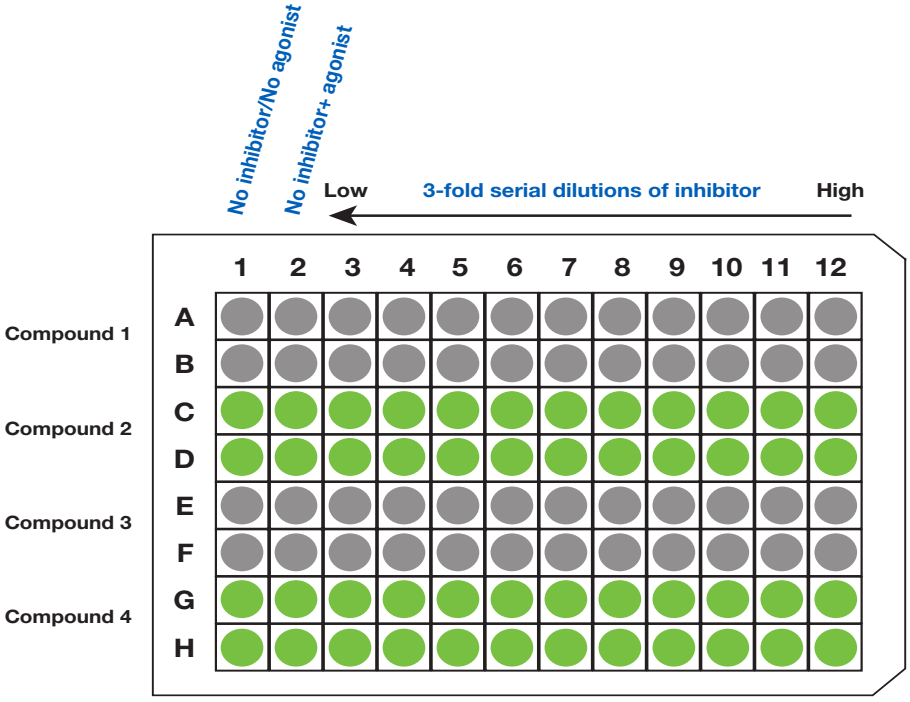


Figure 2. This inhibitor plate map shows a 10 point dose curve with 2 data points each, 4 inhibitors per plate for a total of 80 data points per 96-well plate.

Assay Formats Table

PathHunter Certified Assay Format				
Plate Format	96-well	FV 384-well	LV 384-well	1536-well
Total Volume	220 µL	55 µL	30 µL	8 µL
Cell Numbers	10,000	5,000	5,000	1,250
Cell Plating Reagents*	100 µL	20 µL	12.5 µL	3 µL
Ligand	10 µL	5 µL	2.5 µL	1 µL
Flash Detection Reagents	110 µL	30 µL	15 µL	4 µL

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

Related Products	
Description	**Ordering Information**
PathHunter Flash 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

Troubleshooting Guide

Problem	Cause	Solution
No Response	High DMSO/solvent concentration	Maintain DMSO/solvent at <1% in serial dilutions of compounds
	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 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 buffer containing 0.1% BSA
		Non-binding surface plates may be necessary for hydrophobic compounds
High well-to-well variability in Z' study	Problems with plate type and compound solubility	Z' studies should be performed with automation
		It may be necessary to test plate types and compound stability

For additional information or technical support, please call or email at the numbers listed below.

Limited Use License Agreement

A. This product and/or its use is covered by U.S. patents #8,541,175 and #8,679,832 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 Dimerization 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 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.

2. 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.

3. 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.

4. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.

5. 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.

6. 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 | 1.510.979.1415 x 104
e | info@discoverx.com

Intended Use

Your PathHunter® eXpress Dimerization Assay Kit, when used in conjunction with provided materials, provides a cell-based functional assay for the detection of receptor dimerization. The assays described in this booklet have been validated for use in both 96-well and 384-well microplate formats.