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PathHunter® Receptor Tyrosine Kinase Internalization Assays

For chemiluminescent detection of activated receptor tyrosine kinases

User Manual

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

Simple Solutions for Complex Biology

CONTENTS

NOTES:

NOTES:

LEGAL SECTION

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:

DiscoveRx Corporation
Attn: Licensing Department
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INTENDED USE

PathHunter® Receptor Tyrosine Kinase (RTK) Internalization Assays are non-imaging, non-antibody based chemiluminescent detection assays that provide a direct and quantitative measurement of internalized RTKs localized to early endosomes. Your PathHunter Receptor Tyrosine Kinase Internalization Cell Line, when used in conjunction with a PathHunter Detection Kit (93-0001S, 93-0001 or 93-0001L), provides a cell-based functional assay for receptor tyrosine kinase internalization.

TECHNOLOGY PRINCIPLE

PathHunter cell lines feature novel *in vivo* applications of Enzyme Fragment Complementation (EFC) technology in which the β-galactosidase (β-gal) enzyme has been split into two inactive fragments. In the PathHunter assay approach for receptor tyrosine kinase internalization, the small enzyme fragment of β-galactosidase (ProLink™) is appended to the C-terminus of receptor tyrosine kinase while the larger, complementing enzyme fragment (termed Enzyme Acceptor or EA) is fused to the surface of cellular endosomes. Stimulation of the RTK results in receptor activation, subsequent internalization of the receptor and trafficking to cellular endosomes. Presence of receptor tyrosine kinase in the endosome enables EA and ProLink fragments to complement resulting in the formation of an active β-galactosidase and an increase in activity that is easily measured using PathHunter Detection Reagents.

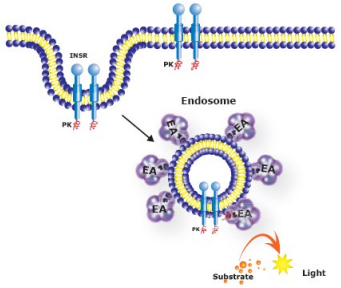


Figure 1. PathHunter Receptor Tyrosine Kinase Internalization Assay Principle.

ASSAY OVERVIEW

To perform PathHunter Receptor Tyrosine Kinase Internalization Assays, you will also need PathHunter Detection Reagents in order to generate the chemilumescent signal. Assays should be run using fresh, low-passage cells that have not been allowed to reach confluency for more than 24 hours. Ideally cells should be grown to ~80% confluence. Following cell treatment, the assay is performed by adding a working solution of PathHunter Detection Reagents to the treated cells in a no-mix, one-addition protocol. After addition of the detection reagents, the samples must be read within 2 hours; longer incubation time will lead to increased background. The reaction is complete in 60 minutes, with no significant further increase in signal strength. The **Assay Procedure** sections and **Quick Start Guides** in this booklet contain detailed information about how to run the assays using an RTK agonist, an antibody, or a receptor antagonist.

RELATED PRODUCTS (CONTINUED)

TARGET	PRODUCT NAME	CATALOG NUMBER	CATALOG NUMBER
		Cell Line	Express
c-MET	PathHunter™ C-MET Functional Assay	93-0632C3	
c-Ret-GFRα2	PathHunter™ c-Ret-GFRα2 Functional Assay	93-0816C3	
DDR1	PathHunter™ DDR1 Functional Assay	93-0578C3	93-0578E3CP16
DDR2	PathHunter™ DDR2 Functional Assay	93-0711C3	
EphB4	PathHunter™ EphB4 Functional Assay	93-0468C3	93-0468E3CP16
ErbB1	PathHunter™ ErbB1 Functional Assay	93-0681C3	
ErbB2/ErbB3	PathHunter™ ErbB2/ErbB3 Functional Assay	93-0535C3	93-0535E3CP16
ErbB4	PathHunter™ ErbB4 Functional Assay	93-0465C3	
FGFR4	PathHunter™ FGFR4 Functional Assay	93-0467C3	93-0467E3CP16
Flt3	PathHunter™ Flt3 Functional Assay	93-0506C3	93-0506E3CP16
IGFR1	PathHunter™ IGFR1 Functional Assay	93-0505C1	93-0505E1CP17
INSR	PathHunter™ INSR Functional Assay	93-0466C3	93-0466E3CP16
INSR	PathHunter® INSR Internalization Assay	93-0808C3	
PDGFRb	PathHunter™ PDGFRb Functional Assay	93-0493C3	93-0493E3CP16
TrkA	PathHunter™ TrkA Functional Assay	93-0462C3	93-0462E3CP16
TrkA-P75	PathHunter™ TrkA - P75 Functional Assay	93-0529C3	93-0529E3CP16
TrkB	PathHunter™ TrkB Functional Assay	93-0463C3	
TrkB-P75	PathHunter™ TrkB - P75 Functional Assay	93-0530C3	93-0530E3CP16
TrkC	PathHunter™ TrkC Functional Assay	93-0464C3	93-0464E3CP16
TrkC-P75	PathHunter™ TrkC - P75 Functional Assay	93-0531C3	93-0531E3CP16

TROUBLESHOOTING GUIDE (continued)

PROBLEM	CAUSE	SOLUTION
High well-to-well variability 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.
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.

RELATED LIGANDS

DiscoveRx Ligand	Ligand Catalog #	Associated Target
Bovine Type II Collagen, Immunization Grade	92-1090	DDR1
Recombinant Mouse EphrinB2-Fc	92-1089	EphB4
Recombinant Human Heregulin-b1	92-1031	ErbB2/ErbB3
Recombinant Human FLT3-Ligand	92-1029	Flt3
Recombinant Human IGF-I	92-1030	IGF1R
Recombinant Human IGF-I	92-1030	IGF1R
Insulin	92-1088	INSR
Insulin	92-1088	INSR
Recombinant Human PDGF-AB	92-1028	PDGFRb
Recombinant Human b-NGF	92-1023	TrkA
Recombinant Human b-NGF	92-1023	TrkA-p75
Recombinant Human BDNF	92-1024	TrkB
Recombinant Human BDNF	92-1024	TrkB-p75
Recombinant Human NT3	92-1025	TrkC
Recombinant Human NT3	92-1025	TrkC-p75

MATERIALS PROVIDED

Please refer to the Product Insert for your PathHunter Receptor Tyrosine Kinase Internalization Assay.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

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

Equipment	Materials
• Single- and multichannel micro-pipettors and pipette tips • Tissue culture disposables and plasticware (T25 and T75 flasks, etc.) • Cryogenic vials for freezing cells • V-bottom 384-well compound dilution plates • Disposable reagent reservoir (Thermo Scientific, Cat. #8094 or similar) • Hemocytometer • White wall, clear bottom 384–well or 96-well microplates • Multimode or luminescence plate reader	• PathHunter® Detection Kit (DiscoveRx Cat. #93-0001S, 93-0001 or 93-0001L) • AssayComplete™ Cell Culture Kit (DiscoveRx Cat. # varies)***** • AssayComplete Cell Plating Reagent (DiscoveRx, Cat. # varies)***** • AssayComplete Cell Detachment Reagent (DiscoveRx Cat. #92-0009) • AssayComplete Revive Reagent (DiscoveRx, Cat. # varies)***** • AssayComplete Preserve Freezing Reagent (DiscoveRx, Cat. # varies)***** • Phosphate buffered saline (PBS) • RTK ligands/agonists, test compounds, antagonists, or antibodies
*****See the Product Insert of your PathHunter cell line for specific tissue culture requirements.	

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Cryovials are shipped in vials on dry ice and contain 4 x10^6 cells per vial in 1 mL of AssayComplete Preserve Freezing Reagent. 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. A face shield, gloves, and a lab coat should be worn during these procedures.

1) PathHunter cells must arrive in a frozen state on dry ice. If cells arrive thawed, do not proceed. Contact technical support.

2) Frozen cells must be 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. **CRYOVIALS ARE NOT RATED FOR STORAGE IN THE LIQUID PHASE OF LIQUID NITROGEN. CRYOVIALS SHOULD BE STORED IN THE VAPOR PHASE.**

3) 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 nitrogen inside the vial to evaporate.

4) Proceed with the thawing protocol in the following section.

SAFETY WARNING: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Some cryovials can leak when submerged in liquid N_2. Upon thawing, the liquid N_2 present in the cryovial converts back to its gas phase which can result in the vessel exploding.

CELL THAWING AND PROPAGATION METHODS

The following procedures are for thawing cells in cryovials, seeding and expanding the cells, and maintaining the cultures once the cells are expanded. Cells are free of contamination prior to shipment and care should be taken in their handling to avoid contaminating them. **Face shield, gloves and a lab coat should be worn during the thawing procedure.**

1) When removing cryovials from 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.

2) Pre-warm 12 mL AssayComplete Revive Media in a 37°C water bath. The cryovial containing 4 x 10^6 cells can be added to 12 mL of AssayComplete Revive Media.

3) Place the frozen cell vial briefly in a 37°C water bath under sterile conditions until only small ice crystals remain and the cell pellet is almost completely thawed (10 sec - 1 min). **Caution: Longer incubation times may result in cell death**.

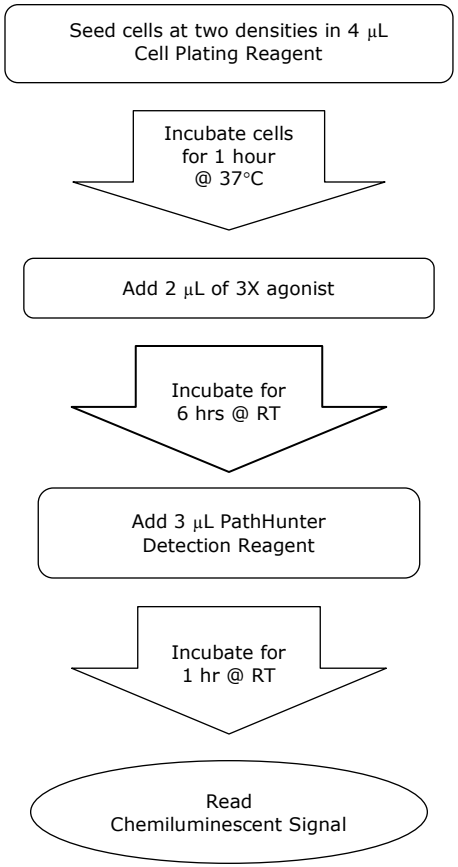
4) Resuspend cells from 1 vial (4 x 10^6 cell per vial) into the tube containing 12 mL of pre-warmed AssayComplete Revive Media, transfer to a T75 flask, and grow for 48 hours. **Do not centrifuge to remove DMSO.**

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No Response	Improper cell growth conditions	See Product Insert for cell culture conditions
	High DMSO/solvent concentration	Maintain DMSO/solvent at < 1% in serial dilutions of compounds.
	Improper ligand incubation time; suboptimal for induction	Optimize ligand incubation time.
Low or No Signal	Improper preparation of detection reagents	Detection reagents should be prepared just prior to use and are sensitive to light.
	Problem with cell growth, cell viability, cell adherence or cell density	See Product Insert for cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 sec/well.
Experimental S:B does not match Product Insert 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.
	Improper preparation of ligand (agonist or ant-agonist)	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.

QUICK-START PROCEDURE: SCREEN-FRIENDLY PROTOCOL FOR 1536 MINIATURIZATION

In a white-walled 1536-well plate, perform the following:

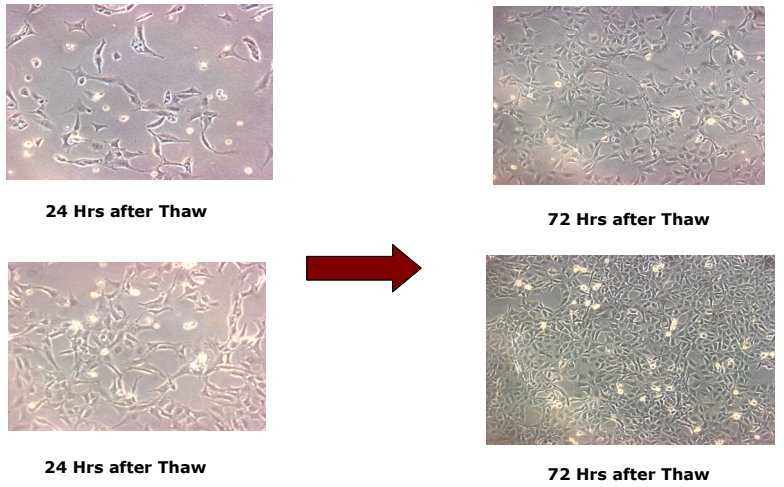


5) After 48 hours, gently remove the AssayComplete Revive Media (being careful not to disturb the cell monolayer) and replace with 12 mL of pre-warmed AssayComplete Cell Culture Media containing the appropriate selection antibiotics.

6) Once cells become confluent in a T75 flask, passage cells every 2–3 days using AssayComplete Cell Detachment Reagent. Dilute passaged cells into a fresh T75 flask at a 1:3 dilution in a total volume of 12 mL of appropriate AssayComplete Cell Culture Media containing selection antibiotics. Once transferred and growing, cells should be maintained in a non-confluent state.

NOTE:
TO MAINTAIN THE LOGARITHMIC GROWTH OF THE CELLS, CULTURES SHOULD BE MAINTAINED IN A SUB-CONFLUENT MONOLAYER.

NOTE:
The 24 and 72 hr post-thaw cells were photographed at different magnifications.



24 Hrs after Thaw **72 Hrs after Thaw**

24 Hrs after Thaw **72 Hrs after Thaw**

7) The clone has been found to be stable for at least 10 passages with no significant drop in assay window or EC_{50} shifts.

CELL FREEZING PROTOCOL

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

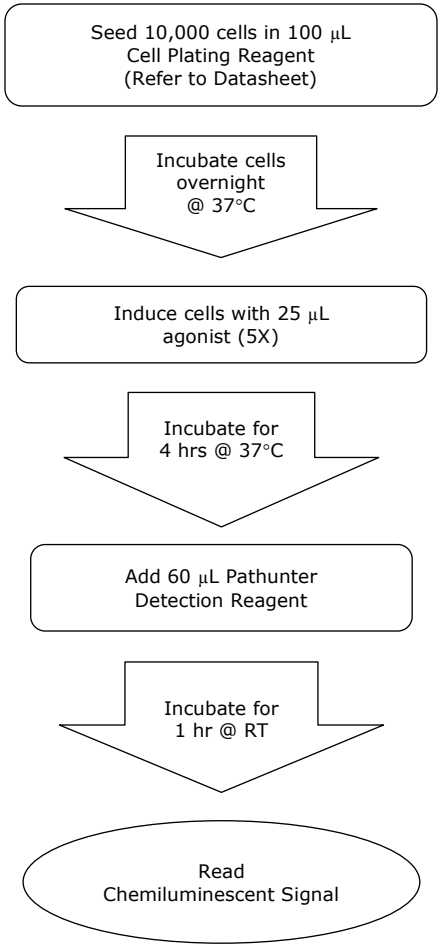
1) Remove T225 flasks from incubator and place in tissue culture hood. Aspirate the media from the flasks.
2) Add 10 mL PBS into each T225 flask and swirl to rinse the cells. Aspirate PBS from flask.
3) Add 3 mL AssayComplete Cell Detachment Reagent to the flask. Rock the flask back and forth gently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

LIGAND HANDLING INSTRUCTIONS

1) Upon arrival if the ligand is not used immediately it should be stored at –20°C. Follow the manufacturer's recommendation for appropriate storage.
2) Reconstitute the ligand in the appropriate solvent as described on the ligand datasheet.
3) Mix solution thoroughly to ensure complete reconstitution of the ligand. Centrifuge the ligand solution for 30 seconds on a regular bench top centrifuge to ensure proper dissolution of the ligand.
4) The ligand is now ready for use.
5) If longer storage is required, we recommend dispensing the stock to smaller, single-use volumes. Store the dispensed ligand solution at –20°C.

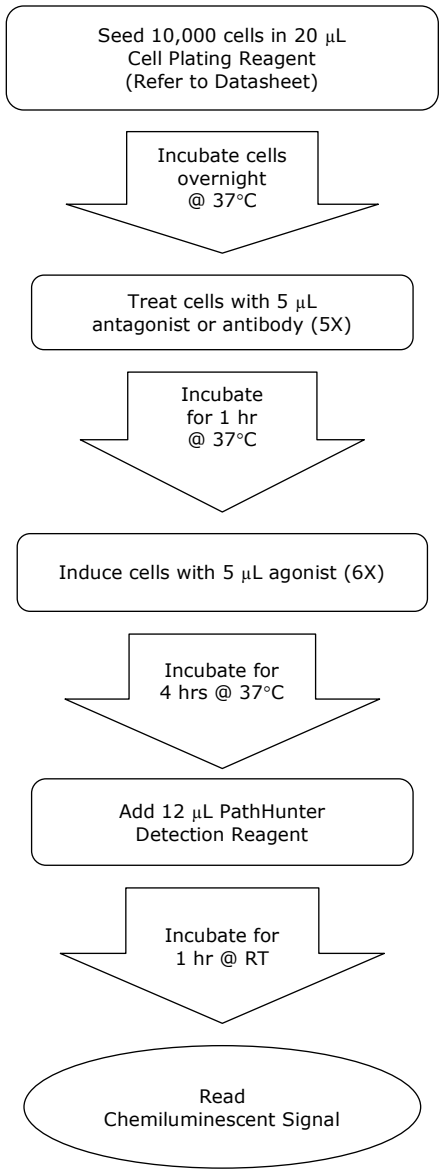
QUICK-START PROCEDURE: 96-WELL AGONIST PROTOCOL

In a white-walled 96-well plate perform the following:



QUICK-START PROCEDURE: ANTAGONIST OR ANTIBODY

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



ASSAY PROCEDURE - LIGAND/AGONIST DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing RTK agonist assays using the PathHunter RTK Internalization Cell Lines and PathHunter Detection Reagents in a 384-well format. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least *duplicate* wells for each dilution.

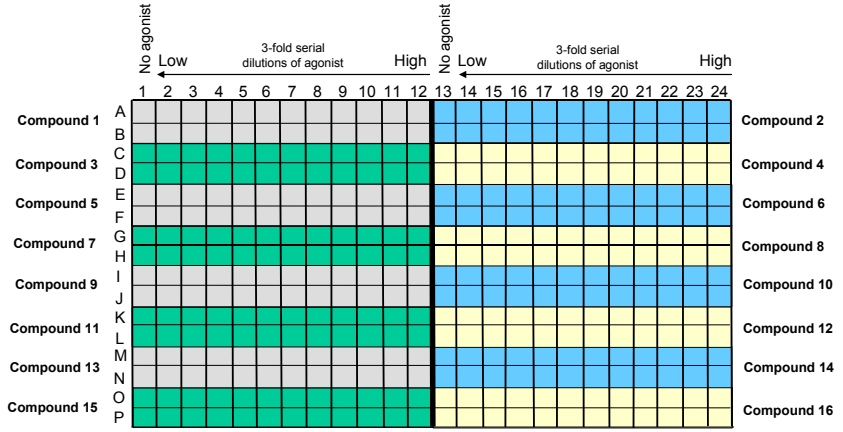


Figure 2. This plate map shows 12-point dose curves with 2 data points at each concentration for 16 compounds per plate for a total of 160 compound dilutions per 384-well plate.

PROTOCOL: LIGAND/AGONIST DOSE RESPONSE

A. DAY 1: PREPARING ASSAY PLATES

1) Harvest cells as follows from a confluent T25 or T75 flask using AssayComplete Cell Detachment Reagent (DiscoveRx, Cat. # 92-0009).
   NOTE:
   DO NOT USE TRYPSIN.
   a) Remove medium.
   b) Wash cells with 5 mL PBS and aspirate.
   c) Add 0.5 mL AssayComplete Cell Detachment Reagent for a T25 flask, or 1 mL AssayComplete Cell Detachment Reagent for a T75 flask.
   d) Rock the flask back and forth gently to ensure surface of the flask is covered.
   e) Place flask in the incubator for 5 minutes, or until cells have detached.
   f) Add 3 mL of AssayComplete Cell Plating Reagent and transfer to a conical tube.
2) Determine cell density using a hemocytometer.
3) Using AssayComplete Cell Plating Reagent, adjust the volume of the suspension to achieve a cell concentration of 500,000 cells/mL (10,000 cells/20 µL).
4) Transfer 20 µL of the cell suspension to each well of a 384-well white-walled microplate.

5) Incubate the plate overnight at 37°C/5% CO_2.

B. DAY 2: LIGAND/AGONIST PREPARATION AND ADDITION

1) Dissolve ligand in the appropriate solvent at the desired stock concentration.

NOTE:
SOLVENTS CAN AFFECT ASSAY PERFORMANCE. PATHHUNTER ASSAYS ARE ROUTINELY CARRIED OUT IN THE PRESENCE OF ≤ 1% SOLVENT (E.G. DMSO, ETHANOL, PBS). IF YOU USE OTHER SOLVENTS OR SOLVENT CONCENTRATIONS, OPTIMIZE THE ASSAY CONDITIONS ACCORDINGLY. DO NOT STORE LIGANDS AT ROOM TEMPERATURE FOR EXTENDED PERIODS OF TIME.

2) Prepare a series of twelve 4-fold serial dilutions of ligand in the appropriate solvent as described below. The concentration of each dilution should be prepared at 5X the final screening concentration (i.e., 5 µL compound + 20 µL of cells). For each dilution, the final concentration of solvent should remain constant.

- To begin the 12-point curve, we recommend targeting a final concentration that is **50X** the expected EC_{50} value for the compound (e.g., **250X** the final screening concentration). **Example:** If the expected EC_{50} is 10 ng/mL, prepare the highest starting concentration of the corresponding dilution at 2.5 µg/mL. This is the working concentration.

a) Label the wells of a dilution plate, #1 through #12.

b) Add 30 µL of ligand vehicle to dilution wells #1–11.

c) Prepare a working concentration of ligand in the appropriate ligand vehicle.

d) Add 40 µL of the working concentration of ligand to well #12.

e) Remove 10 µL of compound from well #12, add it to well #11 and mix gently by pipetting up and down. Discard pipette tip.

f) With a clean pipette tip, remove 10 µL of diluted compound from well #11, add it to well #10 and mix gently by pipetting up and down. Discard the pipette tip.

g) Repeat this process 8 more times in succession to prepare serial dilutions for the remaining wells, or from right to left across the plate.

h) **DO NOT add ligand to well #1**. Add only ligand vehicle. This sample serves as the no agonist control and completes the dose curve.

i) Repeat this process for any additional ligands.

j) Set the dilutions aside until you are ready to add them to the cells.

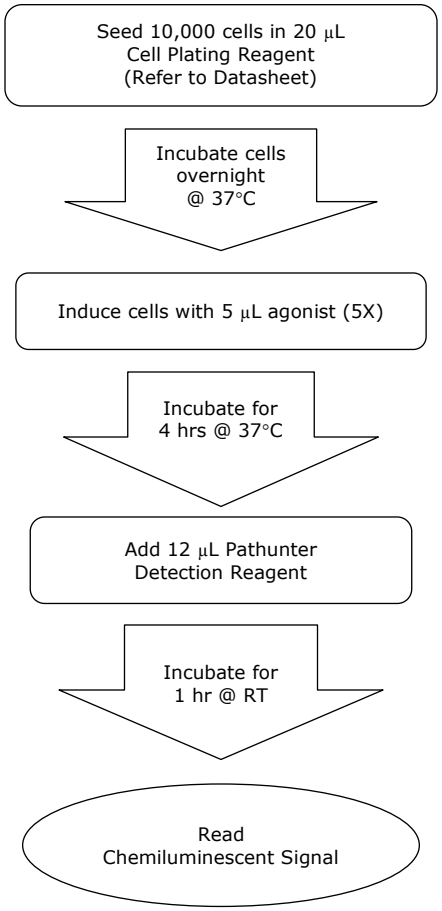
3) Remove PathHunter cells from the incubator (previously plated on day 1).

4) Transfer 5 µL of the control and 5X ligand dilutions from wells #1-12 to assay plate wells according to the plate map shown in Figure 2.

5) Incubate for 4 hours @ 37°C.

QUICK-START PROCEDURE: LIGAND/AGONIST DOSE RESPONSE

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



C. LIGAND/AGONIST PREPARATION AND ADDITION

1) During the antagonist incubation, calculate the EC_{80} concentration of the receptor ligand/agonist from an existing dose response curve (described on pages 8–10). Prepare a solution of the ligand at a concentration equivalent to **6X** the EC_{80} in the appropriate ligand vehicle.

   Example: If the expected EC_{80} of the agonist compound is 10 ng/mL, prepare a stock at 60 ng/mL.

2) When the antagonist incubation is complete, add 5 µL of agonist compound to wells #2–12. Add 5 µL of ligand vehicle to the "No agonist" wells (column 1 in Figure 3).

3) Incubate for 4 hours @ 37°C.

D. DETECTION OF INTERNALIZED RECEPTOR TYROSINE KINASE

1) Prepare PathHunter Detection Reagent by combining 1 part **Substrate Reagent 2** with 5 parts **Substrate Reagent 1**, and 19 parts **Cell Assay Buffer**, as described in the PathHunter Detection Kit (DiscoveRx, Cat. # 93-0001 Series) product insert.

2) Add 12 µL of prepared detection reagent to the appropriate wells. **DO NOT pipet up and down in the well to mix or vortex/shake plates.**

3) Incubate for 1 hr at room temperature.

   NOTE:
   ALL ASSAYS MUST BE PERFORMED AT ROOM TEMPERATURE. WE HAVE TESTED THE EFFECT OF TEMPERATURE ON THE PATHHUNTER ASSAY AND THIS APPEARS TO BE THE OPTIMUM INCUBATION TEMPERATURE RANGE. THE ASSAY WILL NOT WORK AT 37°C. WE RECOMMEND A PLATE INCUBATOR SET TO 22–25°C.

4) Read samples on any standard luminescence plate reader.
   [Compound potencies (EC_{50} or IC_{50}) can be derived from a four-parameter nonlinear iterative curve-fitting analysis.]

5) Use GraphPad Prism® or other comparable program to plot your agonist dose response. See the examples shown in the Assay Validation section.

C. DETECTION OF INTERNALIZED RECEPTOR TYROSINE KINASE

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2) Add 12 µL of prepared detection reagent to the appropriate wells. **DO NOT pipet up and down in the well to mix or vortex/shake plates.**

3) Incubate for 1 hr at room temperature .

   NOTE:
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4) Read samples on any standard luminescence plate reader.
   [Compound potencies (EC_{50} or IC_{50}) can be derived from a four-parameter nonlinear iterative curve-fitting analysis.]

5) Use GraphPad Prism® or other comparable program to plot your agonist dose response. See the examples shown in the Assay Validation section.

ASSAY PROCEDURE - ANTIBODY/ANTAGONIST DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing RTK antagonist or antibody assays using PathHunter RTK Internalization Cell Lines and PathHunter Detection Reagents in a 384-well format. Although plate layouts and experimental designs may vary, we recommend performing a 12-point dose curve for each compound using at least *duplicate* wells for each dilution.

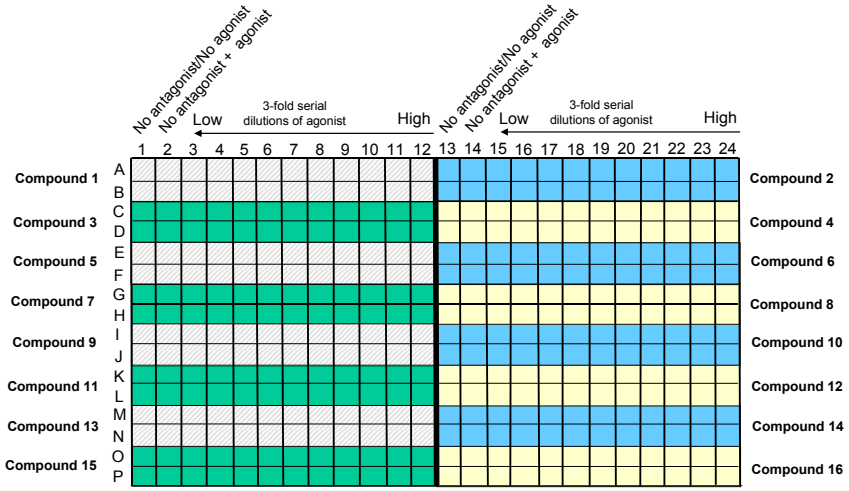

Figure 3. This illustration shows an 11-point dose curve with 2 data points each 16 compounds

PROTOCOL: ANTAGONIST DOSE RESPONSE

A. DAY 1: PREPARING ASSAY PLATES

- Same as page 9.

B. DAY 2: ANTIBODY/ANTAGONIST COMPOUND PREPARATION AND ADDITION

1) Dissolve your antagonist in the vehicle of choice (DMSO, ethanol, water or other), or antibody in PBS + 0.1% BSA, at the desired stock concentration.

   NOTE:
   SOLVENTS CAN AFFECT ASSAY PERFORMANCE. PATHHUNTER ASSAYS ARE ROUTINELY CARRIED OUT IN THE PRESENCE OF ≤ 1% SOLVENT (I.E. DMSO, ETHANOL, PBS OR OTHER). IF YOU USE OTHER SOLVENTS OR SOLVENT CONCENTRATIONS, OPTIMIZE THE ASSAY CONDITIONS ACCORDINGLY.

2) Prepare a series of twelve 3-fold serial dilutions of antagonist in Assay-Complete Cell Plating Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other), as described below. The concentration of each dilution should be prepared at **5X** the final screening concentration (i.e., 5 µL antagonist compound will be used in a final volume of 25 µL). For each dilution, the final concentration of solvent should remain constant.

   - To begin the 12-point curve, we recommend targeting a final concentration that is **50X** the expected IC_{50} value for the compound (e.g., **250X** the final screening concentration). **Example:** If the expected IC_{50} is 10 nM, prepare the highest starting concentration of the corresponding dilution at 2.5 µM. This is the working concentration.

   a) Label wells, or the wells of a dilution plate, #1 through #12.

   b) Add 20 µL of AssayComplete Cell Plating Reagent to wells #1-11.

   c) Prepare a working concentration of antagonist in AssayComplete Cell Plating Reagent.

   d) Add 30 µL of the working concentration of antagonist to the empty well #12.

   e) Remove 10 µL of compound from well #12, add it to well #11 and mix gently by pipetting up and down. Discard pipette tip.

   f) With a clean pipette tip, remove 10 µL of diluted compound from well #11, add it to well #10 and mix gently by pipetting up and down. Discard the pipette tip.

   g) Repeat this process 7 more times in succession to prepare serial dilutions for the remaining wells, or from right to left across the plate.

   h) **DO NOT add antagonist compound to wells #1 and #2**. Add only appropriate AssayComplete Cell Plating Reagent containing solvent. These samples serve as the no antagonist controls and complete the dose curve.

   i) Repeat this process for any additional antagonists.

   j) Set the dilutions aside until you are ready to add them to the cells.

3) Remove PathHunter cells from the incubator (previously plated on day 1).

4) Transfer 5 µL of the controls and 5X antagonist dilutions from wells #1–12 to duplicate wells according to the plate map shown in Figure 3.

5) Incubate the cells with antagonists for 1 hour @ 37°C.

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.

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.