PathHunter® eXpress Receptor Tyrosine Kinase Activity Assay Kit

For chemiluminescent detection of activated tyrosine kinases

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 info@discoverx.com or visit www.discoverx.com.

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Overview

Intended Use

PathHunter eXpress Receptor Tyrosine Kinase (RTK) Activity Assay Kits are functional cell-based assays used for identification, screening or profiling of small molecule inhibitors. This assay format cannot be used for functional testing of biologics.

Technology Principle: PathHunter® RTK Activity Assay

These assays utilize Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, 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.

In the PathHunter assay approach for tyrosine kinases, the ProLink tag is fused to the C-terminus of the receptor. The EA is fused to a phosphotyrosine SH2 domain containing protein that is able to bind the activated RTK. Ligand-induced activation of the receptor results in receptor phosphorylation. The SH2-EA fusion protein binds the phosphorylated receptor, forcing complementation of PK and EA to form an active β-gal enzyme. β-gal enzymatic activity is quantitatively measured using a chemiluminescent substrate in the PathHunter Detection Kit (Figure 1.)

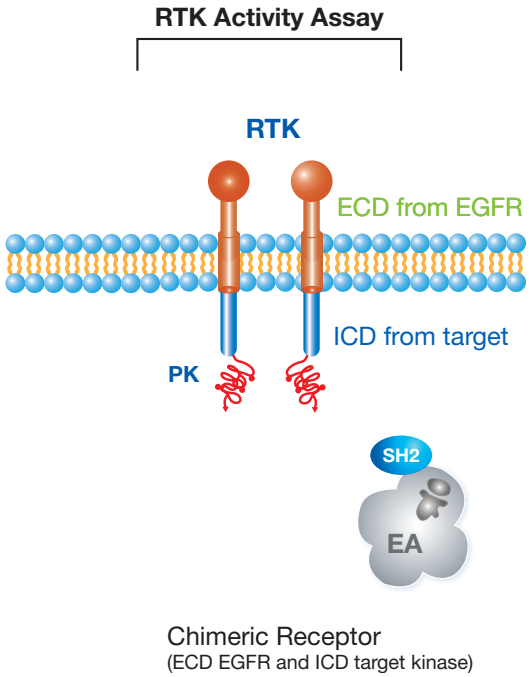


Figure 1: PathHunter Receptor Tyrosine Kinase Activity Assay Principle.

PathHunter RTK Activity assays have partial proteins with only the catalytic domain of the target kinase fused to the extracellular domain of EGFR. Upon activation by EGF, the target kinase domain is activated and phosphorylated leading to the recruitment of an SH2-domain containing protein. By tagging the intracellular domain of the target protein with PK and the SH2-domain protein with EA, we can monitor the activation of the target kinase.

Materials Provided

List of Components			
Description Kit Size	**2-Plate Kit**	**10-Plate Kit**	**Storage**
PathHunter eXpress Receptor Tyrosine Kinase Cells	2 vials (1 x 10^6 cells each)	10 vials (1 x 10^6 cells each)	-80°C (short) Liquid N_2 (long)
PathHunter Detection Kit	200 dp	1000 dp	-20°C
• Cell Assay Buffer	11.4 mL	57 mL	
• Substrate Reagent 1	3 mL	15 mL	
• Substrate Reagent 2*	0.6 mL	3 mL	
Cell Plating Reagent**	1 x 100 mL	2 x 100 mL	
96-well Tissue Culture-Treated Plates	2 plates	10 plates	Room Temperature

*Centrifuge vial before opening to maximize recovery. Replace cap tightly to avoid evaporation.
**Refer to cell-line specific data sheets for 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 and initiate the culture 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 detection reagents and cell plating (CP) reagent: • **Store at -20°C** **Note:** Once thawed, store the PathHunter 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 N2. Upon thawing, if liquid N2 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
Hemocytometer
Multimode or luminescence plate reader

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

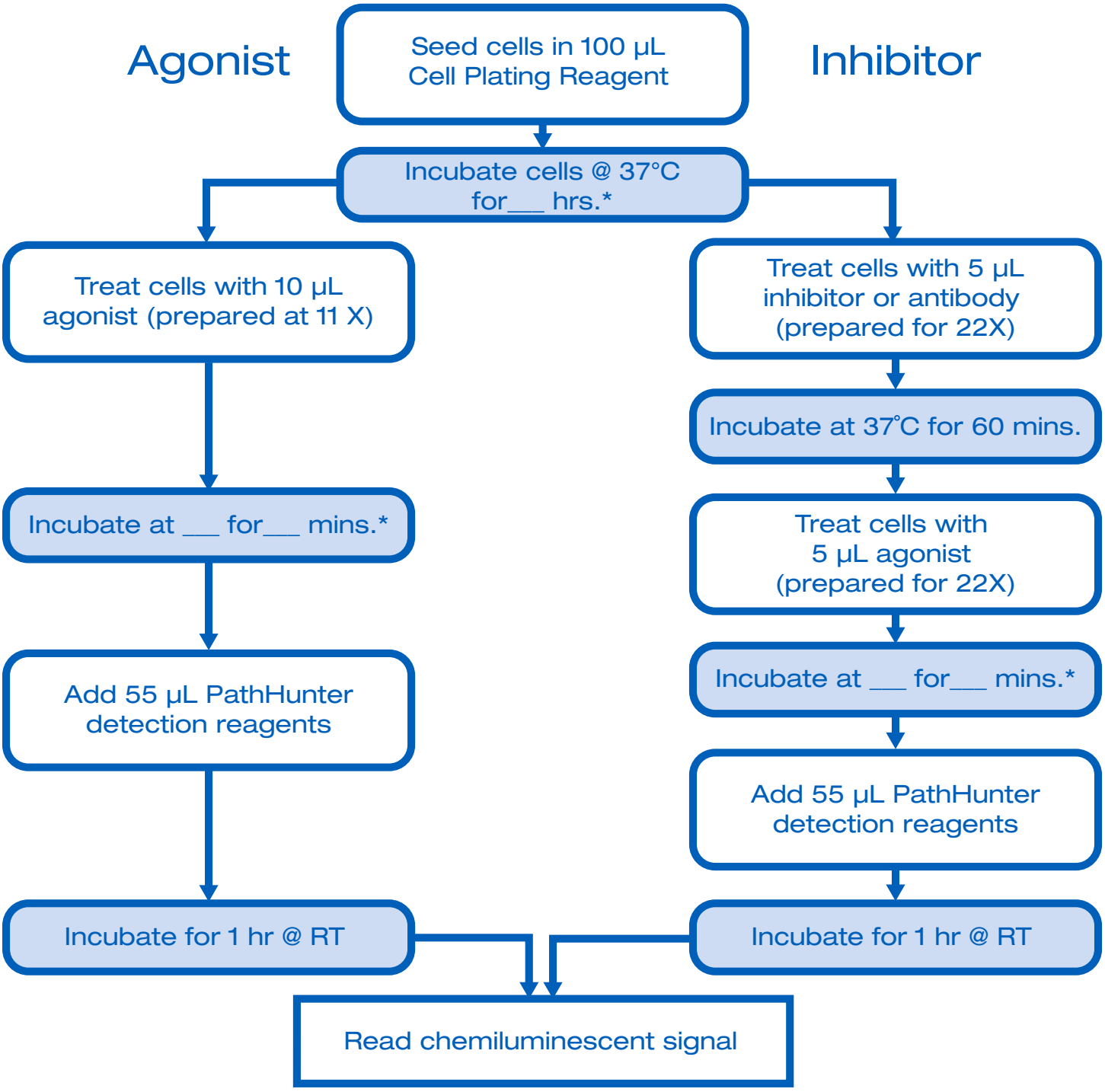
Tip: *Use this sheet to note your assay specific conditions. Post on your bench to use as a quick reference guide.*

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 cells in cryovials and seeding the cultures.

Cell Thawing Method

1. Pre-warm CP reagent in a 37˚C water bath for fifteen minutes to equilibrate pH and temperature.
2. Remove cryovials from storage and place immediately on dry ice.

Caution: 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. Do not touch the bottom of the tubes 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 uL 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.

Assay Protocol

Small Molecules

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-well and 384-well plates. For volumes associated with 384-well format, please see page 8.

Assay Protocol For Small Molecules

	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 (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: 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 hrs @ RT in the dark. These reagents are light sensitive.	
Step 5: Add Detection Reagents	Add 55 µL PathHunter 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, incubation times and temperature, please see product data sheet.

** Agonists and inhibitors prepared at 11X or 22X of final screening concentration. For detailed protocol, see compound datasheet and page 6.

***The specific concetration 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/antagonist preparation and reconstitution, please see Data Sheet for appropriate DiscoveRx Synergy products. For detailed instructions on setting up a dose response curve, please see Supplemental Protocols on page 6.

Supplemental Information

Appropriate Methods to Set Up Dose Curves and Dilutions

1. Dissolve ligand in the provided Reconstitution Buffer (see ligand specific information on data sheet).
2. Prepare a series of 12 three-fold dilutions of molecule in cell plating reagent. The concentration of each dilution should be prepared at 11X or 22X of the final screening concentration (10 µL of compound + 100 µL of cells will give 11X 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 for molecule in cell plating reagent. [We recommend targeting a working concentration 500 times the expected EC50. e.g. For an expected EC50 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 molecule to well #1.
    h. Set up additional dose curves for agonists and antagonists. 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 Cell Plating reagent.
- Compounds in organic solvents like DMSO or ethanol need special handling to ensure accuracy.
    - Prepare compound dilution curves at 110X the final screening concentration in organic solvent.
    - Dilute the 110X compound dilution series in organic solvent from the above step, 10-fold in cell plating reagent to get the working 11X concentration for entire dose curve.
    - Add 10µL of 11X working conc. to 100 µL of cells to get 1X screening concentration. The final solvent concentration will be 1% in each well.
    - Our Tyrosine Kinase assays are typically tolerant of DMSO concentrations up to 2%.
    - Compounds 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 compound at high concentrations in aqueous solution will not affect subsequent lower dilution series.

Representative Plate Maps for Agonist/Inhibitor Curve

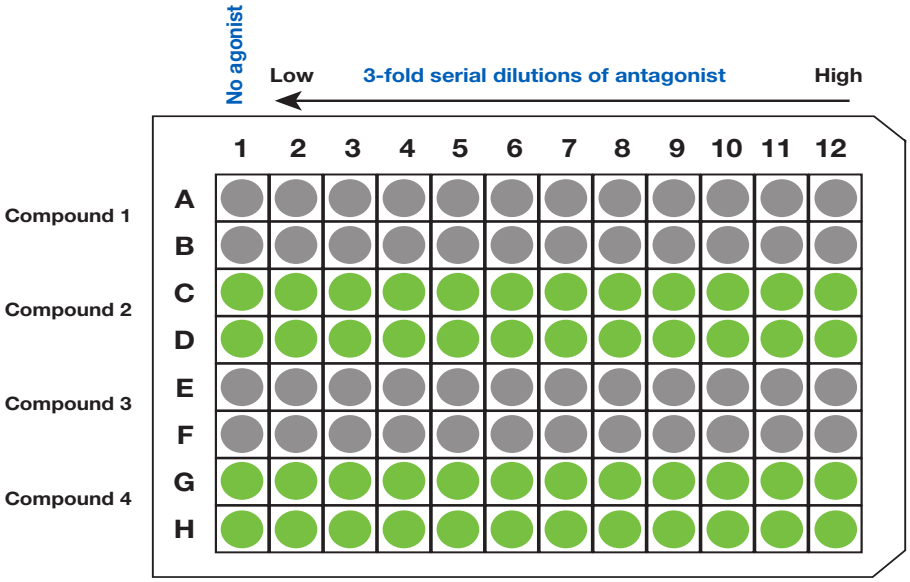


Figure 1. This plate map shows 11-point dose curves with 2 data points at each concentration for 4 compounds per plate for a total of 88 data points per 96-well plate.

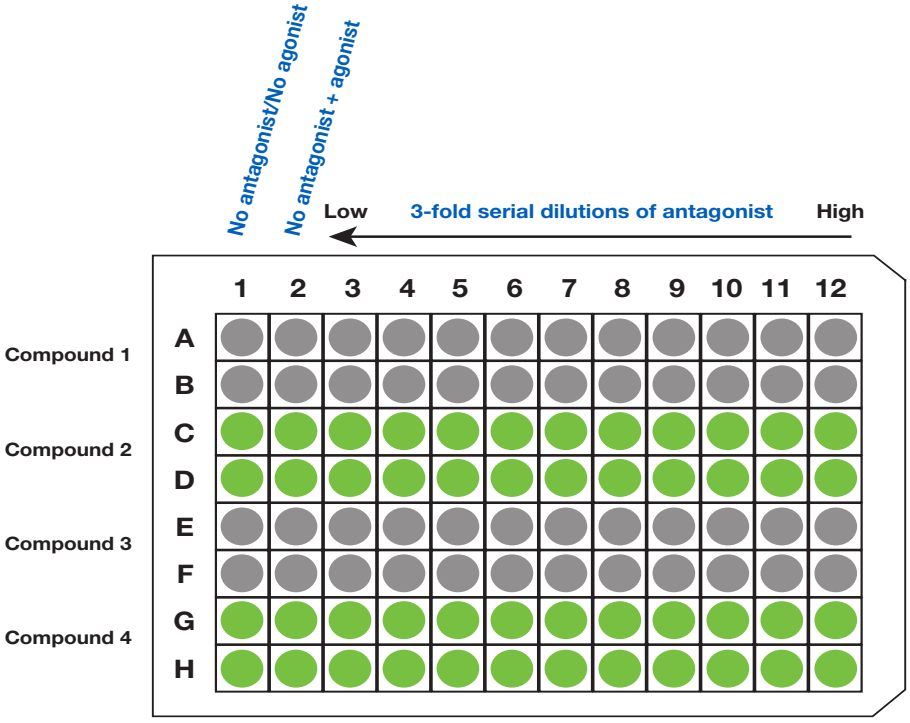


Figure 2. This plate map shows a 10 point dose curve with 2 data points each, 4 compounds per plate.

Assay Formats Table

PathHunter Certified Assay Format				
Plate Format	96-well	FV 384-well	LV 384-well	1536-well
Total Volume	165 µL	40 µL	20 µL	8 µL
Cell Numbers	10,000	10,000	5,000	1,250
Cell Plating Reagents*	100 µL	20 µL	10 µL	4 µL
Ligand	10 µL	5 µL	2.5 µL	1 µL
Detection Reagents	55 µL	12 µL	6 µL	3 µL

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

Related Products	
Description	**Ordering Information**
PathHunter Detection Reagents	www.discoverx.com/detectionreagents
Cell Culture Kits, Reagents & Consumables	www.discoverx.com
PathHunter Cell Plating Reagents	www.discoverx.com
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 **Hamamatzu** FDS6000, FDSS/RayCatcher **Thermo Scientific:** Luminoskan Ascent **Berthold:** CentroLIApc **Biotek:** Synergy 2	Luminescence

Troubleshooting Guide

Problem	Cause	Solution
No Response	High DMSO/solvent concentration	Maintain DMSO/solvent at <2% 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 microscopy
Low or No Signal	Improper preparation of detection reagents	Detection reagents should be ideally 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. The cells and detection reagents (collectively "Materials") purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Cell-Based Kinase 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
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e | info@discoverx.com

Intended Use

Your PathHunter® eXpress Receptor Tyrosine Kinase Kit, when used in conjunction with provided materials, provides a cell-based functional assay for activated receptor tyrosine kinases. The assays described in this booklet have been validated for use in both 96-well and 384-well microplate formats.