# PathHunter® Dimerization Assay

For chemiluminescent detection of protein dimerization

## *Simple Solutions for Complex Biology*

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

DiscoveRx

# Table of Contents



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

# Overview

## Intended Use

PathHunter Dimerization assays provide a robust, highly sensitive and easy-to-use cell-based functional assay to study various protein activities in a cell. The assays allow for the monitoring of receptor-receptor interactions at the cell surface, with high serum tolerance and large signal to noise ratios ideal for lead identification, quality control and development of neutralizing antibody (NAb) assays for a variety of biologics, including bispecific antibodies.

## Technology Principle: PathHunter® Dimerization Assay

These assays utilize Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, ProLink (PK) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

The PathHunter Dimerization assay detects ligand induced dimerization of two subunits of a receptor-dimer pair. The cells have been engineered to co-express one receptor subunit (target protein 1) fused to Enzyme Donor (ED), and a second dimer partner (target protein 2) fused to Enzyme Acceptor (EA). Binding of an agonist to one receptor subunit induces it to interact with its dimer partner, forcing complementation of the two enzyme fragments. This results in the formation of a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.

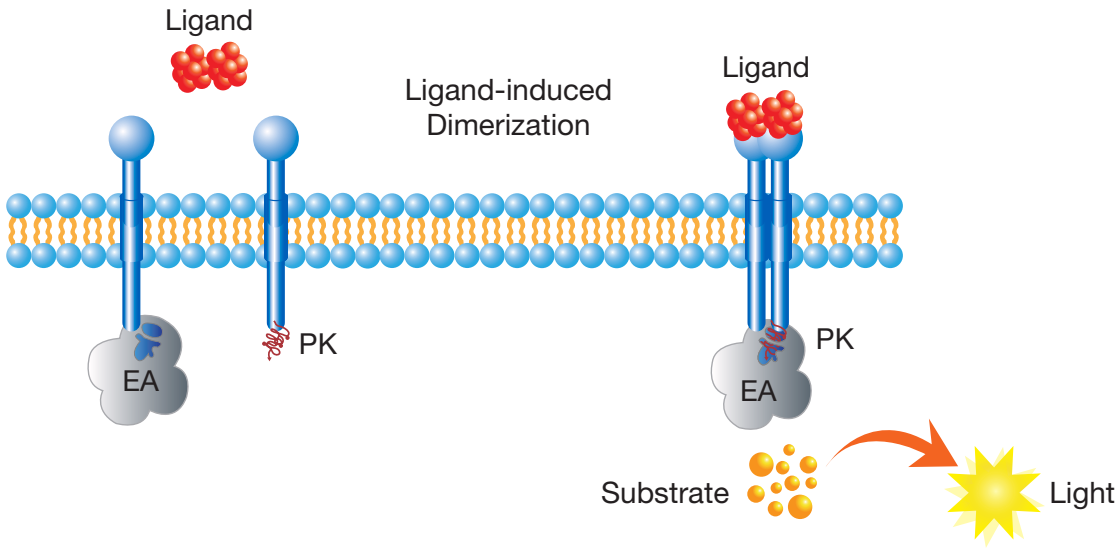



Figure 1: PathHunter Dimerization Assay Principle

# Materials Provided

| List of Components | | | | |
|---|---|---|---|---|
| | **Name** | **Contents** | **Size** | **Catalog Number** |
| **Product** | PathHunter Dimerization Assay Cell Line | 2 vials of 1x10$^6$ cells/vial in 1mL freezing reagent | 1 Set | 93-XXXXXX |
| **Shipping Conditions** | Dry Ice | | | |
| **Storage Conditions of Cells** | Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vial and initiate the culture as soon as possible upon receipt.<br><br>If continued storage of the frozen vials is necessary, store as follows:<br><br>• **Short term** (2 weeks or less): Store vials at -80°C immediately upon arrival. (DO NOT store at –80°C for more than 2 weeks.)<br><br>• **Long term** (greater than 2 weeks): Vials should ONLY be stored in the **vapor phase** of liquid nitrogen ($LN_2$). * | | | |

***Safety Warning:** A face shield, gloves and lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid $N_2$. Upon thawing, if liquid $N_2$ is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the freezer.

# Additional Materials Required (Not Provided)

The following equipment and additional materials are required to perform PathHunter Dimerization Assays:

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

| Product Description | Vendor | Part Number |
|---|---|---|
| AssayComplete™ Revive Media | DiscoveRx | See product datasheet |
| AssayComplete™ Preserve Freezing Reagent | DiscoveRx | |
| AssayComplete™ Cell Detachment Reagent | DiscoveRx | |
| AssayComplete™ Protein Dilution Buffer | DiscoveRx | |
| 96-well Clear bottom TC treated, Sterile (10 plates/pack) | DiscoveRx | |
| 96-well White bottom TC treated, Sterile (10 plates/pack) | DiscoveRx | |
| Green V-bottom Ligand Dilution Plates (10 plates/pack) | DiscoveRx | |

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

## Recommended Materials

The following products* are recommended:

| Product Description | Catalog No. |
|---|---|
| CytoTracker™ Cell Proliferation Kit | 92-2001M |
| CytoTracker™ LDH Quantification Kit | 92-2002 |
| CytoTracker™ Glutathione Quantification Kit | 92-2003 |
| CytoTracker™ DNA Damage Quantification Kit | 92-2004M |
| Control Ligands | www.discoverx.com/controlligands |

* Products not available in all countries. Please inquire.

## Protocol Schematic

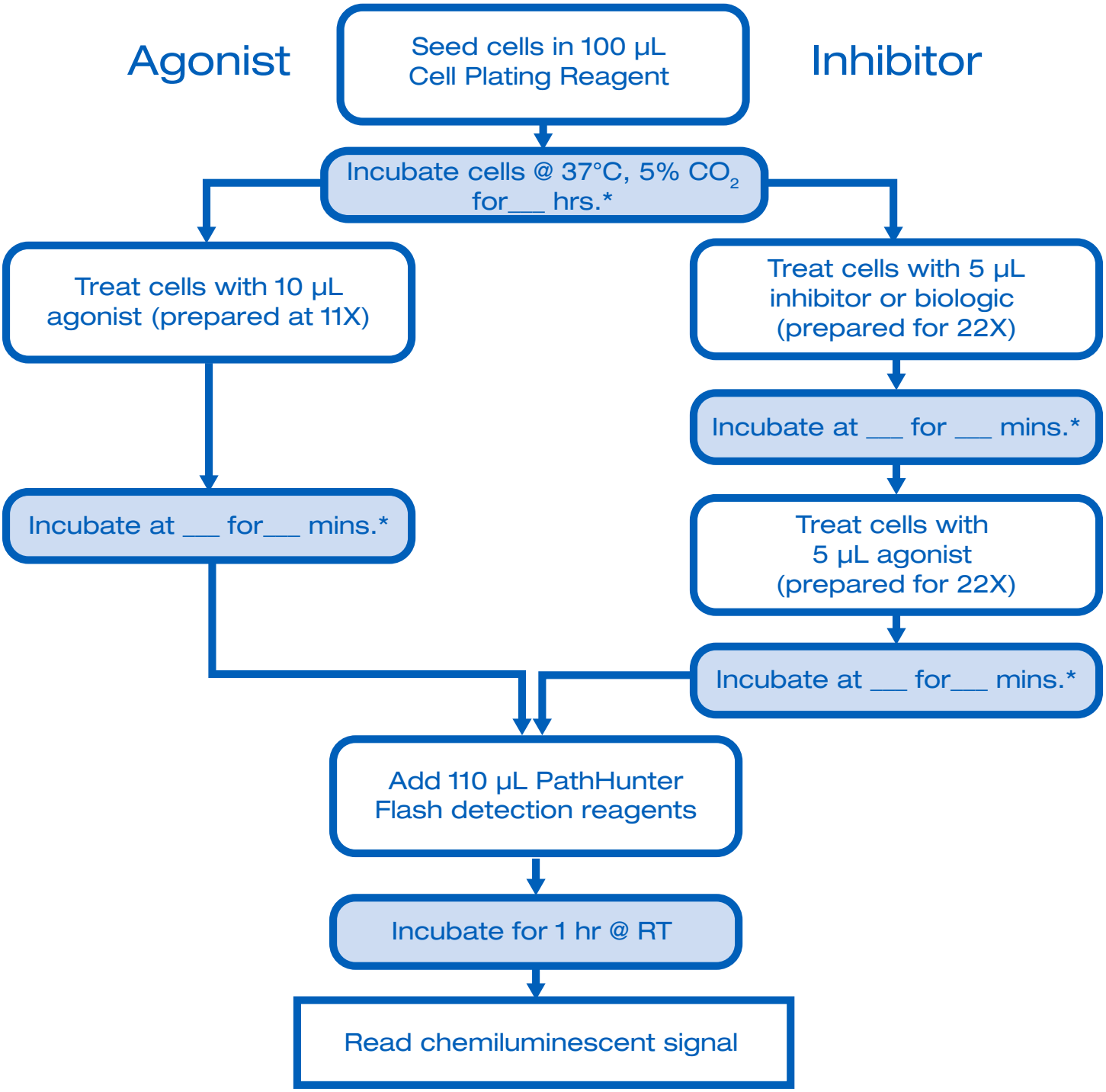
**Tip:** *Use this sheet to note your assay specific conditions. Post on your bench to use as a quick reference guide.*

Assay Name:_____

Product Number: _____

**Quick-Start Procedure:** Agonist Dose Response

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




* Assay specific time and temperature in datasheet

# Detailed Protocols

## Cell Culture and Handling Procedures

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

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

### Cell Thawing Method

1. Place 12 mL AssayComplete Revive Media in T75 flask and pre-warm in a humidified 37˚C/5% CO2 incubator for fifteen minutes to equilibrate pH and temperature.
2. Remove cryovials from liquid $N_2$ storage and place immediately on dry ice.

**Caution:** 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. Do not touch the bottom of the tubes at any time to avoid inadvertent thawing of the cells.

3. Rapidly thaw the cells in a 37˚C water bath for 30-45 seconds with gentle agitation. (Do not submerge the vial when thawing. Longer incubation times may result in cell death.)
4. Decontaminate the vial by wiping with 70% ethanol.
5. Gently transfer the contents of the vial into the pre-warmed medium from step one and allow cells to recover for at least 48 hours (or until 80% confluent).

   **Caution:** Do not centrifuge to remove DMSO.

6. After 48 hours, gently remove the thawing media without disturbing the cell monolayer. If confluent, continue to passage the cells as described below. If not confluent, replace with 12mL of appropriate pre-warmed AssayComplete Cell Culture Media.

### Cell Propagation Method

1. Passage cells as necessary. Typically, you can passage cells every 2-3 days with a 1:3 split ratio. Maintain cells between 30% and 90% confluence (e.g. once the cells are 90% confluent, split 1:3 for 30% confluency). Heavier split ratios (e.g. >1:5) are NOT recommended for these cell lines.

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

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

- 6-well dish = 0.2mL AssayComplete Cell Detachment Reagent
- T25 = 1.0 mL AssayComplete Cell Detachment Reagent
- T75 = 2.0 mL AssayComplete Cell Detachment Reagent
- T225 = 3.0 mL AssayComplete Cell Detachment Reagent

3. Cells usually detach in 2-5 minutes. Verify cell detachment under microscope. Add 3-5 mLs of AssayComplete Cell Culture Media or AssayComplete Cell Plating Reagent, depending on subsequent application. For continuing culture, use AssayComplete Cell Culture Media and split cells as described in step 1. For performing the assay, use AssayComplete Cell Plating Reagent and follow the instructions in the Assay Protocol section of this manual.

## Cell Freezing Method

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

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

# Assay Protocol

## Small Molecules and Purified Biologics

After harvesting cells with AssayComplete Cell Detachment Reagent, spin down (300g for 5min.) and resuspend the cells in appropriate AssayComplete Cell Plating Reagent to exactly the correct cell concentration, suitable for plating (e.g. for 10,000 cells/100µL one needs a 100,000 cells/mL concentration). Please see the datasheet for assay-specific information. The PathHunter assays are routinely carried out in the presence of <1% solvent (eg. DMSO, ethanol, etc.). Solvents may affect the assay performance, so please optimize assay conditions when using other solvents/solvent concentrations. These assays are optimized for 384-well and 96-well white-walled plates. For volumes associated with 384-well format, please see page 10.

**Assay Protocol For Small Molecules And Purified Biologics**

| | Agonist Assay Protocol | Inhibitor Assay Protocol |
|---|---|---|
| **Step 1:** Plate Cells and Incubate | Spin down, resuspend and seed specified number of cells (indicated on assay-specific datasheet) in 100 µL AssayComplete Cell Plating Reagent. Incubate for indicated amount of time at indicated temperature.* | |
| **Step 2:** Pre-treat with Inhibitor and Incubate | NA | Treat cells with 5 µL inhibitor (22X)** in dose curve.<br>Incubate for indicated amount of time at indicated temperature.* |
| **Step 3:** Treat Cells with Agonist and Incubate | Induce cells with 10 µL agonist (11X)** in dose curve.<br>Incubate at appropriate temperature for indicated amount of time. | Induce cells with 5 µL agonist challenge (22X)** at $EC_{90}$.***<br>Incubate at appropriate temperature for indicated amount of time. |
| **Step 4:** Add Detection Reagents | Immediately after the ligand incubation, add 110 µL PathHunter Flash Detection Reagents (working detection solution) to each well. Refer to the PathHunter Flash Detection Kit user manual for instructions on preparing the working detection solution.<br>Incubate plate at room temperature for 1 hour in the dark. | |
| **Step 5:** Read Samples | Read sample on a standard luminescence plate reader at 0.1 to 1 sec/well for PMT readers or 5-10 seconds for imager. | |

*** Note:** For product-specific information on AssayComplete Cell Culture Media, Cell Plating Reagent, control agonist, control inhibitor (if available), incubation times and temperature, please see product data sheet.

** Agonists are prepared at 11X of final screening concentration for agonist assays. The inhibitor is prepared at 22X of final screening concentration and the agonist challenge dose is prepared at 22X final concentration for inhibitor assays. These concentrations will result in 1x concentration when added to the assay plate.

*** The specific concentration of agonist challenge to be used can vary from $EC_{75}$ to $EC_{100}$, based on the target and assay conditions. For an anti-receptor antibody, antibody and agonist can be pre-incubated together before combined addition to the assay plate. The pre-incubation time should be optimized empirically. 0-60 min. pre-incubation times are typical.

For more information on agonist/inhibitor preparation and reconstitution, please refer to the product datasheet for the appropriate DiscoveRx Synergy products.

For detailed instructions on setting up a dose response curve, please refer to Supplemental Protocols on page 8.

### Non-Purified Biologics

After harvesting cells with AssayComplete Cell Detachment Reagent, spin down (300g for 5min.) and resuspend the cells in appropriate AssayComplete Cell Plating Reagent to exactly the correct cell concentration, suitable for plating (e.g. for 10,000 cells/100µL one needs a 100,000 cells/mL concentration). Please see the datasheet for assay-specific information. The PathHunter assays can be run in the presence of high levels of serum or plasma without significantly impacting assay performance, therefore, standard curves of control can be prepared in neat serum or plasma and added directly to cells without further dilution. These assays are optimized for 384-well and 96-well white-walled plates. For volumes associated with 384-well format, please see page 10.

**Assay Protocol For Non-Purified Biologics**

| | Agonist Assay Protocol | Biologic Sample Assay Protocol |
|---|---|---|
| **Step 1:** Plate Cells and Incubate | Spin down, resuspend and seed specified number of cells (indicated on assay-specific datasheet) in 100 µL AssayComplete Cell Plating Reagent. Incubate for indicated amount of time at indicated temperature.* | |
| **Step 2:** Aspirate Media | NA | Gently remove the Cell Plating Reagent from the cells. |
| **Step 3:** Pre-treat with Crude Biologic and Incubate | NA | Treat cells with 100 µL crude biologic samples (1X)** in dose curve, diluted in AssayComplete Protein Dilution Buffer as necessary. Incubate for indicated amount of time at indicated temperature.* |
| **Step 4:** Treat Cells with Agonist and Incubate | Induce cells with 10 µL agonist (11X)** in dose curve. Incubate at appropriate temperature for indicated amount of time. | Induce cells with 10 µL agonist challenge (11X)** at $EC_{90}$.*** Incubate at appropriate temperature for indicated amount of time. |
| **Step 5:** Aspirate Incubation Mixture | Immediately after the ligand incubation, gently remove the incubation mixture from the cells and add 110 µL of fresh Cell Plating reagent to each well. | |
| **Step 6**: Add Detection Reagents | Add 110 µL of PathHunter Flash Detection Reagents (working detection solution) to each well. Refer to the detection kit user manual for instructions. Incubate plate at room temperature for 1 hour in the dark. | |
| **Step 7:** Read Samples | Read plate on a standard luminescence plate reader at 0.1 to 1 sec/well for PMT readers or 5-10 seconds for imager. | |

*** Note:** For product-specific information on AssayComplete Cell Culture Media, Cell Plating Reagent, control agonist, control inhibitor (if available), incubation times and temperature, please see product data sheet.

** Agonists are prepared at 11X of final screening concentration. Crude biologic samples are added at 1X of final screening concentration. These concentrations will result in 1X concentration when added to the assay plate.

*** The specific concentration of agonist challenge to be used can vary from $EC_{75}$ to $EC_{100}$, based on the target and assay conditions.

# Supplemental Information

## Appropriate Methods to Set Up Dose Curves and Dilutions

1. Dissolve test article(s) in the appropriate solvent (see specific information on data sheet).
2. Prepare a series of 12 three-fold dilutions of test article in Cell Plating Reagent or appropriate solvent. The concentration of each dilution should be prepared at 11X of the final screening concentration (10µL of 11X test article + 100µL of cells will give 1X final screening concentration).
    a. Label the wells of the dilution plate.
    b. Add 30µL of vehicle to dilution wells #1 to #11 (the dilution volume needs to be adjusted according to the number of duplicate wells).
    c. Prepare the highest concentration of test article in appropriate solvent. [We recommend targeting a working concentration 550 times the expected $EC_{50}$. e.g. For an expected $EC_{50}$ of 10ng/mL, prepare the highest concentration in working dilution at 5500ng/mL. This is 11X the screening or final concentration of 500ng/mL].
    d. Add 45µL of highest concentration to well #12.
    e. Remove 15µL from well #12 and add it to well #11 and mix gently.
    f. With clean tip, remove 15µL from well #11 and add it to well #10. Mix gently.
    g. Repeat process until well #2 is reached. DO NOT add test article to well #1.
    h. Set up additional dose curves for agonists and inhibitors. See figures 1 and 2 on the following page for sample plate maps with agonist and inhibitor dose curves.

## Important Tips for Preparing Accurate Dilution Curves

- Peptides and proteins can be diluted in AssayComplete Protein Dilution Buffer.
- Test articles in organic solvents like DMSO or ethanol need special handling to ensure accuracy.
    - Prepare dilution curves at 110X the final screening concentration in organic solvent.
    - Dilute the 110X dilution series in organic solvent from the above step, 10-fold in AssayComplete Protein Dilution Buffer to get the working 11X concentration for entire dose curve.
    - Add 10µL of 11X working concentration to 100µL of cells to get 1X screening concentration. The final solvent concentration will be 1% in each well.
    - Test articles that are soluble in organic solvents will precipitate out of aqueous solution at higher concentrations, decreasing accuracy in subsequent dilutions. This protocol ensures that insufficient solubility of molecule at high concentrations in aqueous solution will not affect subsequent lower dilution series.

## Representative Plate Maps for Agonist/Inhibitor Curve

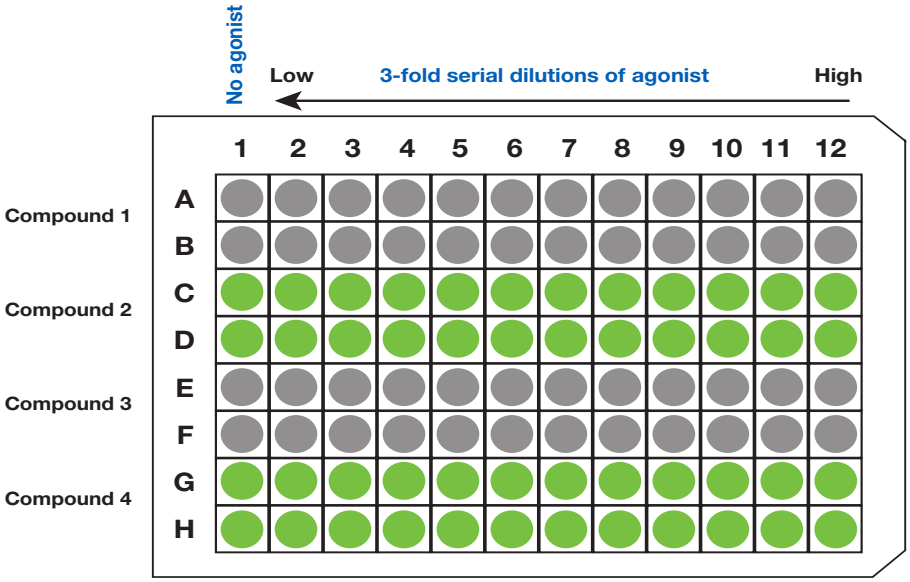



Figure 1. This agonist plate map shows 12-point dose curves with 2 data points at each concentration for 4 agonists per plate for a total of 24 data points per 96-well plate.

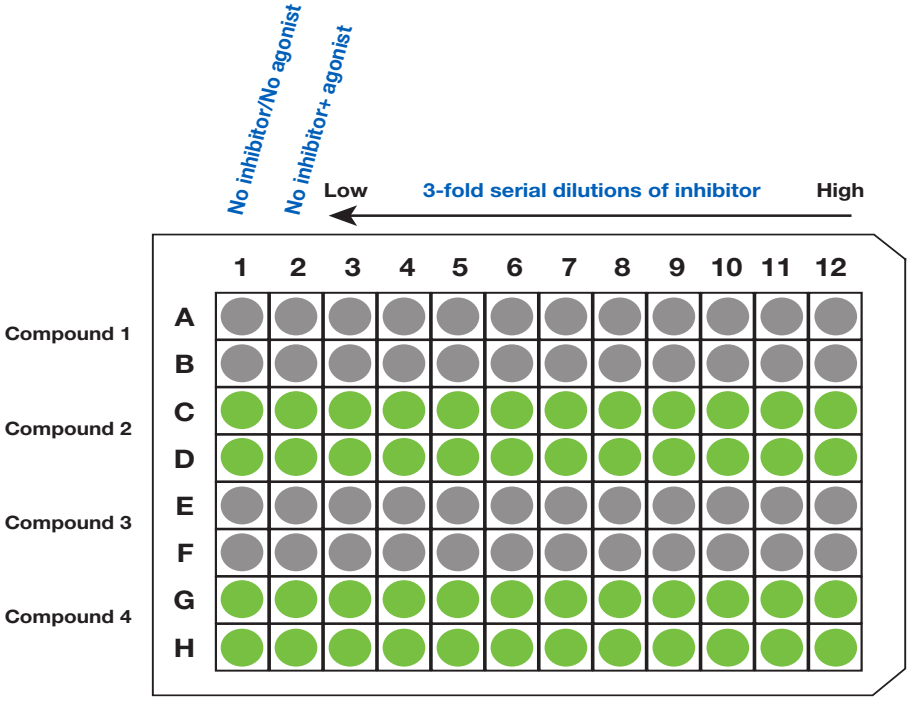



Figure 2. This inhibitor plate map shows 11-point dose curves with 2 data points at each concentration for 4 inhibitors per plate for a total of 24 data points per 96-well plate.

## Assay Formats Table

| PathHunter Certified Assay Format | | | | |
|---|---|---|---|---|
| Plate Format | 96-well | FV 384-well | LV 384-well | 1536-well |
| Total Volume | 220 µL | 55 µL | 30 µL | 8 µL |
| Cell Numbers | 10,000 | 5,000 | 5,000 | 1,250 |
| Cell Plating Reagents* | 100 µL | 20 µL | 12.5 µL | 3 µL |
| Ligand | 10 µL | 5 µL | 2.5 µL | 1 µL |
| Flash Detection Reagents | 110 µL | 30 µL | 15 µL | 4 µL |

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

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

| Instrument Compatibility Chart | | | |
|---|---|---|---|
| **Assay** | **Instrument** | **Read-Out** | **Specifications** |
| All PathHunter® assays<br>HitHunter® cAMP<br>HitHunter® cGMP | **COMPATIBLE WITH ANY LUMINOMETER**<br>**BMG:** PheraStar, Cytostar, LumiStar<br>**Perkin Elmer:** TopCount, Victor 2 or V, Fusion, LumiCount, Envision, Micro-beta (Trilux), Viewlux, Northstar<br>**GE:** LEAD seeker, Farcyte<br>**Molecular Devices:** FLIPR, SpectraMax M3/ M4/M5/M5e, FlexStation 3, SpectraMax L<br>**Tecan:** Ultra, Evolution<br>**Turner BioSystems:** Modulus Microplate<br>**Caliper:** LabChip 3000 & EZ Reader<br>**Berthold Technologies**: Mithras LB940, CentroLIApc<br>**Hamamatzu:** FDS6000, FDSS/RayCatcher<br>**Thermo Scientific:** Luminoskan Ascent<br>**Biotek:** Synergy 2 | Luminescence | Excitation Filter Fluorescein- 485 nm<br>Emission Filter Fluorescein- 530 nm<br>Dichroic Fluorescein- 505 nm |

# Troubleshooting Guide

| Problem | Cause | Solution |
|---|---|---|
| **No Response** | Improper cell growth conditions | See datasheet for cell culture conditions |
| | High DMSO/solvent concentration | Maintain DMSO/solvent at <1% in serial dilutions of compounds |
| | Improper ligand used or improper ligand incubation time | See datasheet for recommended ligand and assay conditions |
| | Improper preparation of ligand (agonist or antagonist) | Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand |
| | Improper time course for induction | Optimize time course of induction with agonist and inhibitor |
| **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 according to the User Manual instructions 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** | Incorrect incubation temperature | Check and repeat assay at correct incubation temperature as indicated on the assay datasheet |
| | Improper preparation of ligand (agonist or antagonist) | Some ligands are difficult to handle. Confirm the final concentration of ligands |
| **Cells growing slowly** | U2OS grows slower than CHO-K1 or HEK 293 | Average doubling time for U2OS cells is 3 days, so please observe cells under microscope and monitor cell health |
| | Slow growing clones | Use of DiscoveRx functionally validated and optimized media and reagents improves assay performance |
| **$EC_{50}$ is right-shifted** | Improper ligand handling or storage | Check ligand handling requirements |
| | Difference in agonist binding affinity | Confirm that the ligand used is comparable to the ligand in the Product Insert |
| | Problems with plate type and compound stability | Hydrophobic compounds should be tested for solubility and may be diluted in buffer containing 0.1% BSA |
| | | Non-binding surface plates may be necessary for hydrophobic compounds |
| **High well-to-well variability in Z' study** | Problems with plate type and compound solubility | Z' studies should be performed with automation |
| | | It may be necessary to test plate types and compound stability |

For additional information or technical support, please call or email at the numbers listed below.

# Limited Use License Agreement

**A.** This product and/or its use is covered by U.S. patents #8,541,175 and #8,679,832 and/or related foreign patents and pending applications and trade secrets that are either owned by or licensed to DiscoveRx corporation. The cells and detection reagents (collectively "Materials") purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Cell-Based Dimerization assay ("Assay") that employs genetically modified cells and vectors (collectively, the “Cells”), and related detection reagents (the “Reagents”) (collectively referred to as “Materials”). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

1. Purchaser is permitted to use and propagate the Cells only for use in the Assay and in connection with Reagents purchased from DiscoveRx Corporation or its authorized distributor.

2. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.

3. The Reagents contain or are based upon the proprietary and valuable know-how developed by DiscoveRx, and the Reagents have been optimized by DiscoveRx to function more effectively with the Cells in performing the Assay. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser’s behalf.

4. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.

5. Purchaser will not use the Cells with any other reagents or substrates, other than the Reagents that are provided by or purchased from DiscoveRx or an authorized DiscoveRx distributor, in connection with the Materials.

6. The number of Assays performed will not exceed the authorized number for which Materials were purchased.

**B.** The use of this cell line may require third party licenses. Purchaser shall have the sole responsibility for obtaining any such licenses prior to use of the cell lines.

If the purchaser has any further questions regarding the rights conferred with purchase of the Materials, please contact:

**Licensing Department**
DiscoveRx Corporation
42501 Albrae Street
Fremont, CA 94538 USA
t | 1.510.979.1415 x 104
e | info@discoverx.com

## Intended Use

Your PathHunter Dimerization Cell Line, when used in conjunction with a PathHunter Flash Detection Kit (93-0247 or 93-0247L), provides a cell-based functional assay for the detection of receptor dimerization. The assays described in this booklet have been validated for use in both 96-well and 384-well microplate formats.

# Technical Bulletin

# Updated Cell Culture Protocol and Handling Procedure

Applies to: Cell Lines Only

Product Numbers: Several

Date: February 28, 2017

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

Dear Valued Customer,

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

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

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

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

Sincerely,

The DiscoverX team

# Cell Culture and Handling Procedure

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

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

Contact technical support immediately if cells are thawed upon arrival.

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

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

## Cell Culture Protocol

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

Care should be taken in cell handling to avoid contamination.

## Cell Thawing

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

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

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

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

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.