This two-page spread is a cover: the left half (contact information) is the back cover and the right half is the front cover.

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PathHunter® Activated GPCR Internalization Assay

For Chemiluminescent Detection of Activated and Internalized GPCRs

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

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NOTES:

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LEGAL SECTION

This product and/or its use is covered by one or more of U.S. patents #7, 135, 325 B2, #8, 101, 373 B2, and/or foreign patent applications, and trade secrets that are either owned by or licensed to DiscoveRx® Corporation. This product is for *in vitro* use only and in no event can this product be used in whole animals.

LIMITED USE LICENSE AGREEMENT

The designated cells and reagents purchased from DiscoveRx are restricted in their use. DiscoveRx has developed an assay for translocation and internalization ("Assay") employing genetically modified cells ("Cells") and detection reagents ("Reagents") (collectively referred to as "Materials"). The Cells and Reagents are designed and optimized to be used together in the Assay. DiscoveRx wishes to ensure that these Cells and Reagents are used properly and effectively. By purchasing the Materials you recognize and agree to the restrictions.

1) 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.
2) Purchaser will not analyze the Reagents nor have them analyzed on Purchaser's behalf.
3) Purchaser will use only the Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Assays.

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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

PathHunter® Activated GPCR Internalization Assays are non-imaging, non-antibody based chemiluminescent detection assays that provide a direct and quantitative measurement of internalized GPCRs localized to early endosomes. This allows the internalization of GPCRs to be quantitatively measured in live cells without the need for expensive microscopy. The PathHunter system combines engineered clonal cell lines with optimized PathHunter Detection Reagents (Cat. #93-0001, 93-0001L and 93-0001XL). Each cell line has been characterized for appropriate GPCR pharmacology, specificity and stability in cell culture. Whether you are studying receptor recycling, identifying functional antagonists, or determining mechanism of action of your lead compounds, a simple, one-step addition protocol and standard chemiluminescent detection makes these assays ideally suited for 96-well, 384-well, or 1536-well compound screening.

TECHNOLOGY PRINCIPLE

PathHunter Activated GPCR Internalization Assays provide a quantitative measurement of internalized GPCR protein localized in early endosome using β-galactosidase (β-gal) enzyme fragment complementation (EFC, Figure 1). In this system, the GPCR of interest is untagged; the small, 42 amino acid enzyme fragment of β-gal called ProLink™ (PK) is localized to the endosomes and the larger, complementing enzyme fragment termed Enzyme Acceptor, or EA, is fused to β-Arrestin. Stimulation of the receptor results in Arrestin binding to the activated GPCR, followed by internalization and trafficking of the receptor/Arrestin-EA complex in PK-tagged endosomes. This action forces complementation of the two enzyme fragments, resulting in the formation of a functional enzyme that is capable of hydrolyzing substrate and generating a chemiluminescent signal using PathHunter Detection Reagents.

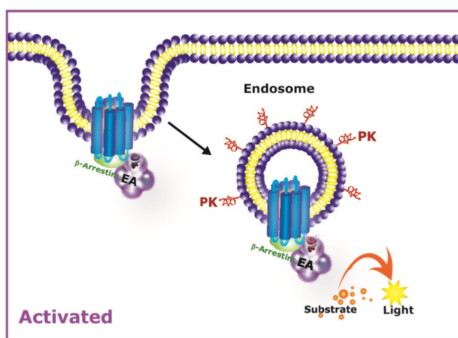


Figure 1. PathHunter® Activated GPCR Internalization Assay Principle. Activation of the GPCR results in internalization of the receptor/Arrestin-EA complex in endosomes and formation of a functional β-gal enzyme capable of hydrolyzing substrate and generating chemiluminescent signal.

NOTES:

APPENDIX A: ASSAY FORMATS

PathHunter® Certified Assay Format				
Plate Format	96-well	FV 384-well	LV 384-well	1536-well
Total Volume	150 µL	40 µL	20 µL	8 µL
Cell Numbers	10,000	5,000	2,500	1,250
Cell Plating Reagents*	90 µL	20 µL	10 µL	4 µL
Ligand	10 µL	5 µL	2.5 µL	1 µL
Detection Reagents	50 µL	12 µL	6 µL	3 µL

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

APPENDIX B: RELATED PRODUCTS

Description	Ordering Information
Control Ligand	www.discoverx.com/pathway_assays/control_ligands.php
AssayComplete™ Cell Plating Reagent	www.discoverx.com/certified/cell_plating_reagents.php
AssayComplete Cell Culture Kit AssayComplete Revive Reagent AssayComplete Preserve Freezing Reagent	www.discoverx.com/certified/PH_cell-culture_reagents.php
PathHunter® Detection Reagents	www.discoverx.com/certified/PH_detection_reagents.php
Microplates	www.discoverx.com/certified/microplates.php
PathHunter eXpress β-Arrestin GPCR Assays	www.discoverx.com/gpcrs/express_arrestin.php
PathHunter eXpress β-Arrestin Orphan GPCR Assays	www.discoverx.com/gpcrs/express_orphan.php
PathHunter eXpress β-Arrestin Ortholog GPCR Assays	www.discoverx.com/gpcrs/express_ortholog.php

ASSAY OVERVIEW

Please read the entire protocol completely before running the assay. Successful results depend on performing these steps correctly. The **Assay Procedure** sections and **Quick Start Guides** in this booklet contain detailed information about how to run the assays. Refer to the cell-line specific datasheet for additional information on the optimized Cell Plating Reagent and reference ligand recommended for the assay.

Assays should be run using a fresh split of low-passage cells that have not been allowed to reach confluency for more than 24 hours. Following treatment of the cells with compound, GPCR activity is detected by adding a working solution of chemiluminescent PathHunter Detection reagents using a simple, mix-and-read protocol.

The following steps are required to monitor GPCR activity using a PathHunter Activated GPCR Internalization Assay System (Figure 2).

1. Plate cells (page 9).
2. Dilute and add compounds or antibodies.
3. Perform functional assay in agonist (p.10), antagonist (p.14) or allosteric modulator mode (p.18).

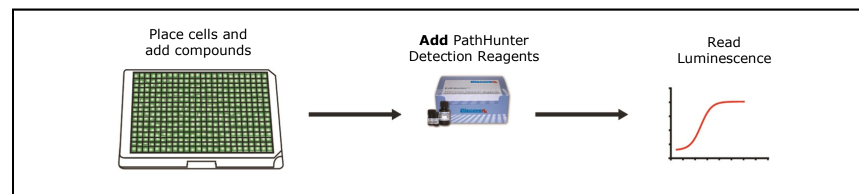


Figure 2. Simple chemiluminescent assay protocol for monitoring GPCR internalization in response to compound challenge.

MATERIALS PROVIDED

Description	Contents	Storage
PathHunter Activated GPCR Internalization Cells*	2 vials	Liquid N_2 (vapor phase)

***Please refer to the cell line specific datasheet for detailed information on the PathHunter Activated GPCR Internalization cell line you are testing.

FROZEN CELL HANDLING PROCEDURE

To ensure maximum cell viability, thaw the vial and initiate the culture as soon as possible upon receipt. If continued storage of the frozen vials is necessary, store vials in the vapor phase of liquid nitrogen (N_2). **DO NOT** store at –80°C for extended periods as this could result in significant loss in cell viability.

ADDITIONAL MATERIALS REQUIRED (NOT PROVIDED)

The following additional materials are required to perform PathHunter Activated GPCR Internalization Assays:

Equipment	Materials
• Green V-Bottom PP Ligand Dilution Plates, 10 plates/pack (DiscoveRx, Cat. #92-0011) • 96-well Clear Bottom TC treated, Sterile WCB, FB w/lid, 10 plates/pack (DiscoveRx, Cat. #92-0014) • 384-well Clear Bottom TC treated, Sterile WCB, FB w/lid, 10 plates/pack (DiscoveRx, Cat. #92-0013) • 384-well White Bottom TC treated, Sterile w/lid, 10 plates/pack (DiscoveRx, Cat. #92-0015) • Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar) • Hemocytometer • Cryogenic Freezing Container (Nalgene, Cat. #5100-0001 or similar) • Cryogenic Freezer Vials (Fisher Scientific, Cat. #375418 or similar) • Multimode or luminescence plate reader • Single and multi-channel pipettors and pipette tips • Tissue culture disposables and plasticware (T25 and T75 flasks, etc.)	• PathHunter® Detection Kit (DiscoveRx, Cat. #93-0001, #93-0001L or #93-0001XL) • 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) • Phosphate buffered saline (PBS) • GPCR control agonist • GPCR test compound(s) and/or antagonists

±Please refer to the cell line specific datasheet to determine catalog numbers for the media and reagent requirements for the PathHunter Activated GPCR Internalization cell line you are testing.

CELL PLATING REAGENT REQUIREMENTS

Each PathHunter Activated GPCR Internalization Cell Line has been validated for optimal assay performance using the recommended Cell Plating (CP) Reagent and control ligand as indicated in the cell line specific datasheet. **For optimal performance using this PathHunter Certified System, always use the CP Reagent recommended for the cell line and DO NOT substitute at any time.**

TROUBLESHOOTING GUIDE (CONTINUED)

PROBLEM	CAUSE	SOLUTION
Cells growing slowly	U2OS grows slower than CHO-K1 or HEK 293	Average doubling time is 3 days, so please observe cells under microscope and monitor cell health
	Slow growing clones	Use of DiscoveRx functionally validated and optimized media and reagents improves assay performance
EC_{50} is right-shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in buffer containing 0.1% BSA
		Non-binding surface plates may be necessary for hydrophobic compounds
High well-to-well variability in Z' study	Problems with plate type and compound solubility	Z' studies should be performed with automation
		It may be necessary to test plate types and compound stability

For additional information or technical support, please call **1.866.448.4864** (US) **+44.121.260.6142** (Europe) or email info@discoverx.com

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No Response	Improper cell growth conditions	See datasheet for cell culture conditions
	High DMSO/solvent concentration	Maintain DMSO/solvent at <1% in serial dilutions of compounds.
	Improper ligand used or improper ligand incubation time	See datasheet for recom-mended ligand and assay conditions
	Improper preparation of ligand (agonist or antagonist)	Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased Response	Higher passages give reduced performance	PathHunter cells are stable up to 10 passages. Use low passage cells whenever possible
	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscopy
Low or No Signal	Improper preparation of detection reagents	Detection reagents should be prepared just prior to use and are sensitive to light.
	Problem with cell growth, cell viability, cell adherence or cell density	See datasheet for cell culture conditions.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 1 sec/well.
Experimental S:B does not match datasheet value	For cell pools, S:B may vary greatly from passage to passage or day to day	Prepare a clonal cell line or use lower passage number cells.
		Repeat the assay
		Confirm assay conditions
	Improper preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands

SOLVENTS AND PREPARATION OF COMPOUND DILUTIONS

PathHunter Activated GPCR Internalization Assays are routinely carried out in the presence of ≤ 1% solvent (i.e. DMSO, ethanol, PBS or other). As solvents can affect assay performance, optimize the assay conditions accordingly if other solvents or solvent concentrations are required.

To validate each PathHunter Activated GPCR Internalization Assay, reference ligand was diluted in Cell Plating (CP) Reagent containing appropriate solvent. For preparation of test compounds, we recommend preparing the dilutions using the CP Reagent recommended for the cell line (containing the appropriate solvent). For antibodies or other compounds that may be sensitive to serum and/or other assay components, dilutions can be prepared in either Hanks Buffered Salt Solution (HBSS) + 10 mM HEPES + 0.1% Bovine Serum Albumin (BSA) or OptiMEM® + 0.1% (BSA) without affecting assay performance.

USE OF PLASMA OR SERUM CONTAINING SAMPLES

PathHunter Activated GPCR Internalization Assays can be run in the presence of high levels of serum or plasma without negatively impacting assay performance. Standard curves of control ligand can be prepared in neat, heparinized plasma and added directly to the cells (without further dilution, ie. 100% plasma in the well). After ligand stimulation, the samples should be removed and replaced with fresh CP Reagent before the addition of the PathHunter Detection Reagents.

NOTE:
EDTA anti-coagulated plasma samples do not give a positive response in the assay. Therefore, the choice of anti-coagulant treatment is very important.

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Cells are shipped in 2 vials on dry ice and contain approximately 1 x 10^6 cells per vial in 1 mL of AssayComplete Preserve Freezing Reagent. The following procedures are for safely storing and removing cryovials from liquid nitrogen storage.

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 immediately transferred to liquid N_2 storage or thawed and put in culture immediately upon arrival.
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 N_2 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

The following procedures are for thawing, seeding and expanding the cells, and for maintaining the cultures once the cells have been expanded. Cells are free of contamination prior to shipment and care should be taken in their handling to avoid contamination.

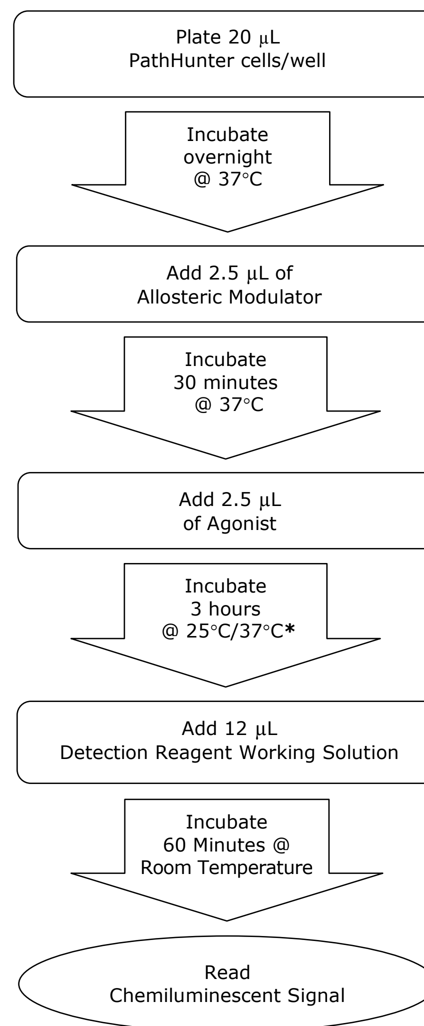
NOTE:
Face shield, gloves and a lab coat should be worn during the thawing procedure.

1. Pre-warm 15 mL AssayComplete Revive Media in a 37°C water bath.
2. Place the frozen cell vials ***briefly*** (10 seconds to 1 min) in a 37°C water bath under sterile conditions until only small ice crystals remain and the cell pellet is almost completely thawed. Caution: Longer incubation may result in cell death.
3. To remove DMSO from the media, carefully transfer the thawed cells to a sterile 15 mL tube and then fill tube with 10 mL pre-warmed AssayComplete Revive Media. Centrifuge at 300 x g for 4 minutes to pellet cells.
4. Remove media without disturbing the cell pellet and resuspend cell pellet in 5 mL of pre-warmed AssayComplete Revive Media. Transfer cells to a T25 flask and incubate for 24 hours at 37°C, 5% CO_2.

   NOTE:
   Cell recovery is greatly improved when selection antibiotics are omitted for the first 24 hours.
5. After 24 hours, gently remove AssayComplete Revive Media (being careful not to disturb the cell monolayer) and replace with 5 mL of pre-warmed complete AssayComplete Cell Culture Media.
6. Once the cells become >70% confluent in the T25 flask, aspirate media and wash with 5 mL PBS. Aspirate PBS and dissociate cells with 0.5 mL AssayComplete Cell Detachment Reagent and resuspend in 5 mL of AssayComplete Cell Culture Media. Transfer the entire cell suspension to a T75 flask containing 15 mL of AssayComplete Cell Culture Media for continued growth.
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 10 mL AssayComplete Cell Culture Media. Transfer 5 mL of the diluted cells to each new T75 flask.

   NOTE:
   To maintain logarithmic growth of the cells, cultures should be maintained in a sub confluent monolayer.
8. Each PathHunter Activated GPCR Internalization Cell Line has been found to be stable for at least 10 passages with no significant drop in assay window or shift in EC_{50}.
9. Assay performance and cellular response can be assessed by treating the cells with reference agonist. **Refer to the cell line specific datasheet for the recommended control agonist for your PathHunter Activated GPCR Internalization Cell Line.** For antagonist assays, cells can be pretreated with varying doses of antagonist/inhibitor compounds followed by agonist challenge, typically at an EC_{80} concentration.

QUICK-START PROCEDURE: ALLOSTERIC MODULATOR DOSE RESPONSE



***Please refer to the cell line specific datasheet for any variations in assay conditions.**

SUBSTRATE PREPARATION AND ADDITION

1. Prepare PathHunter Detection Reagent by combining 1 part **Substrate Reagent 2** Substrate with 5 parts **Substrate Reagent 1**, and 19 parts of **PathHunter Cell Assay Buffer**.

Component	Entire Plate (384 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 2	1.25 mL
Substrate Reagent 1	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 12 μL of prepared detection reagent to the appropriate wells and incubate for 60 minutes at room temperature (23°C). **DO NOT pipette up and down in the well to mix or vortex/shake plates**.
3. Incubate for 60 minutes at room temperature (23ºC).
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your allosteric modulator dose response.

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 the 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 AssayComplete Cell Detachment Reagent to the flask. Rock the flask back and forth gently to ensure the surface of the flask is covered. Incubate at 37°C, 5% CO_2 for 2–5 minutes or until the cells have detached.
4. 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.
5. Add 8–10 mL of AssayComplete Revive Media to each T225 flask. Rinse the cells from the surface of the flask using the added media. Remove the cells from the flask, 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 from Step 5, dilute the resuspended cells to a concentration of 1.2×10^6 cells/mL using AssayComplete Preserve Freezing Reagent.
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 N_2 tank for long-term storage.

PREPARATION OF ASSAY PLATES

Each PathHunter Activated GPCR Internalization Assay has been validated for optimal assay performance using the specific AssayComplete Cell Plating Reagent. **Always use the CP Reagent recommended for the cell line and DO NOT substitute at any time.**

1. Harvest the cells as follows from a confluent T25 or T75 flask using AssayComplete Cell Detachment Reagent. **Do not use Trypsin.**
   a) Remove AssayComplete Cell Culture Media.
   b) Gently wash cells with 5 mL PBS and aspirate.
   c) Add 0.5 mL AssayComplete Cell Detachment Reagent to each T25 flask, or 1 mL to each T75 flask.

d) Place the flask in the incubator for 5 minutes or until cells have detached.

e) Add 3 mL of CP Reagent and transfer to a 15 mL conical tube.

2. Determine the cell density using a hemocytometer. Centrifuge the cells at 300 x g for 4 minutes to pellet cells. Remove supernatant.

3. Resuspend cells in CP Reagent at a concentration of 250,000 cells/mL (5,000 cells/20 μL). Transfer 20 μL of the cell suspension to each well of a 384-well microplate. Please refer to Appendix A for cell numbers and volumes for alternate formats.

4. Incubate the plate for 24 hours at 37°C, 5% CO_2.

ASSAY PROCEDURE — AGONIST DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing GPCR agonist assays using the PathHunter Activated GPCR Internalization Cell Lines and PathHunter Detection Reagents in a 384-well format. Refer to Appendix A for cell numbers and volumes for alternate formats. 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.

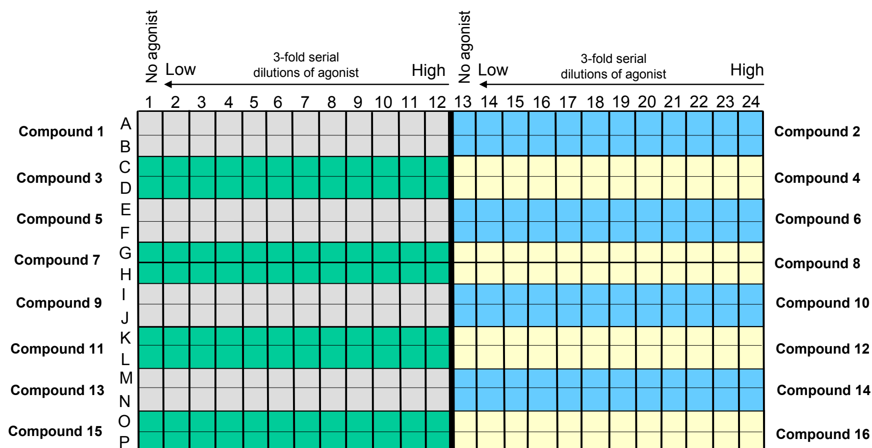


Figure 3. This plate map shows 12-point dose curves with 2 data points at each concentration. Plate map allows 16 compounds to be tested in duplicate per 384-well plate.

DAY 1: PREPARATION OF ASSAY PLATES

Plate PathHunter cells in the appropriate number of wells in a 384-well plate as described in the "Preparation of Assay Plates" section on page 9. Allow cells to incubate overnight.

for the compound (e.g. **500X** IC_{50} would be the final working concentration).

Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration of the corresponding dilution at 5 μM. This is the working concentration.

a) For each compound tested, label the wells of a 384-well dilution plate #1 through #12.

b) Add 20 μL of CP Reagent containing appropriate solvent to wells #1-11.

c) Prepare a working concentration of modulator compound in CP Reagent containing appropriate solvent.

d) Add 30 μL of the working concentration of modulator 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 the pipet tip.

f) With a clean pipet 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 pipet 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. **DO NOT add modulator compound to wells #1 and 2**. These samples serve as the no modulator controls and complete the dose curve.

h) Repeat this process for any additional modulator compounds to be tested.

i) Set compounds 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 2.5 μL from wells #1–12 to duplicate wells according to the plate map on p.18.

5. Incubate cells with modulator compounds for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the modulator compound incubation, determine the EC_{10}/EC_{90} concentration of the agonist from the agonist dose response curve (described on pages 10-13). Prepare a **10X** EC_{10} concentration (PAM) or **10X** EC_{90} concentration (NAM) of agonist compound in the appropriate CP Reagent/solvent as shown below:

   Example: If the expected EC_{10}/EC_{90} of the agonist compound is 10 nM, prepare a stock at 100 nM.

2. When the modulator incubation is complete, add 2.5 μL of agonist compound to well #2-12. Add 2.5 μL of CP Reagent containing appropriate solvent to the "No modulator/No agonist" wells (columns 1 & 13 in Figure 6).

3. Incubate for 3 hours @ 25ºC/37ºC*****.

   NOTE:
   ***** Please refer to cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE — ALLOSTERIC MODULATOR DOSE RESPONSE

The steps outlined below provide the assay volumes and procedure for performing allosteric modulator assays using PathHunter activated internalization GPCR Cell Lines and PathHunter Detection Reagents in a 384-well format. Refer to Appendix A for cell numbers and volumes for alternate formats. Although plate layouts and experimental designs may vary, we recommend performing a 11-point dose curve for each compound using at least *duplicate* wells for each dilution. The protocol and volumes described below are designed for a complete 384-well plate.

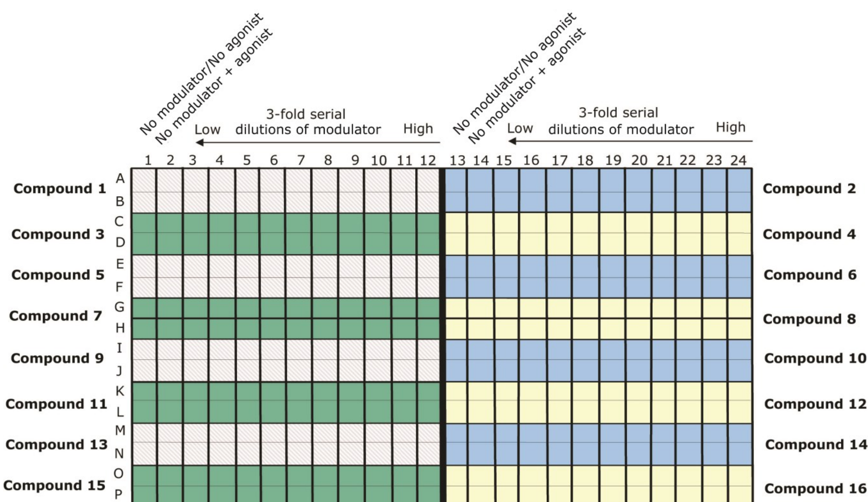


Figure 6. This plate map shows 11-point dose curves with 2 data points at each concentration. Plate map allows 16 modulator compounds to be tested in duplicate per 384-well plate.

DAY 1: PREPARATION OF ASSAY PLATES

Plate PathHunter cells in the appropriate number of wells in a 384-well plate as described in the "Preparation of Assay Plates" section on page 9. Allow cells to incubate overnight.

DAY 2: MODULATOR COMPOUND PREPARATION AND ADDITION

1. Dissolve your allosteric modulator compound in the vehicle of choice (DMSO, ethanol, PBS or other) at the desired stock concentration.
2. Prepare a series of eleven 3-fold serial dilutions of modulator compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other) as described below. The concentration of each dilution should be prepared at **10X** the final screening concentration (i.e. 2.5 μL modulator compound will be used in a final volume of 25 μL). For each dilution, the final concentration of solvent should remain constant. To prepare the 11-point dose curve serial dilutions, we recommend starting with a concentration that is **50X** the expected IC_{50} value

DAY 2: AGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve agonist compound in the vehicle of choice (DMSO, ethanol, PBS or other) at the desired stock concentration.
2. Prepare a series of twelve 3-fold serial dilutions of agonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other) as described below. The concentration of each dilution should be prepared at **5X** of 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 prepare the 12-point dose curve serial dilutions, we recommend starting with a concentration that is **50X** the expected EC_{50} value for the compound (e.g. **250X** EC_{50} would be the final screening concentration).

   Example: If the expected EC_{50} is 10 nM, prepare the highest starting concentration of the corresponding dilution at 2.5 μM. This is the working concentration.

   a) For each compound tested, label the wells of a 384-well dilution plate #1 through #12.
   b) Add 20 μL of CP Reagent containing appropriate solvent to wells #1-11.
   c) Prepare a working concentration of agonist compound in CP Reagent containing appropriate solvent.
   d) Add 30 μL of the working concentration of agonist 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 pipet tip.
   f) With a clean pipet 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 pipet tip.
   g) Repeat this process 8 more times in succession to prepare serial dilutions for the remaining wells, from right to left across the plate. **DO NOT add agonist compound to well #1.** This sample serves as the no agonist control and completes the dose curve.
   h) Repeat this process for each additional agonist compound to be tested.
   i) Set compounds aside until agonist compounds are ready to be added.
3. Remove PathHunter cells from the incubator (previously plated on day 1).
4. Transfer 5 μL from wells #1-12 to duplicate wells according to the plate map shown on p.10.
5. Incubate for 3 hours @ 25ºC or 37ºC*****.

   NOTE:
   *****Please refer to cell line specific datasheet for any variations in assay conditions.

SUBSTRATE PREPARATION AND ADDITION

1. Prepare PathHunter Detection Reagent by combining 1 part **Substrate Reagent 2** Substrate with 5 parts **Substrate Reagent 1**, and 19 parts of **PathHunter Cell Assay Buffer**.

Component	Entire Plate (384 wells)
Cell Assay Buffer	4.75 mL
Substrate Reagent 2	1.25 mL
Substrate Reagent 1	0.25 mL

NOTE:
The working solution is stable for up to 8 hours at room temperature.

2. Add 12 uL 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 60 minutes at room temperature (23ºC).
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your agonist dose response. See the example shown in Figure 4.

REPRESENTATIVE DATA AND DATA ANALYSIS

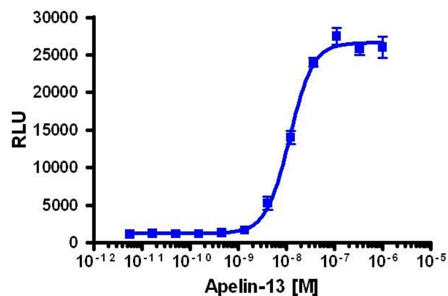
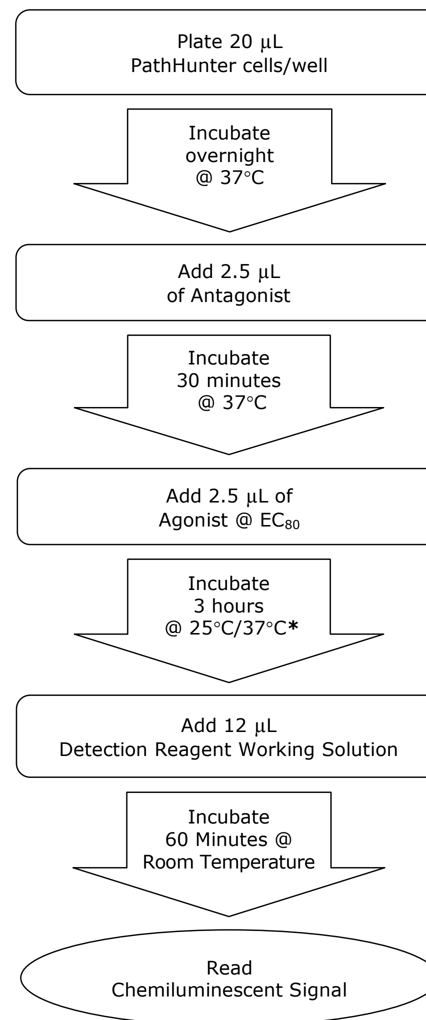


Figure 4. PathHunter® U2OS AGTRL1 Activated GPCR Internalization Cells (93-0710C3). Cells were plated in a 384-well plate at 5,000 cells/well and stimulated with the known agonist Apelin-13 (DiscoveRx, Cat. #92-1093) for 3 hours. Signal was detected using the PathHunter Detection Kit (93-0001) and the recommended protocol. An assay window of 22.7 was achieved in this example, and the EC_{50} for agonist was estimated at 11.7 nM.

QUICK-START PROCEDURE: ANTAGONIST DOSE RESPONSE



***Please refer to the cell line specific datasheet for any variations in assay conditions.**

SUBSTRATE PREPARATION AND ADDITION

1. Prepare PathHunter Detection Reagent by combining 1 part **Substrate Reagent 2** Substrate with 5 parts **Substrate Reagent 1**, and 19 parts of **PathHunter Cell Assay Buffer**.

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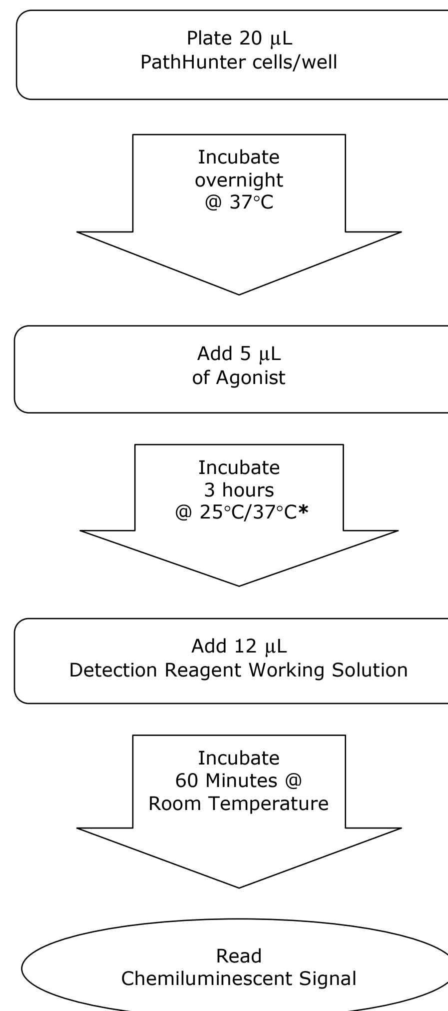
NOTE:
The working solution is stable for up to 8 hours at room temperature.

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 60 minutes at room temperature (23°C).
4. Read samples on any standard luminescence plate reader.
5. Use GraphPad Prism® or other comparable program to plot your antagonist dose response.

QUICK-START PROCEDURE: AGONIST DOSE RESPONSE



*Please refer to the cell line specific datasheet for any variation in assay conditions.

ASSAY PROCEDURE — ANTAGONIST DOSE RESPONSE CURVE

The steps outlined below provide the assay volumes and procedures for performing GPCR antagonist assays using the PathHunter Activated GPCR Internalization cell line and PathHunter Detection Reagents in a 384-well format. Refer to Appendix A for cell numbers and volumes for alternate formats. Although plate layouts and experimental designs may vary, we recommend performing a 11-point dose curve for each compound using at least duplicate wells for each dilution. The protocol and volumes described below are designed for a complete 384-well plate.

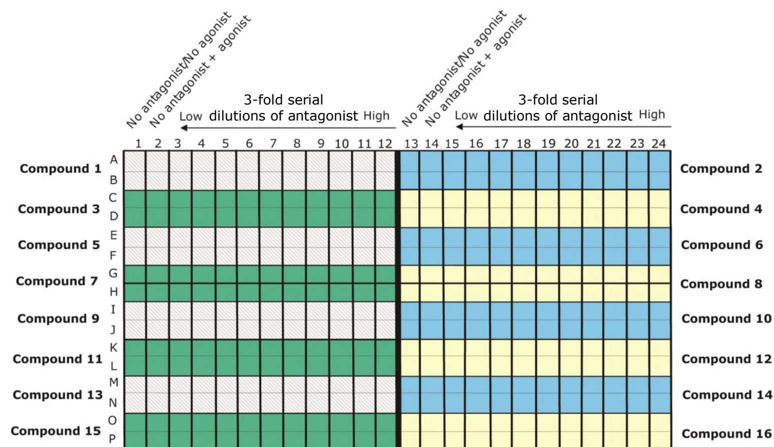


Figure 5. This plate map shows 11-point dose curves with 2 data points at each concentration. Plate map allows 16 compounds to be tested in duplicate per 384-well plate.

DAY 1: PREPARATION OF ASSAY PLATES

Plate PathHunter cells in the appropriate number of wells in a 384-well plate as described in the "Preparation of Assay Plates" section on page 9. Allow cells to incubate overnight.

DAY 2: ANTAGONIST COMPOUND PREPARATION AND ADDITION

1. Dissolve your antagonist compound in the vehicle of choice (DMSO, ethanol, PBS or other) at the desired stock concentration.
2. Prepare a series of eleven 3-fold serial dilutions of antagonist compound in CP Reagent containing the appropriate solvent (DMSO, ethanol, PBS or other) as described below. The concentration of each dilution should be prepared at **10X** the final screening concentration (i.e. 2.5 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 prepare the 11-point dose curve serial dilutions, we recommend starting with a concentration that is **50X** the expected IC_{50} value for the compound (e.g. **500X** IC_{50} would be the final working concentration).

Example: If the expected IC_{50} is 10 nM, prepare the highest starting concentration of the corresponding dilution at 5 μM. This is the working concentration.

a) For each compound tested, label the wells of a 384-well dilution plate #1 through #12.
b) Add 20 μL of CP Reagent to wells #1-11.
c) Prepare a working concentration of antagonist compound in CP Reagent containing appropriate solvent.
d) Add 30 μL of the working concentration of antagonist 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 the pipet tip.
f) With a clean pipet 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 pipet 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. **DO NOT add antagonist compound to wells #1 and #2**. These samples serve as the no antagonist controls and complete the dose curve.
h) Repeat process for any additional antagonist compounds to be tested.
i) Set compounds 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 2.5 μL from wells #1–12 to duplicate wells according to the plate map on page 14.
5. Incubate cells with antagonist compounds for 30 minutes @ 37°C.

AGONIST COMPOUND PREPARATION AND ADDITION

1. During the antagonist incubation, determine the EC_{80} concentration of the agonist from the agonist dose response curve (described on pages 10-13). Prepare a **10X** EC_{80} concentration of agonist compound in the appropriate CP Reagent/ solvent as shown below:

   Example: If the expected EC_{80} of the agonist compound is 10 nM, prepare a stock at 100 nM.

2. When the antagonist incubation is complete, add 2.5 μL of agonist compound to wells #2–12. Add 2.5 μL of CP Reagent to the "No agonist" wells (columns 1 & 13 in Figure 5).
3. Incubate for 3 hours @ 25°C/37°C*****.

   NOTE:
   *****Please refer to cell line specific datasheet for any variations in assay conditions.

DiscoverX

Technical Bulletin

Updated Cell Culture Protocol and Handling Procedure

Applies to: Cell Lines Only

Product Numbers: Several

Date: February 28, 2017

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

Dear Valued Customer,

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

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

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

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

Sincerely,

The DiscoverX team

Cell Culture and Handling Procedure

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

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

Contact technical support immediately if cells are thawed upon arrival.

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

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

Cell Culture Protocol

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

Care should be taken in cell handling to avoid contamination.

Cell Thawing

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

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

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

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

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

Cell Propagation

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

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

Prolonged treatment with Trypsin-EDTA may compromise cell viability

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

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

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

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

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

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

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

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

Cell Freezing

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

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

Care should be taken in handling to avoid contamination.

2. Gently aspirate the media from the flasks.

3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.

4. Gently aspirate PBS from the flask.

5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).

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

6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.

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.