PathHunter® Receptor Tyrosine Kinase Activity Assay

For chemiluminescent detection of activated tyrosine kinases

Simple Solutions for Complex Biology

Please refer to the updated
"Cell Culture and Handling Procedure"
attached at the end of this user manual

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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DiscoveRx

Overview

Intended Use

PathHunter Receptor Tyrosine Kinase (RTK) Activity Assays 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.)

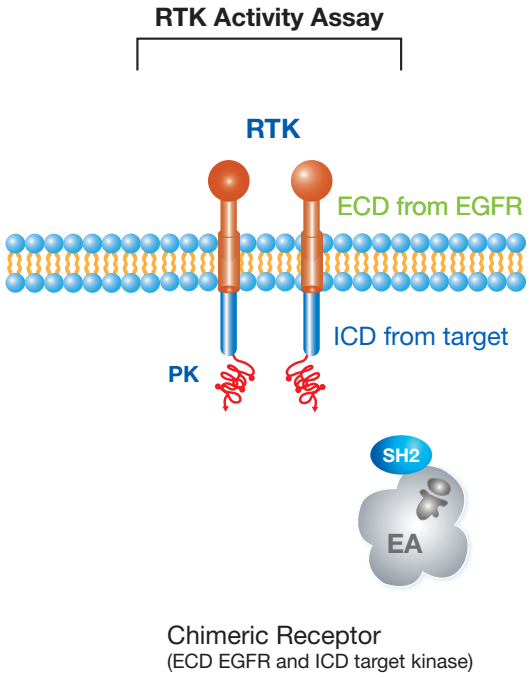


Figure 1: PathHunter Receptor Tyrosine Kinase Activity Assay Principle.

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				
	Name	**Contents**	**Size**	**Catalog Number**
Product	PathHunter RTK Activity Assay	2 vials of $4x10^6$ cells/vial in 1mL freezing media	1 Set	93-XXXXXX
Shipping Conditions	Dry Ice			
Storage Conditions of Cells	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 (LN2). *			

***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 freezer.

Additional Materials Required (Not Provided)

The following equipment and additional materials are required to perform PathHunter Receptor Tyrosine Kinase Assays:

Product Description	Vendor	Part Number
AssayComplete™ Cell Culture Kit	DiscoveRx	See product datasheet
AssayComplete™ Cell Plating Reagent	DiscoveRx	
PathHunter Detection Kit	DiscoveRx	

Product Description	Vendor	Part Number
AssayComplete™ Revive Media	DiscoveRx	See product datasheet
AssayComplete™ Preserve Freezing Reagent	DiscoveRx	
AssayComplete™ Cell Detachment Reagent	DiscoveRx	
384-well Clear bottom TC treated, Sterile (10 plates/pack)	DiscoveRx	
384-well White bottom TC treated, Sterile (10 plates/pack)	DiscoveRx	
Green V-bottom Ligand Dilution Plates (10 plates/pack)	DiscoveRx	

Equipment
Single and multichannel micro-pipettors and pipette tips
Tissue culture disposables and plasticware (T25 and T75 flasks, etc.)
Cryogenic vials for freezing cells
Hemacytometer
Multimode or luminescence plate reader

Recommended Materials

The following products* are recommended:

Product Description	Catalog No.
CytoTracker™ Cell Proliferation Kit	92-2001M
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

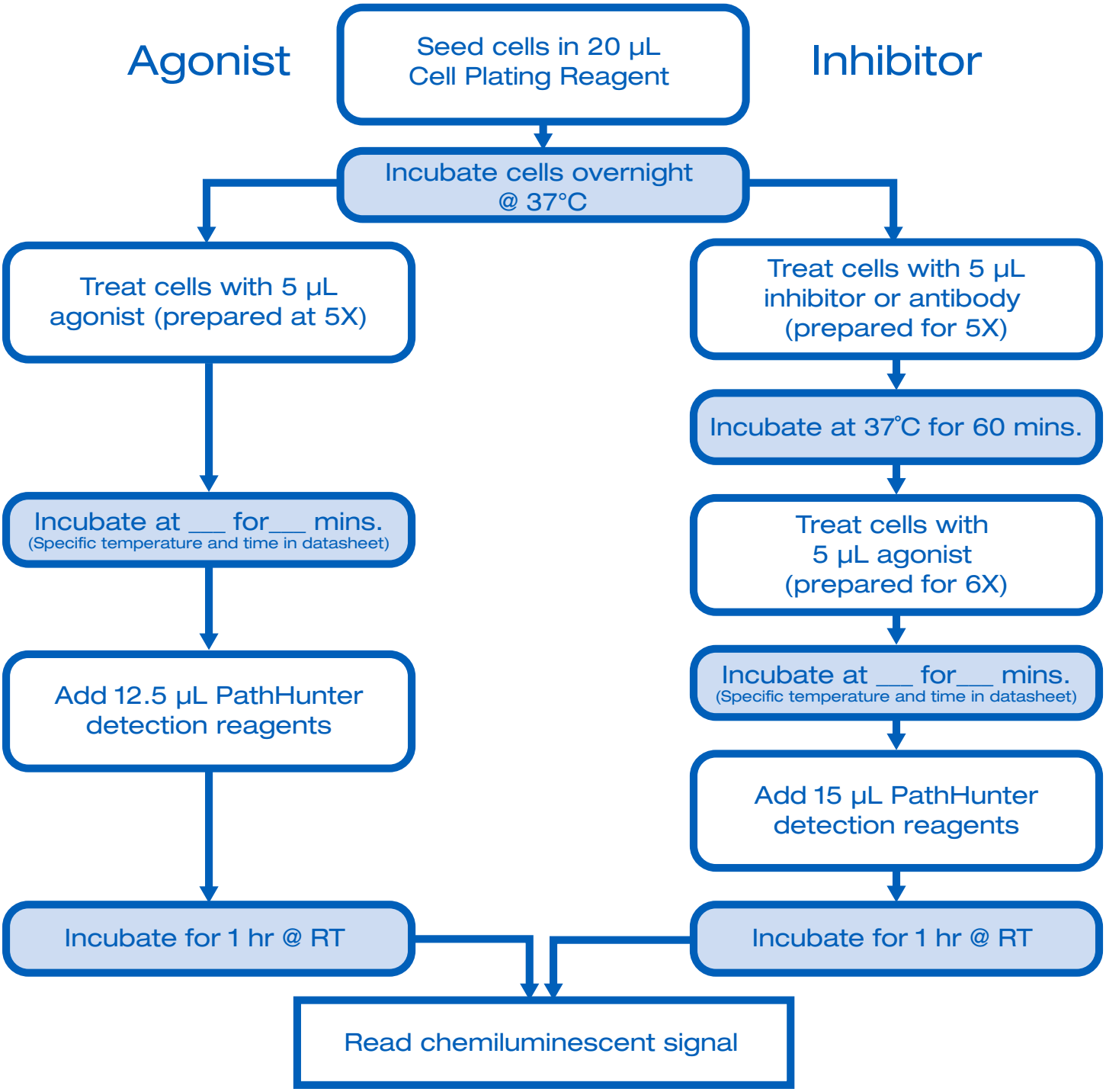
Tip: *Use this sheet to note your assay specific conditions. Post on your bench to use as a quick reference guide.*

Assay Name:_____

Product Number: _____

Quick-Start Procedure: Agonist Dose Response

In a white-walled 384-well plate perform the following:



Detailed Protocols

Cell Culture and Handling Procedures

The following protocols are for thawing cells in cryovials, seeding and maintaining the cultures once the cells are expanded.

Note: For product-specific information on AssayComplete Revive Media, Cell Culture Media, CellPlating Reagent, Preserve Freezing Reagent and Control Agonist, please see product data sheet.

Cell Thawing Method

1. Place 15 mL AssayComplete Revive Media in T75 flask and pre-warm in a humidified 37˚C/5% CO2 incubator 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. Rapidly thaw the cells in a 37˚C water bath for 30-45 seconds with gentle agitation. (Do not submerge the vial when thawing. Longer incubation times may result in cell death.)
4. Decontaminate the vial by wiping with 70% Ethanol.
5. Gently transfer the contents of the vial into pre-warmed from step one and allow cells to recover for at least 48 hours (or until 90% confluent).

Caution: Do not centrifuge to remove DMSO.

6. After 48 hours, gently remove the thawing media without disturbing the cell monolayer. If confluent, continue to passage the cells as described below. If not confluent, replace with 20mL of appropriate pre-warmed AssayComplete Cell Culture Media.

Cell Propagation Method

1. Passage cells as necessary. Typically, you can passage cells every 2-3 days with a 1:3 split ratio. Maintain cells between 30% and 80% confluence.

Caution: For the first 2-3 passages, maintain cells above 50% confluency to ensure successful growth.

2. To passage cells, aspirate medium, rinse once in PBS, add AssayComplete Cell Detachment Reagent (DiscoveRx, Cat. # 92-0009) as below.

- 6-well dish = 0.2mL AssayComplete Cell Detachment Reagent
- T25 = 1.0 mL AssayComplete Cell Detachment Reagent
- T75 = 2.0 mL AssayComplete Cell Detachment Reagent
- T225 = 6.0 mL AssayComplete Cell Detachment Reagent

Caution: Do not use Trypsin on cells

3. Cells usually detach in 2-5 minutes. Verify cell detachment under microscope. Add 3-5 mL of AssayComplete Cell Culture Media or AssayComplete Cell Plating Reagent, depending on subsequent application.

Cell Freezing Method

Note: The following procedures are for freezing cells from confluent T225 flasks. If smaller flasks are used, adjust the volumes accordingly.

1. Aspirate media from the T225 flasks. Wash cells gently with 10 mL PBS and aspirate.
2. Add 3 mL AssayComplete Cell Detachment Reagent to the flask and swirl gently to cover cells. Cells usually detach in 2-5 minutes.
3. Rinse cells with 8 mL of AssayComplete Cell Culture Media in T225 flask. Count the cells and centrifuge cells at 300xg for 5 minutes. Discard supernatant.
4. Resuspend cells in AssayComplete Preserve Freezing Reagent to a density of 4x10^6 cells/mL.
5. Dispense 1mL aliquots into cryogenic vials that are kept on ice.
6. Transfer cryogenic vials to –80°C in an insulated container (we recommend Thermo Scientific Cat. #5100-0001) for slow cooling and store overnight. The following day, transfer cryogenic vials to liquid nitrogen for long-term storage (beyond 2 weeks).

Assay Protocol

Small Molecules

After harvesting cells with AssayComplete Cell Detachment Reagent, resuspend the cells in appropriate AssayComplete Cell Plating Reagent to the correct cell concentration, suitable for plating (e.g. for 10,000 cells/20µL you need 500,000 cells/mL concentration). Please see the datasheet for assay-specific information. 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 solvents/solvent concentrations. These assays are optimized for 384-well white walled plates. For volumes associated with 96-well format, please see page 11.

Assay Protocol For Small Molecules And Purified Biologics

	Agonist Assay Protocol	Inhibitor Assay Protocol
Step 1: Plate Cells and Incubate	Seed specified number of cells (indicated on assay-specific datasheet) in 20 µL AssayComplete Cell Plating Reagent. Incubate overnight at 37°C	
Step 2: Pre-treat with Inhibitor and Incubate	NA	Treat cells with 5 µL inhibitor (5X) ** in dose curve. Incubate for indicated amount of time at indicated temperature.*
Step 3: Treat Cells with Agonist and Incubate	Induce cells with 5 µL agonist (5X) ** in dose curve. Incubate at appropriate temperature for indicated amount of time.	Induce cells with 5 µL agonist (6X) ** at EC_{90}. *** Incubate at appropriate temperature for indicated amount of time.
Step 4: Add Detection Reagents	Add 12.5 µL PathHunter Detection reagents to each well. Incubate plate at room temperature for 1 hr in the dark.	Add 15 µL PathHunter Detection Reagents to each well. Incubate plate at room temperature for 1hr in the dark.
Step 5: 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 AssayComplete Cell Culture Media, Cell Plating Reagent, control agonist, control inhibitor, incubation times and temperature, please see product data sheet.

** Agonists and inhibitors prepared at 5X or 6X of final screening concentration. For detailed protocol, see compound datasheet and page 11.

***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 9.

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 AssayComplete Cell Plating Reagent. The concentration of each dilution should be prepared at 5X of the final screening concentration (5µL of compound + 20µL of cells will give 1X final screening concentration).
    a. Label the wells of the dilution plate.
    b. Add 20µL 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 AssayComplete Cell Plating Reagent. [We recommend targeting a working concentration 250 times the expected EC50. e.g. For an expected EC50 of 10ng/mL, prepare the highest concentration in working dilution at 2500ng/mL. This is 5X the screening or final concentration of 500ng/mL].
    d. Add 30µL of highest concentration to well #12.
    e. Remove 10µL from well #12 and add it to well #11 and mix gently.
    f. With clean tip, remove 10µL from well #11 and add it to well #10. Mix gently.
    g. Repeat process until well #2. DO NOT add molecule to well #1.
    h. Set up additional dose curves for agonists and antagonists. See figures x and y 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 Cell Plating Reagent.
- Compounds in organic solvents like DMSO or Ethanol need special handling to ensure accuracy.
    - Prepare compound dilution curves at 100X the final screening concentration in organic solvent.
    - Dilute the 100X compound dilution series in organic solvent from the above step, 20-fold in AssayComplete Cell Plating Reagent to get the working 5X concentration for entire dose curve.
    - Add 5µL of 5X working conc. to 20µL of cells to get 1X screening conc. 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

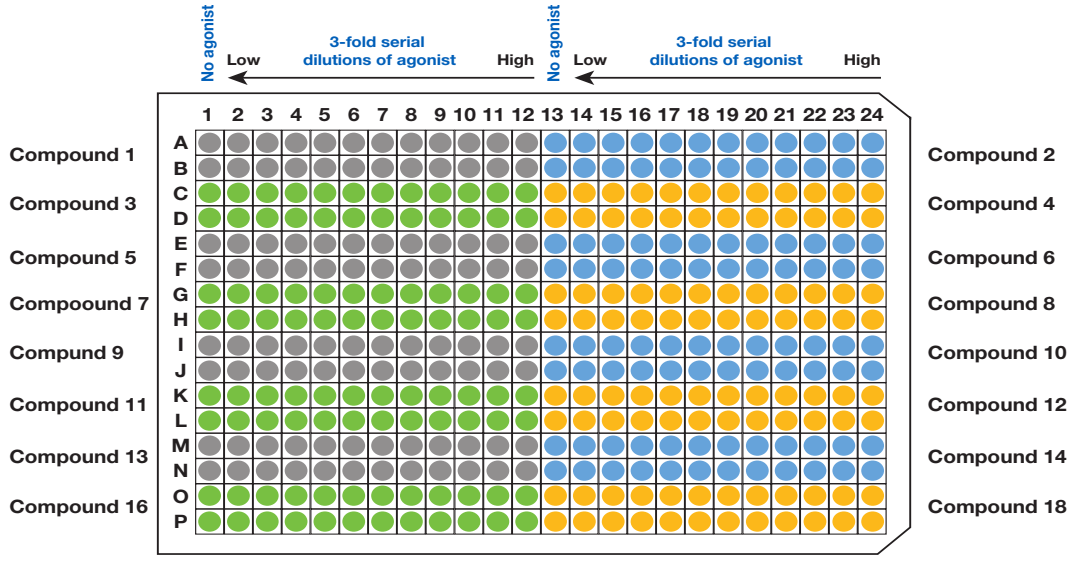


Figure 1. This plate map shows 12-point dose curves with 2 data points at each concentration for 16 compounds per plate for a total of 192 data points per 384-well plate.

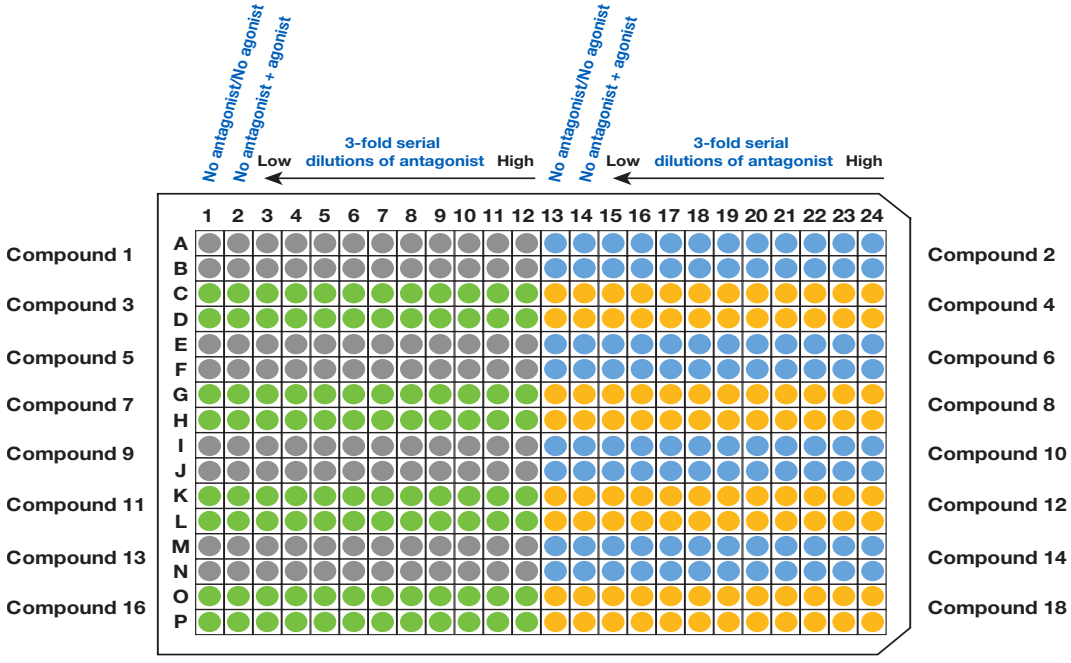


Figure 2. This plate map shows an 11-point dose curve with 2 data points each 16 compounds per plate for a total of 176 data points per 384-well plate.

Assay Formats Table

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	10,000	5,000	1,250
Cell Plating Reagent*	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

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

Instrument Compatibility Chart			
Assay	**Instrument**	**Read-Out**	**Specifications**
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	Excitation Filter Fluorescein- 485 nm Emission Filter Fluorescein- 530 nm Dichroic Fluorescein- 505 nm

Troubleshooting Guide

Problem	Cause	Solution
No Response	Improper cell growth conditions	See datasheet 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 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	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 datasheet for cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 sec/well.
Experimental S:B does not match datasheet value	For cell pools, S:B may vary greatly from passage to passage or day to day	Prepare a clonal cell line or use lower passage number cells.
		Repeat the assay
		Confirm assay conditions
	Incorrect incubation temperature	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
Cells growing slowly	U2OS grows 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
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 and propagate 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® Receptor Tyrosine Kinase Activity Assay, when used in conjunction with a PathHunter Detection Kit (93-0001, 93-0001L or 93-0001XL), 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.

Technical Bulletin

Updated Cell Culture Protocol and Handling Procedure

Applies to: Cell Lines Only

Product Numbers: Several

Date: February 28, 2017

Related Documents: AssayComplete™ Product Lines Product Change Notification
discoverx.com/reagents

Dear Valued Customer,

DiscoverX constantly strives to deliver simple experimental workflows to ensure best performance of our assays. To ensure that these protocols work equally well in your hands, we have updated the “Cell Culture and Handling Procedure” for our cell lines.

The protocol is included in this bulletin and a summary of the updates is provided in Appendix 1. We are currently updating all our cell line user manuals to incorporate this updated protocol.

In the meantime, please use the attached protocol for thawing, propagation, and freezing of your cell lines until the revised cell line user manuals become available.

If you have any questions, please contact our technical support team at SupportUS@discoverx.com (in North America and Asia-Pacific) or SupportEurope@discoverx.com (in Europe).

Sincerely,

The DiscoverX team

Cell Culture and Handling Procedure

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells shipped in the vial). Upon receipt, the vials should be transferred to liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.
2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.
3. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid nitrogen inside the vial to evaporate.
4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line product datasheet for the specific AssayComplete products listed in the protocol below.

Contact technical support immediately if cells are thawed upon arrival.

A face shield, gloves, and a lab coat should be worn during these procedures.

Cryovials should be stored in the vapor phase. They are not rated for storage in the liquid phase of liquid nitrogen.

Cell Culture Protocol

The following procedures are for thawing cells in cryovials, seeding and expanding the cells, maintaining the cell cultures once the cells have been propagated, and preparing them for assay detection.

Care should be taken in cell handling to avoid contamination.

Cell Thawing

The following is a protocol for thawing cells in a T75 flask.

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.
2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask in a sterile tissue culture hood. Set aside for step 6 below. DO NOT add selection antibiotics to the thawing reagent.

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

3. Remove the cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them on dry ice prior to thawing.
4. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.
5. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.

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6. With a pipette, gently transfer the thawed cells to the pre-filled T75 flask and incubate at 37°C and 5% CO_2.
7. Maintain the cells in culture until they are >70% confluent. Then proceed to "Cell Propagation" instructions. Do not split if cells are below this confluency or growth issues may occur.

Cell Propagation

The following is a protocol for propagating cells once they become ≥70% confluent in a T75 flask.

1. Pre-warm AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.
2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate media from the T75 flask.
4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire surface of the flask to rinse the cells.
5. Gently aspirate PBS from flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
7. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with trypsin.
8. Incubate the flask at 37°C, 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

Prolonged treatment with Trypsin-EDTA may compromise cell viability

10. Add 4 mL of AssayComplete Thawing Reagent to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
12. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are included in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK 293	1:3
U2OS	1:2

For example, for CHO-K1 cells, add 4 mL of AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5th of the total reagent in the flask) into each new tissue culture flask.

13. Fill a new T75 or T225 flask with AssayComplete Thawing Reagent until the total volume equals 12 mL for T75 flasks (or 45 mL for T225 flasks), and add cell suspension (added volume determined at step 12) to the media in the flask. Transfer flask to a tissue culture incubator and incubate cells for 24 hours at 37°C and 5% CO_2.

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

14. After 24 hours, examine the cells under a microscope. If the cells appear to be healthy (adhering to the surface of the flask with only a small number of cells remaining in suspension) exchange the AssayComplete™ Thawing Reagent with AssayComplete Cell Culture Reagent supplemented with the recommended concentration of selection antibiotic (refer to cell line datasheet to determine the correct Cell Culture Kit and recommended antibiotic and antibiotic concentration for your cell line); 12 mL for T75 flasks (or 45 mL for T225 flasks). Then return the flask to a tissue culture incubator. If the cells do not appear to be healthy or if confluency is <25%, incubate for an additional 24 to 48 hours to allow for additional cell recovery before executing this step.

15. Once the cells become ≥70% confluent in the flask, split the cells every 2 to 3 days, based on the doubling time of the cell line, using AssayComplete Cell Culture Reagent supplemented with the proper selection antibiotics. Typical split ratios for common cell backgrounds are included in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:10
HEK 293	1:5
U2OS	1:3

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 1 X 10^6 per vial).

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.

Care should be taken in handling to avoid contamination.

2. Gently aspirate the media from the flasks.
3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).
6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.

Keep cells on ice during this process and transfer to a cryogenic container.

7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. 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.
9. Add 5 mL AssayComplete Cell Culture Kit media to each T75 flask (or 15 mL to each T225 flask).
10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.
11. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional AssayComplete Cell Culture

Kit media to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.

12. For the purpose of determining the concentration of cells in the suspension:
    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.
    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.
13. Centrifuge the collected cells at 300 X g for 4 minutes.
14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.
15. Based on the total cell number calculated in step 12 above, re-suspend cells to the desired concentration (e.g. 1 X 10^6 to 2 X 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent.
16. Make 1 mL aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.
17. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

Keep cells on ice during this process to protect cell viability.

Appendix 1

Summary of updates in the “Cell Culture and Handling Procedure”

- When thawing cells, DO NOT centrifuge or vortex freshly thawed cells.
- Once cells are thawed, incubate cells in the AssayComplete Thawing Reagent without any selection antibiotics. Do not use selection antibiotics for the first passage to ensure robust recovery.
- For subsequent passages, supplement the cell line-specific cell culture media with appropriate selection antibiotic.
- For routine propagation and maintenance of adherent cell lines, use Trypsin-EDTA for detaching cells. Use of the AssayComplete Cell Detachment Reagent for routine cell passaging is not necessary.

Additionally, the recommendation for use of the Cell Detachment Reagent has been revised.

When preparing cells for the assay:

- Use AssayComplete Cell Detachment Reagent (Part number 92-0009) for detaching cells. Do not use trypsin for this step, especially in assays interrogating membrane-anchored receptors (e.g. GPCRs). Use of trypsin at this step can negatively affect assay results.
- Also, it is important to rinse the cells with 2 mL AssayComplete Cell Detachment Reagent, rather than with PBS, as rinsing with PBS may inhibit the detachment of cells from the flask surface.