Contact Information

70-265 DRx_UM_PH_PATHWAY_0216V6A

DiscoveRx Corporation
(World Wide Headquarters)
42501 Albrae Street
Fremont, CA 94538
United States

t | 1.510.771.3500
f | 1.510.979.1650
toll-free | 1.866.448.4864

DiscoveRx Corporation Ltd.
(Europe Headquarters)
Faraday Wharf, Holt Street
Birmingham Science Park Aston
Birmingham, B7 4BB
United Kingdom

t | +44.121.260.6142
f | +44.121.260.6143

KINOME*scan*®
A division of DiscoveRx
11180 Roselle Street, Suite D
San Diego, CA 92121
United States

t | 1.800.644.5687
f | 1.858.630.4600

BioSeek®
A division of DiscoveRx
310 Utah Avenue, Suite 100
South San Francisco, CA 94080
United States

t | 1.650.416.7600
f | 1.650.416.7625

www.discoverx.com

PathHunter® Pathway Assay

For chemiluminescent detection of protein function

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

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

NOTES:

FREQUENTLY ASKED QUESTIONS

Q: I did not see a signal with my control compound.
R: There may be differences in compounds purchased from different vendors. Confirm that the control compound used is the same as used in the dose response shown in the provided target-specific data sheet.

Q: I did not see a response with my compound.
R1: The concentration of DMSO or Ethanol used for dilution is too high. Maintain concentration of the compound/antagonist diluent at ≤ 1%.
R2: Confirm that the final ligand concentration is correct. Some ligands are "sticky" and difficult to dissolve.
R3: Confirm that the cell line responds to the control compound.
R4: Repeat the experiment using a new lot of control compound.

Q: My cells arrived thawed. Can I use them?
R: No. Call technical support for a replacement.

Q: How long is the prepared detection reagent good for?
R: The working detection reagent solution must be used within 8 hours of mixing.

Q: What instruments can I use to read the plates?
R: Any bench top luminometer will work with the PathHunter eXpress assays.

Q: How long is the signal stable for?
R: The signal is stable for 5 hours after addition of detection reagent.

Q: My adherent cells are floating after the 24 hours incubation.
R: The cells are not viable, contact technical support for a replacement.

Q: Can I switch plates or should I use the plate provided?
R: You can use any clear bottom white or opaque walled plate.

LEGAL SECTION

LIMITED USE LICENSE AGREEMENT

DiscoveRx Corporation
Attn: Licensing Department
42501 Albrae Street, Suite 100
Fremont, CA 94538
tel | 510.771.3527
Agreements@discoverx.com

For some products/cell lines, certain 3rd party gene specific patents may be required to use the cell line. It is the purchaser's responsibility to determine if such patents or other intellectual property rights are required.

INTENDED USE

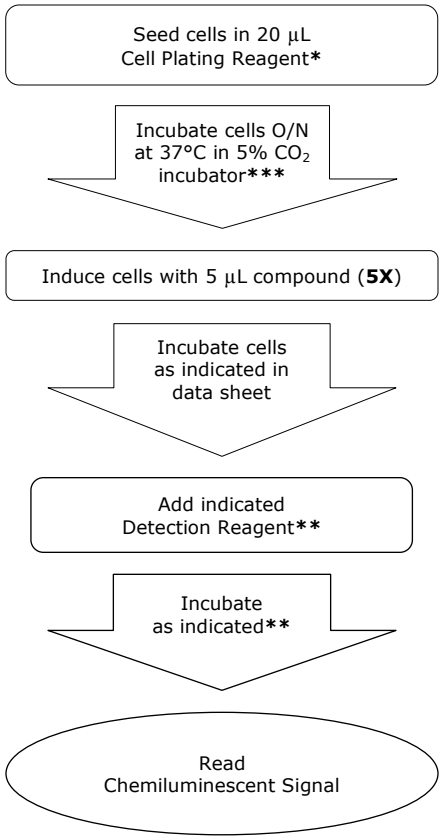
Your PathHunter® Pathway Assay Cell Line, when used in conjunction with the respective PathHunter Detection kit (indicated in the target specific data sheet) provides a cell-based functional assay to study various protein activities in a cell. The assays described in this booklet have been validated for use in 384-well microplate formats.

TECHNOLOGY PRINCIPLE

EFC is a homogeneous, non-radioactive detection technology based on two genetically engineered β-galactosidase fragments - a large protein fragment (Enzyme Acceptor, EA) and a small peptide fragment Enzyme Donor (ED). ED exists in different forms like Prolabel® (PL), Prolink™ (PK) and enhanced Prolabel® (ePL). Separately, the β-galactosidase fragments are inactive, but in solution, they rapidly recombine to form active β-galactosidase enzyme that hydrolyzes substrate; to produce an easily detectable chemiluminescent signal.

QUICK-START PROCEDURE: COMPOUND DOSE RESPONSE

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



***Refer to target specific datasheet for specific assay condition, cell numbers, cell incubation times and temperatures, compound incubation times and temperatures, Cell Plating and Detection Reagent requirements.

****Refer the user manual of Detection Reagents for volumes, incubation times and temperatures.

*****Different handling for suspension cells. Refer target specific data sheet for details.

ASSAY DETECTION METHODS

1. Refer target specific data sheet for the required PathHunter® Detection Reagent kit. The following table lists all the detection reagent part numbers that are relevant for the PathHunter Pathway Assays.
2. Prepare working solution of the detection reagents as indicated in the respective user manuals. Please refer below for the catalog number for the appropriate user manual.

Catalog Number	Description	Assay Principle	Volume of working detection reagent solution/well (384-well plate)
93-0001 Series	PathHunter Detection Kit	Nuclear Translocation; Protein Interaction	12 µL
93-0247 Series	PathHunter Flash Detection Kit	Protein Dimerization	25 µL
93-0273 Series	PathHunter Secretion Detection Kit	Protein Secretion	10 µL
93-0812 Series	PathHunter PK/PL Detection kit	Protein Degradation	30 µL

3. Add the indicated volumes of the detection reagents and proceed exactly as denoted in the user manual of the PathHunter Detection Reagents.

NOTE:

1. Read samples on any standard luminescence plate reader. [Compound potencies can be derived from a four-parameter nonlinear curve-fitting analysis.]
2. Use GraphPad Prism® or other comparable program to plot your compound dose response.

ASSAY OVERVIEW

PathHunter EFC technology has been applied to study several signaling pathways based on the following assay principles (Refer to schematic on page 5):

Protein Activity	Principle
Nuclear Translocation	In this assay the translocating protein is tagged to the ED fragment; the EA fragment is localized in the nucleus. Pathway activation results in the ED-tagged protein translocation into the nucleus. This results in the complementation of the two enzyme fragments that form active β-galactosidase enzyme.
Protein Translocation (with TAZ)	In this assay the translocating protein is tagged to the ED fragment; the EA fragment is fused to the TAZ domain (derived from CBP/p300) and is localized in the nucleus. Pathway activation results in the ED-tagged protein translocation into the nucleus. This results in the complementation of the two enzyme fragments that form active β-galactosidase enzyme.
Protein Degradation	In this assay the target protein is tagged to the ED fragment. Pathway activation results in the degradation of the target protein. This is detected by the addition of the cell lysis buffer and EA fragment present in the detection kit. The two enzyme fragments now complement to form active β-galactosidase enzyme.
Protein Interaction	In this assay one interacting target protein is tagged with ED and the second target protein is tagged with the EA fragment. Pathway activation results in the interaction of both the target proteins. This forces the complementation of the two enzyme fragments that form active β-galactosidase enzyme.
Protein Dimerization	This assay detects ligand induced dimerization of two subunits of a receptor-dimer pair. One receptor subunit fused to ED and a second dimer partner is fused to EA. Binding of a compound to one receptor subunit induces it to interact with its dimer partner, forcing complementation of the two enzyme fragments that form active β-galactosidase enzyme.
Protein Secretion	In this assay the target protein is tagged to the ED fragment. Pathway activation results in the secretion of the target protein. This is detected by the exogenous addition of the EA fragment present in the detection kit. The two enzyme fragments now complement to form active β-galactosidase enzyme.

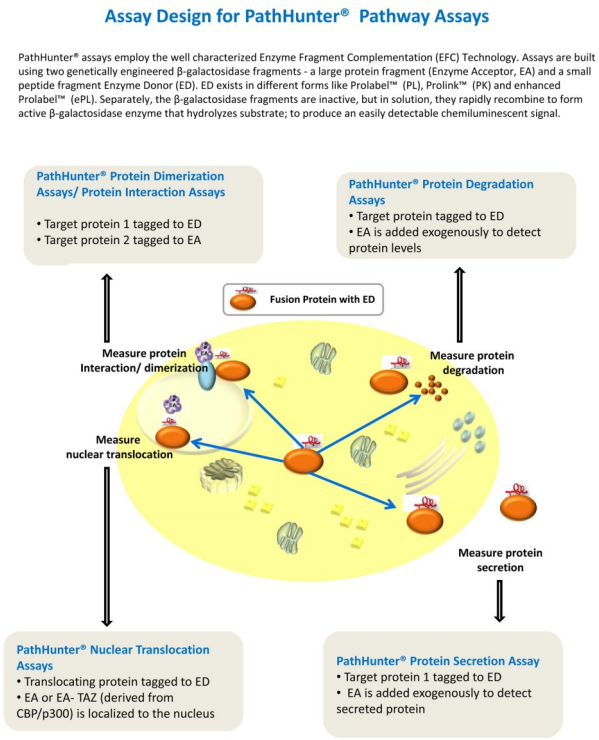
MATERIALS PROVIDED

Description	Storage
PathHunter Pathway Cell Line (2 vials)	Liquid N_2 (Vapor phase)

NOTE:
Please refer to the target specific data sheet for detailed information on the cell line that you are testing.

PATHWAY ASSAY SCHEMATIC

PathHunter assays employ the well characterized Enzyme Fragment Complementation (EFC) Technology. Assays are built using two genetically engineered β-galactosidase fragments - a large protein fragment (Enzyme Acceptor, EA) and a small peptide fragment Enzyme Donor (ED). ED exists in different forms like Prolabel (PL), Prolink (PK) and enhanced Prolabel (ePL). Separately, the β-galactosidase fragments are inactive, but in solution, they rapidly recombine to form active β-galactosidase enzyme that hydrolyzes substrate; to produce an easily detectable chemiluminescent signal.



DAY 2: COMPOUND PREPARATION AND ADDITION

1. Dissolve compound in the required solvent, 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 4-fold serial dilutions of compound in vehicle 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 vehicle to dilution wells #1–11.
   c) Prepare a working concentration of compound in the appropriate vehicle.
   d) Add 40 µL of the working concentration of compound 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 compound to well #1.** Add only vehicle. This sample serves as the no compound control and completes the dose curve.
   i) Repeat this process for any additional compounds.
   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** compound dilutions from wells #1-12 to assay plate wells according to the plate map shown on page 14.
5. Incubate cells with the compounds for the indicated times and temperatures in the target specific data sheet.
6. Proceed to page number 17 for details on assay detection methods.

ASSAY PROCEDURE (SUSPENSION CELLS) - COMPOUND DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing assays using PathHunter Cell Lines and the respective 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.

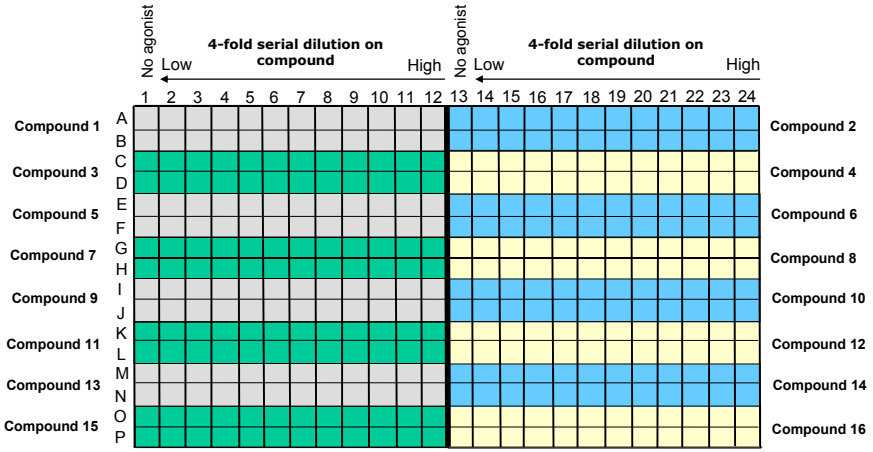


Figure 1. This plate map shows 12-point dose curves with 2 data points at each concentration for 16 compounds per plate for a total of 160 compound dilutions per 384-well plate.

PROTOCOL: COMPOUND DOSE RESPONSE

DAY 1: PREPARING ASSAY PLATES

1. Harvest cells as follows from a confluent T25 or T75 flask.
   a) Transfer cells to a 15 or 50 mL conical tube from the flask.
   b) Rinse the flask with additional 5 mL AssayComplete Cell Plating Reagent and transfer it to the tube.
   c) Centrifuge cells at 300 x g for 4 minutes to pellet cells, gently remove media (being careful not to disturb the cell pellet).
   d) Add 3 mL of appropriate AssayComplete Cell Plating Reagent (denoted in the datasheet) and resuspend the cells gently.
2. Determine cell density using a hemocytometer.
3. Using the AssayComplete Cell Plating Reagent , adjust the volume of the suspension to achieve a cell concentration per well indicated in the target specific data sheet.
4. Transfer 20 µL of the cell suspension to each well of a 384-well white-walled microplate.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

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 (DiscoveRx, Cat. #92-0011 or similar) • Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar) • Hemocytometer • White wall, clear bottom 384–well microplates (DiscoveRx, Cat. #92-0013 or similar) • Multimode or luminescence plate reader	• PathHunter® Detection Kit (Please refer to target specific data sheet) • AssayComplete™ Revive Reagent (DiscoveRx, Cat. #92-0016RM Series) • AssayComplete Cell Culture Kit (DiscoveRx, Cat. #92-0018/19/20/21/22G Series) • AssayComplete Preserve Freezing Reagent (DiscoveRx, Cat. #92-0017FR Series) • AssayComplete Cell Detachment Reagent (DiscoveRx, Cat. #92-0009) • AssayComplete Cell Plating (CP) Reagent (DiscoveRx, Cat. #93-0563R Series) • Control compound • Phosphate buffered saline (PBS) • Ligands/compounds, test compounds, antagonists, or antibodies
*****See the target specific datasheet for specific Cell Plating Reagents, Detection Reagents, Control Compounds other and tissue culture requirements.	

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Two cryovials are shipped on dry ice and in 1 mL of freezing medium. Upon receipt, the vials can be stored for up to 2 weeks at -80°C or transferred to the vapor phase of liquid nitrogen. **DO NOT** store at -80°C for extended periods, as this could result in significant loss in cell viability. 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. 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 liquid nitrogen storage or a –80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours after reciept, 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.

PROCEDURES FOR ADHERENT CELLS: HEK 293, U2OS, CHO-K1 AND NCI-H460 CELL THAWING AND PROPAGATION METHODS

The following procedures are for thawing cells in cryovials, seeding, 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. Pre-warm 5-10 mL of appropriate AssayComplete Revive Medium in a 37°C water bath.
2. Place the frozen cell vials 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**.
3. Transfer thawed cells to a sterile 15 mL conical tube containing the 5-10 mL of pre-warmed AssayComplete Revive Medium. Centrifuge at 300 x g for 4 minutes to pellet cells. Remove media.
4. Resuspend cell pellet in 5 mL of pre-warmed AssayComplete Revive Medium. Transfer cells to a T25 flask and incubate for 24 hours at 37°C/5% CO_2.
5. After 24 hours, gently remove media (being careful not to disturb the cell monolayer) and replace with 5 mL of appropriate AssayComplete Cell Culture Media (Refer target specific data sheet for media requirements).
6. Once the cells become >70% confluent in the T25 flask, detach using Assay-Complete Cell Detachment Reagent and transfer the cells to a T75 flask containing 5 mL of AssayComplete Cell Culture Media.

   NOTE:
   To maintain the logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.
7. Passage the cells every 2-3 days, based on the doubling time of the cell line, using AssayComplete Cell Detachment Reagent. For routine passaging, prepare a 1:3 dilution of cells in a total volume of 15 mL of cell culture media. Transfer 5 mL of the diluted cells to each of three new T75 flasks.
8. The clone has been found to be stable for at least 10 passages with no significant drop in assay window and EC_{50}.

4. Centrifuge the collected cells at 300 x g for 4 minutes.
5. After centrifugation, discard the supernatant. Resuspend the cell pellet in appropriate AssayComplete Preserve Freezing Reagent. Based on the cell number obtained from Step 5, dilute the resuspended cells to a concentration of 1.0-2.0 x 10^6 cells/mL.
6. Transfer 1.2 mL cells to each 2 mL cryogenic tube. (Keep tubes on ice during this process and transfer to a cryogenic container pre-chilled at 4°C).
7. Transfer tubes to –80°C and store overnight. Transfer tubes into the vapor phase of a liquid nitrogen tank for long-term storage.

TIPS FOR OPTIMAL PERFORMANCE: FOR ADHERENT AND SUSPENSION CELLS

1. Cells must be maintained in selective media at all times to maintain expression of fusion protein.
2. Ideally cells should be maintained at approximately 70% confluence. Cells should not be allowed to grow at confluence for more than 24 hours.
3. Allow adherent cells to grow and attach to the plate overnight prior to any assay is recommended.
4. Suspension cells may stick at the bottom of the plate. Rinse the surface of the plate with media under such conditions.
5. Some cell types like THP-1 are slow growers and may require longer doubling times.

PROCEDURES FOR SUSPENSION CELLS: EXAMPLE THP-1 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. Pre-warm 5-10 mL of appropriate AssayComplete Revive Medium in a 37°C water bath.
2. Place the frozen cell vials 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**.
3. Transfer thawed cells to a sterile 15 mL conical tube containing the 5-10 mL of pre-warmed AssayComplete Revive Medium. Centrifuge at 300 x g for 4 minutes to pellet cells. Remove media.
4. Resuspend cell pellet in 5 mL of pre-warmed AssayComplete Revive Medium. Transfer cells to a T25 flask and incubate for 48 hours at 37°C/5% CO_2.
5. After 48 hours transfer cells to a 15 mL conical tube, centrifuge cells at 300 x g for 4 minutes to pellet cells, gently remove media (being careful not to disturb the cell pellet) and add 5 mL of appropriate AssayComplete Cell Culture Media and transfer to a T25 flask. Refer target specific data sheet for media requirements.
6. Once the cells obtain the desired confluency by visual inspection transfer the cells to a T75 flask containing 15 mL of AssayComplete Cell Culture Media.

   NOTE:
   To maintain the logarithmic growth of the cells, cultures should be maintained in a sub-confluent state.
7. Passage the cells every 2-3 days, based on the doubling time of the cell line. For routine passaging, transfer 1:3 times of the total cell volume into a fresh T75 flask containing 10 mL media.
8. The clone has been found to be stable for at least 10 passages with no significant drop in assay window and EC_{50}.

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.
2. Transfer cells to a 50 mL conical tube.
3. Rinse the cells from the surface of the flask using 5-10 mL of AssayComplete Revive Medium and transfer to the conical tube. Remove 0.5 mL of the resuspended cells and count the cells using a hemocytometer.

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 5 mL of 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 Revive Medium 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 AssayComplete Revive Medium 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 5 minutes.
7. After centrifugation, discard the supernatant. Resuspend the cell pellet in appropriate AssayComplete Preserve Freezing Reagent. Based on the cell number obtained from Step 5, dilute the resuspended cells to a concentration of 1.0 - 2.0 x 10^6 cells/mL.
8. Transfer 1.2 mL cells to each 2 mL cryogenic tube. (Keep tubes 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.

ASSAY PROCEDURE (ADHERENT CELLS) - COMPOUND DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing assays using PathHunter Cell Lines and the respective 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.

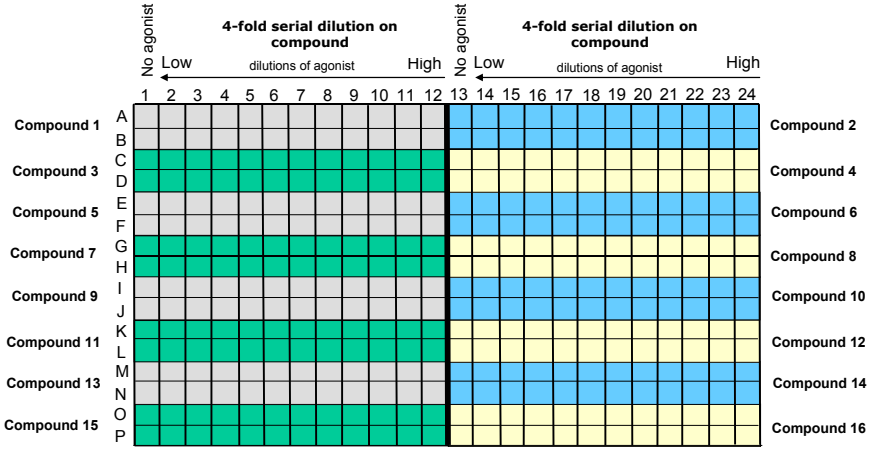


Figure 1. This plate map shows 12-point dose curves with 2 data points at each concentration for 16 compounds per plate for a total of 160 compound dilutions per 384-well plate.

PROTOCOL: COMPOUND DOSE RESPONSE

DAY 1: PRPEPARING ASSAY PLATES

1. Harvest cells as follows from a confluent T25 or T75 flask using AssayComplete Cell Detachment Reagent.
   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) Place flask in the incubator for 5 minutes, or until cells have detached.
   e) 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 per well indicated in the target specific data sheet.

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.

DAY 2: COMPOUND PREPARATION AND ADDITION

1. Dissolve compound in the required solvent, 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 4-fold serial dilutions of compound in vehicle 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 vehicle to dilution wells #1–11.
   c) Prepare a working concentration of compound in the appropriate vehicle.
   d) Add 40 µL of the working concentration of compound 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 compound to well #1**. Add only vehicle. This sample serves as the no compound control and completes the dose curve.
   i) Repeat this process for any additional compounds.
   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** compound dilutions from wells #1-12 to assay plate wells according to the plate map shown on page 9.
5. Incubate cells with the compounds for the indicated times and temperatures in the target specific data sheet.
6. Proceed to page number 17 for details on assay detection methods.

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.