DiscoverX

User Manual

PathHunter®

Pharmacotrafficking Cell Lines

For Measuring the Trafficking of Properly Folded Proteins in Mammalian Cells

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

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Overview

Transmembrane proteins (such as receptors and ion channels) are translated and folded in the endoplasmic reticulum (ER), and then translocated to the cell surface. However, defects, such as a mutation, deletion, or truncation, can lead to protein misfolding and prevent trafficking to the surface. This renders the protein non-functional and leads to disease. Compounds called pharmacochaperones (also known as pharmacological chaperones) provide a way to rescue these misfolded proteins. PathHunter cell-based pharmacotrafficking assays provide a simple, quantitative screening and profiling tool for the discovery and characterization of pharmacochaperones that correct protein misfolding related diseases.

- Detect trafficking and functionally restored GPCRs or ion channels
- Simple, rapid cell-based assay with a 1-step addition protocol
- Identify and rank potential antagonists, agonists, and allosterics
- Directly measure pharmacotrafficking using a standard plate reader

PathHunter Pharmacotrafficking assays provide a direct and quantitative measurement of pharmacologically rescued targets with trafficking disabling mutations. This simple and effective trafficking assay, with its one-step addition protocol and standard chemiluminescent detection, makes it ideally suited for 96-well or 384-well compound screening.

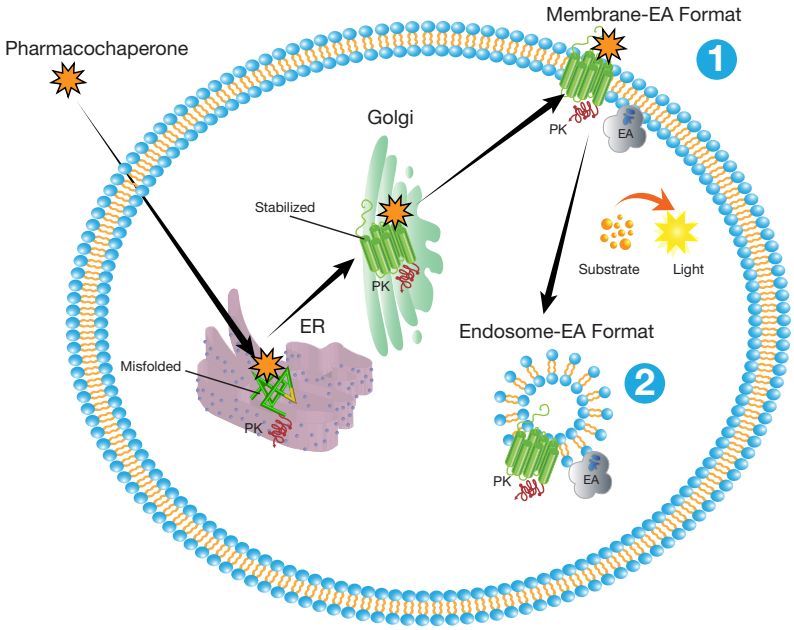
To perform a PathHunter Pharmacotrafficking assay with your cell line, you will also need DiscoverX's chemiluminescent detection reagents to measure proper trafficking. The cell assay protocols have been optimized for use with detection reagents found in our PathHunter Detection Kit or PathHunter Flash Detection Kit, removing the need for extensive in-house assay development. When used in conjunction with one of these detection kits, these cells provide a complete out-of-the-box solution for identification, screening, or profiling of pharmacochaperones. Please refer to the PathHunter Pharmacotrafficking Cell Line datasheet for additional information on the optimized AssayComplete™ Cell Plating Reagent, the appropriate Detection Kit, and the reference compound recommended for the assay.

Assays should be run using fresh, low-passage cells that have not been allowed to reach confluency for more than 24 hours. Each PathHunter pharmacotrafficking clone has been found to be stable for at least 10 passages with no significant drop in assay window, expression level, or shift in potency. After cell treatment, the assay is performed by adding the detection reagents to the treated cells, following the provided homogeneous easy-to-use protocol, and reading the resulting chemiluminescent signal on a standard luminometer.

Technology Principle

PathHunter Pharmacotrafficking cell lines are based on the enzymatic fragment complementation technology. The cells are expressed as a ProLink™ (PK)-tagged mutant transmembrane protein that is retained in the endoplasmic reticulum (ER) due to misfolding and co-expresses an Enzyme Acceptor (EA) tag localized either to (see figure below) (1) the cell membrane (Membrane-EA format), or (2) the early endosomes (Endosome-EA format). In either system, binding of a small molecule pharmacochaperone to the misfolded, PK-tagged protein stabilizes the protein in a conformation that enables its trafficking through the Golgi, then onward to the cell membrane. In the Membrane-EA format, complementation of the two β-galactosidase enzyme fragments (EA and PK) occurs at the membrane; while in the Endosome-EA format, protein re-localized to the cell membrane subsequently internalizes (either passively or actively) into endosomes, forcing complementation of EA-PK. The resulting functional, complemented β-galactosidase enzyme hydrolyzes substrate to generate a chemiluminescent signal.

PathHunter Pharmacotrafficking Assay Schematic Protocol



1. Cells lines co-express a ProLink (PK)-tagged trans-membrane protein retained in the ER (due to protein misfolding), and an Enzyme Acceptor (EA) tag localized to the cell membrane (Membrane-EA format).

2. Cells lines co-express a ProLink (PK)-tagged trans-membrane protein retained in the ER (due to protein misfolding), and an Enzyme Acceptor (EA) tag localized to the early endosomes (Endosome-EA format).

Materials Provided

List of Components	Contents
2 vials	Refer to cell line-specific datasheet

Storage Conditions

Cells must arrive in a frozen state on dry ice and should 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.

Additional Materials Needed

Refer to the cell line specific datasheet to determine catalog numbers for the media and reagent requirements for the specific PathHunter Pharmacotrafficking cell line you are testing. Each PathHunter cell line has been validated for optimal assay performance using the recommended AssayComplete™ Cell Plating (CP) Reagent and its ligand, as indicated on the cell line datasheet.

Do not substitute Cell Plating Reagent from an alternate kit at any time.

Required Materials	
Product	**Catalog Number and Ordering Information**
PathHunter Detection Kit or PathHunter Flash Detection Kit	93-0001 series or 93-0247 series (refer to cell-line datasheet for specific detection kit)
AssayComplete Cell Plating Reagent	Refer to cell line-specific datasheet
AssayComplete Thawing Reagent	Refer to cell line-specific datasheet
AssayComplete Cell Culture Kit	Refer to cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Freezing Reagent	Refer to cell line-specific datasheet
Microplates	discoverx.com/microplates
Disposable Reagent Reservoir	Thermo Scientific, Cat. No. 8094 or similar
Compound Dilution Buffer (Protein Dilution Buffer)	92-0023 Series
15 mL polypropylene tubes and 1.5 mL microtubes	
Tissue culture disposables (pipettes 1 mL to 25 mL) and plastic ware (T25 and T75 flasks, etc.)	
Cryogenic vials for freezing cells	
Hemocytometer	
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micro-pipettors and pipette tips (10 µL – 100 µL)	
Multimode or Luminescence reader	discoverx.com/instrument-compatibility

Recommended Materials	
Product	**Catalog Number and Ordering Information**
PathHunter Anti-PK/PL Antibody	92-0010
Compounds (ligands)	discoverx.com/ligands

Unpacking Cell Cryovials

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 provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for 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.

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 of liquid nitrogen. They are not rated for storage in the liquid phase.

4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line-specific datasheet for appropriate AssayComplete products mentioned in the protocol below.

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 while handling cells 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.
3. Remove the cell cryovials from -80°C or liquid nitrogen storage, and immediately place them on dry ice prior to thawing.

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.

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 >70-80% 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 >70-80% 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 and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm that the cells have detached by viewing them under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells have not detached, return the flask to the incubator for an additional 1 to 2 minutes, and repeat this step until all 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 indicated 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 12 mL for T75 flasks (or 45 mL for T225 flasks) cell culture media supplemented with the recommended concentration of selection antibiotic. Refer to cell line-specific datasheet to determine the correct Cell Culture Kit and the recommended antibiotic and antibiotic concentration for your cell line.

The cell culture media comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic.

15. Return the flask to a tissue culture incubator. If the cells do not appear to be healthy or if confluency is <25%, incubate for additional 24 to 48 hours to allow for additional cell recovery before splitting cells.

16. 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 cell culture media containing appropriate selection antibiotics. Typical split ratios for common cell backgrounds are indicated 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 cell culture media containing appropriate selection antibiotic, 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 cell culture media containing appropriate selection antibiotic, 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 maintain cell viability.

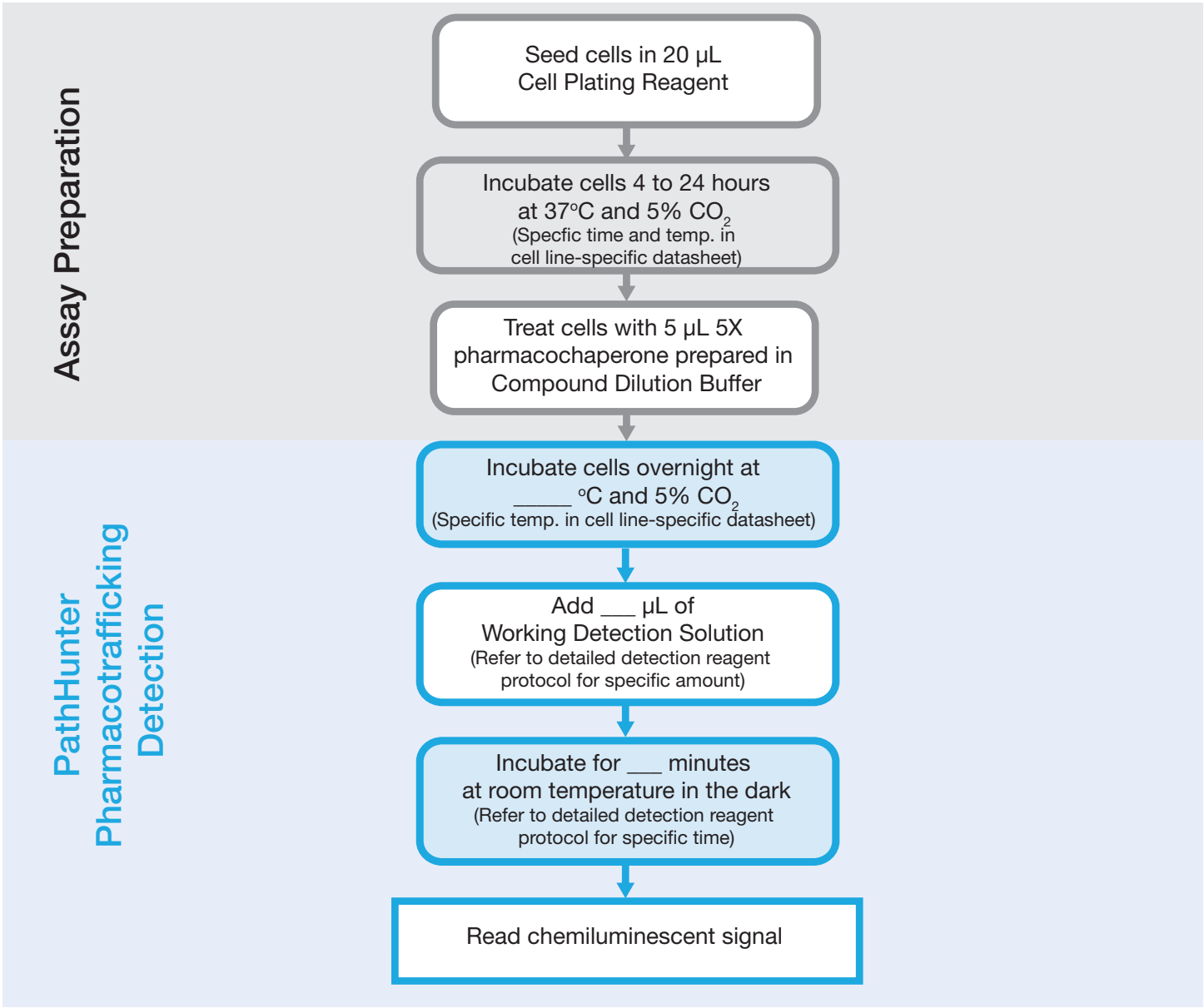
Protocol Schematic

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

Assay Name:_____ Date:_____

Product Details: _____

Quick-Start Procedure: In a 384-well tissue culture treated plate, perform the following:



Detailed Assay Protocol

The following is a procedure for determining the dose-dependent compound response in a 384-well tissue culture plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes.

Volumes for 96- and 384-Well Plates		
Assay Reagents	**96-Well Plate (Volume per Well)**	**384-Well Plate (Volume per Well)**
Number of Cells	10,000	5,000
AssayComplete™ Cell Plating Reagent	100 µL	20 µL
Compound	10 µL	5 µL
Working Detection Solution*	55 µL for PathHunter Detection (or 110 µL PathHunter Flash Detection)	12.5 µL PathHunter Detection (or 25 µL PathHunter Flash Detection)
Final Assay Volume	165 µL for PathHunter Detection (or 220 µL PathHunter Flash Detection)	37.5 µL for PathHunter Detection (or 50 µL PathHunter Flash Detection)

* Refer to cell-line datasheet to determine the specific detection kit needed to create the working detection solution.

Section I: Cell Preparation and Plating

The following is a protocol for harvesting cells from a confluent T75 or T225 flask, and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that cells have been cultured in AssayComplete Cell Culture Media. The cell culture media comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic.

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

1. Aspirate the media from the flask.
2. Add 2 mL AssayComplete Cell Detachment Reagent into each T75 flask (or 3 mL to T225 flasks). Swirl to rinse the cells.

Do not use trypsin for this step; especially with assays interrogating membrane-anchored receptors (e.g. GPCRs). Use of trypsin at this step can negatively affect assay results. Additionally, it is important to rinse the cells with AssayComplete Cell Detachment Reagent. It is not recommended to use PBS to rinse cells as PBS may inhibit the detachment of cells from the plate.

3. Gently aspirate AssayComplete Cell Detachment Reagent from flask.
4. Add 1 mL fresh AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL to the T225 flasks).
5. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with AssayComplete Cell Detachment Reagent.
6. Incubate at 37°C and 5% CO_2 for 2 to 5 minutes or until the cells have detached.
7. 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.
8. Add 4 mL AssayComplete Cell Plating Reagent to T75 flasks (or 7 mL to T225 flasks). Note: Refer to datasheet to determine the correct Cell Plating Reagent for this cell line.

9. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
10. Remove the entire amount of cells from the flask and transfer to a 15 mL conical centrifuge tube.
11. 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.

Keep suspended cells on ice to maintain cell viability until ready for transfer to the assay plate.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.
    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.
12. Centrifuge the collected cells at 300 X g for 4 minutes.
13. After centrifugation, discard the supernatant and re-suspend the cell pellet in AssayComplete™ Cell Plating Reagent. Based on the cell number obtained in step 11 above, dilute the resuspended cells to the desired concentration (e.g. 250,000 cells/mL or 5,000 cells/20 µL).
14. Transfer 20 µL/well of the cell suspension to a 384-well (or 100 µL/well to a 96-well) assay plate.
15. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line datasheet for recommended cell incubation time.
16. Proceed to compound preparation and addition.

Section II: Pharmacochaperone Preparation

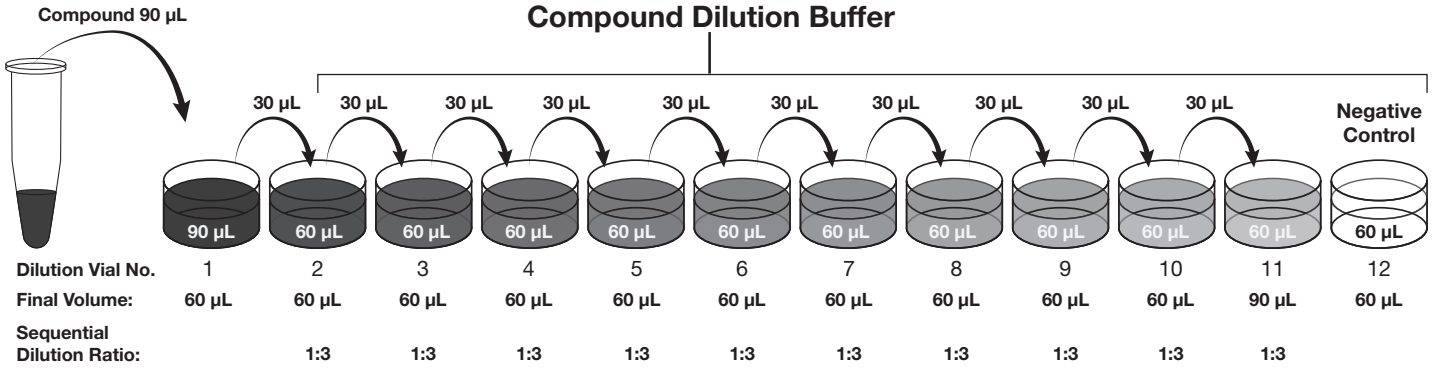
The following is a procedure for setting up dilution series for a compound dose-response curve.

1. Prepare small molecule pharmacochaperone (must be membrane permeable) serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of pharmacochaperone in Compound Dilution Buffer. The concentration of each dilution should be prepared at 5X of the final screening concentration.
    a. For each pharmacochaperone compound, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
    b. Add 60 µL of Compound Dilution Buffer to dilution wells No. 2 through No. 12. This is enough volume for over 4 rows, and the dilution volume may be adjusted according to the number of wells desired.
    c. Prepare the highest concentration of pharmacochaperone in Compound Dilution Buffer. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the pharmacochaperone. Therefore, prepare a working concentration that is 1250X the expected EC_{50} per well to get a 5 X working pharmacochaperone concentration (e.g. for an expected EC_{50} of 1 nM, prepare the highest working concentration at 1250 nM. This is 5X the screening or final top concentration of 250 nM, and the expected EC_{50} will lie near the center of the dose response curve).
    d. Add 90 µL of the highest concentration of Compound Dilution Buffer to well No. 1 (see figure below).
    e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

g. Repeat this process until well No. 11 is reached. Do not add pharmacochaperone to well No. 12 since this is the negative control well.

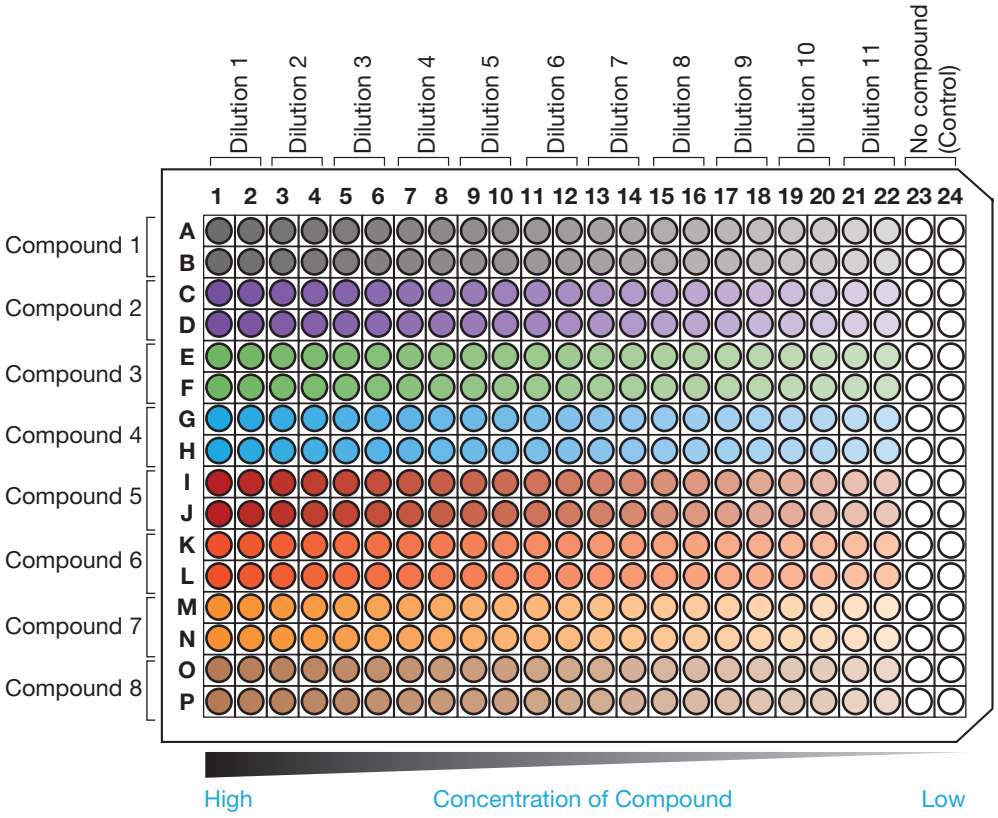
2. Set up serial dilutions for any additional pharmacochaperones in a similar manner.



Compound Dilution Buffer: Make eleven 3-fold serial dilutions of pharmacochaperone compound in a separate dilution plate or vials.

Section III: Pharmacochaperone Addition

The following is a procedure for adding the dilution series to the assay plate, to generate dose-response curves.



Assay Plate Map: Create 11-point curves for eight different compounds (pharmacochaperones) in quadruplicate.

1. Add 5 µL of each 5X pharmacochaperone serial dilution in quadruplicate to the designated pharmacochaperone rows (e.g. pharmacochaperone 1 in Rows A and B, Columns 1 and 2; pharmacochaperone 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated on the previously described assay plate map.
2. Incubate assay plate at the indicated time and temperature for the specific cell line (refer to the specific cell line datasheet for conditions). For most cell lines, incubate overnight at 37°C and 5% CO_2. For the best results, the optimal incubation time should be empirically determined.

Section IV: Detection Reagent Addition

The working detection solution is unique to specific cell lines. Please refer to the Pharmacotrafficking cell line datasheet to determine the specific detection kit to use (PathHunter Detection Kit or PathHunter Flash Detection Kit) before proceeding to one of the two detection steps below.

Refer to your Pharmacotrafficking cell line datasheet for the appropriate detection kit before proceeding.
Use the working stock solution within 24 hours.

Instructions for using the PathHunter Detection Kit (Catalog No. 93-0001 Series)

1. Prepare a stock of Working Detection Solution in a separate 15 mL polypropylene tube or reagent reservoir by mixing nineteen parts of Cell Assay Buffer, five parts of Substrate Reagent 1, and one part Substrate Reagent 2.
2. Add 12.5 µL of Working Detection Solution to all wells of a 384-well assay plate.

Working Detection Solution		
Components	**Volume Ratio**	**Volume per Plate (mL)**
Cell Assay Buffer	19	5.7
Substrate Reagent 1	5	1.5
Substrate Reagent 2	1	0.3
Total Minimum Volume		**7.5**

3. Incubate assay plate for 1 hour at room temperature in the dark for the reaction to occur.

Do not agitate or vortex plates after adding detection reagents.
Working Detection Solution is light sensitive, thus incubation in the dark is necessary.

Instructions for using the PathHunter Flash Detection Kit (Catalog No. 93-0247 Series)

1. Prepare a stock of Working Detection Solution (Flash) in a separate 15 mL polypropylene tube or reagent reservoir by mixing one part of Cell Assay Buffer and four parts Flash Substrate.
2. Add 25 µL of Working Detection Solution (Flash) to all wells of the assay plate.
3. Incubate assay plate for 30 minutes at room temperature in the dark for the reaction to occur.

Working Detection Solution (Flash)		
Components	**Volume Ratio**	**Volume per Plate (mL)**
Flash Cell Assay Buffer	1	2.5
Flash Substrate	4	10
Total Minimum Volume		**12.5**

Do not agitate or vortex plates after adding detection reagents.
Working Detection Solution is light sensitive, thus incubation in the dark is necessary.

Section V: Assay Plate Reading

1. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well with photomultiplier tube readers or 5 to 10 seconds with image based readers. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

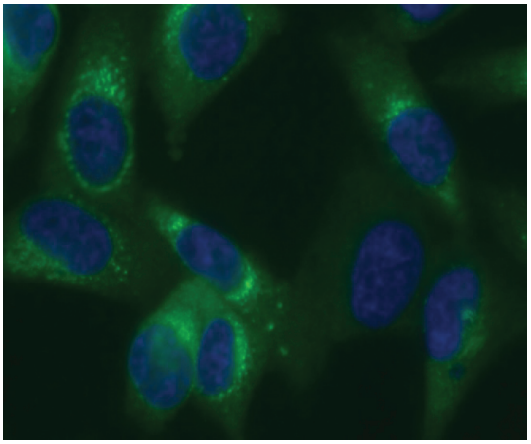
The assay plate should be read within 2 hours after adding the detection reagent solution.

2. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, Biotek Instruments Gen5, Microsoft Excel, etc.).

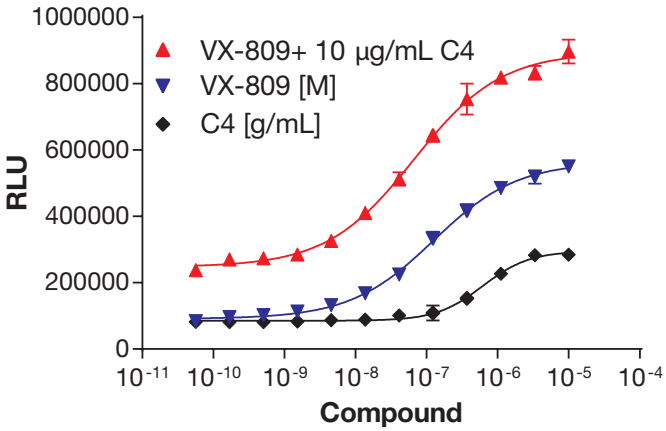
Typical Results

Typical results shown below from PathHunter Pharmacotrafficking assays using different mutated GPCR and ion channel cell lines.

CFTR-ΔF508 Immunostaining

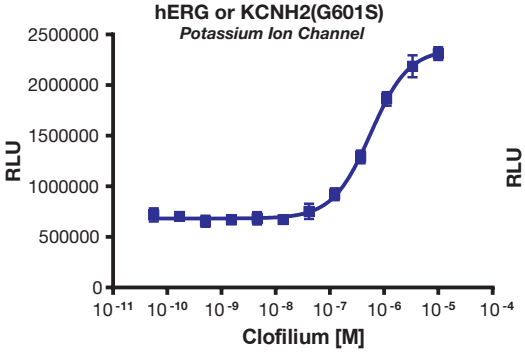


CFTR-ΔF508 Pharmacotrafficking Assay

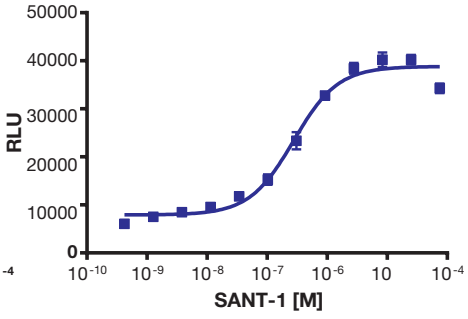


Analysis of a mutant form of the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) containing a single point deletion CFTR-ΔF508 was conducted. This common deletion in cystic fibrosis patients causes the protein to misfold, thus preventing efficient trafficking and leading to ER retention (immunofluorescence image, left). Using the PathHunter CFTR-ΔF508 Pharmacotrafficking assay with a combination of 2 corrector (or pharmacochaperone) compounds, C4 and VX-809, stabilizes the mutant receptor, allowing for proper trafficking. Dual treatment results in elevated signal (right, red curve) indicating an additive effect, which is the expected behavior of the combination of the two compounds.

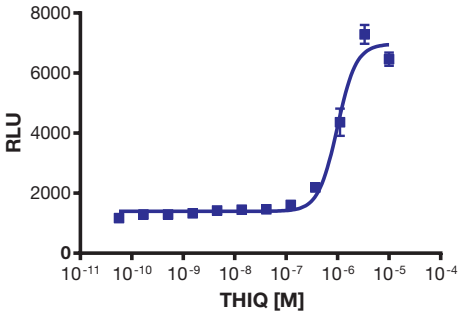
Long QT Syndrome (Cardiac Arrhythmias)
hERG or KCNH2(G601S)
Potassium Ion Channel



Basal Cell Carcinoma
SMO(W535L)
Frizzled class



Severe Early-Onset Obesity
MC4R(T162I)
Class A: Melanocortin



Examples of disease relevant mutant ion channel and GPCRs using various small molecule pharmacochaperones to stabilize the receptors and allow for their proper trafficking to the membrane as detected by the PathHunter Pharmacotrafficking Assay.

Frequently Asked Questions

Do you perform any quantitative expression analysis of your recombinant cell lines?

- No. Adequate expression is crucial for detection of a response to control compound. We select clones according to response to the compound rather than expression level.

At what passage number do you freeze down your inventory lots?

- Passage 2-3.

I understand that you generate your cell lines via retroviral infection, as it is a very efficient system. Do the final cell lines produce any viable retroviral particles?

- No. We have confirmed the lack of retroviral particles using a Marker Rescue Assay.

What is the general liquid dispensing speed that you would recommend when using robotic dispensing instruments?

- This will depend on the particular instrument and has to be determined empirically, but in general, we would recommend a speed of 15-20 µL/second.

Can your assays be run in 96-, 384-, and 1536-well formats?

- Yes. Optimization for 1536-well format would be needed.

What if there is no or low signal?

- When using clear bottom plates, visually inspect the cells before and after compound incubation to ensure that they are healthy. Make sure proper cell plating procedure is followed to ensure assay performance.
- Make sure proper detection reagent is used (refer to the cell-line datasheet).
- Make sure detection reagents are stored and prepared properly.
- Make sure pharmacochaperone compounds are membrane permeable.
- Make sure the plate reader is setup properly to read luminescence.
- High levels of protein in the wells may interfere with enzyme fragment complementation. If high levels are present, a media exchange step could be performed just prior to the detection reagent addition. A mild detergent (e.g. pluronic acid) may also help decrease protein aggregation.
- White-walled assay plates should be used; black-walled plates may decrease signal.

What if the response is lower than expected (lower than expected S/B)?

- Make sure that the compound is prepared properly, take extra care to observe compound solubility.
- Make sure DMSO and other solvent concentrations are not too high (not more than 1% final concentration).
- When testing compounds at high concentrations, make sure the vehicle is compatible.
- Make sure compound is incubated for the designated time and at the designated temperature.
- Make sure plates are protected from light during incubation.

What if the EC_{50} does not match reported values?

- Make sure compounds are incubated at the proper temperature. Refer to the cell-line datasheet for additional protocol information.
- The source of a compound, particularly for biologics, can have a large effect on the observed pharmacology.
- Changing tips during serial dilutions can help to avoid carryover.

What if the variability between replicates is high?

- Make sure dispensing equipment is properly calibrated and proper pipetting technique is used.
- Make sure to avoid cell clumps when plating. Uneven number of cells in the wells can cause high variabilty.

Do I need to use clear bottom plates?

- No, but clear bottom plates are desirable for allowing cellular visualization after plating. White bottom plates generate brighter signals and reduce signal bleed to other wells.

What if my compound is in media containing high concentrations of serum, can I use it as-is or will the serum interfere with the assay?

- Typically, our assays are highly tolerant to high serum content. However, there may be other compounds present in the serum that may raise the assay background, which can be target specific.
- We recommend that you aspirate the high serum media prior to adding detection reagents. Aspirating the media can help increase S/B, but it may not affect altered potency from compounds present in the serum or elevated background.

During the Assay Preparation step, can I use shorter incubation times than overnight?

- This is not recommended. Short incubation times typically lead to a limited dynamic range.

My EC_{50}s are very right shifted?

- It is possible that your compounds have poor membrane permeability. Membrane permeability is required so that the compound will reach the targeted protein trapped in the ER.

Can the rescued target protein be tested for functional activity?

- Yes, in most cases this is feasible.
- For mutant GPCRs in the Endosome EA format, the following procedure is recommended: After overnight compound incubation with a non-activating pharmacochaperone, remove compound media by washing cells once with 50 µL of the same Cell Plating Reagent used to plate the cells. Next remove the Cell Plating Reagent and add 20 µL fresh Cell Plating Reagent. Then add 5 µL of an 5X compound dilution curve, incubate 3 hours @ 37°C, and continue with the working detection reagent procedure as indicate in the protocol.
- For ion channels: Activity can be tested using an appropriate functional assay.

For additional information or technical support, see contact information below.

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