DiscoveRx

InCELL Hunter™ Epigenetic Cell-Based Assay

For chemiluminescent detection of protein levels

User Manual

Simple Solutions for Complex Biology

CONTENTS

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

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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

InCELL Hunter™ Epigenetic assay cell Line, when used in conjunction with a InCELL Hunter™ Detection Kit (96-0002, 96-0002L or 96-0002XL), provides a cell based assay to look at protein stability upon compound binding. The assay described in this booklet have been validated for use in 384-well microplate formats.

TECHNOLOGY PRINCIPLE

InCELL Hunter™ cell lines feature a novel in vivo application of the Enzyme Fragment Complementation (EFC) technology in which the β-galactosidase enzyme has been split into two inactive fragments, the enhanced ProLabel (ePL) and the enzyme acceptor (EA). The platform measures compound-protein binding using a novel β-galactosidase tag, ePL. In this system the protein of interest is tagged to ePL. The cellular amount of protein is detected by the addition of enzyme acceptor (EA) which complements with ePL to form a fully active β-galactosidase enzyme, that can be quantitatively detected using the chemiluminescent substrate. The amount of enzyme activity obtained is proportional to the amount of ePL tagged protein present in the well. Cells expressing the designated fusion protein will be tested for changes in protein levels, in response to compound treatment.

ASSAY OVERVIEW

To perform InCELL Hunter™ Assays, you will also need the InCELL Hunter™ Detection Kit (96-0002, 96-0002L or 96-0002XL) in order to generate the chemiluminescent signal. Assays should be run using fresh, low-passage cells that have not been allowed to reach confluency for more than 24 hours. Ideally cells should be grown to 70-80% confluence. Following cell treatment, the assay is performed by adding a working solution of InCELL Hunter™ Detection Reagents to the treated cells in a no-mix, one-addition protocol. After addition of the detection reagents, the samples must be read after 30 minutes. The **Assay Procedure** sections and **Quick Start Guide** in this booklet contain detailed information about how to run the assay.

MATERIALS PROVIDED

Description	Storage
InCELL Hunter™ cells (2 vials)	Liquid N_2 (Vapor phase)

NOTE:
Please refer to the datasheet of the InCELL Hunter™ cell line for detailed information on the target that you are testing.

MATERIALS NOT PROVIDED (REQUIRED)

The following equipment and additional materials are required to perform InCELL Hunter™ Assays:

Equipment	Materials
• Single- and multichannel micro-pipettors and pipette tips • Tissue culture disposables and plasticware (T25 and T75 flasks, etc.) • Cryogenic vials for freezing cells • V-bottom 384-well compound dilution plates (DiscoveRx, Cat. #92-0011 or similar) • Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar) • Hemocytometer • White wall, clear bottom 384–well microplates (DiscoveRx, Cat. #92-0013 or similar) • Multimode or luminescence plate reader (LumiLite; DiscoveRx Cat. #75-0001 or similar)	• InCELL Hunter™ Detection Kit (DiscoveRx, Cat. #96-0002 series) • Revive™ Media (DiscoveRx, Cat. #92-0016RM Series) • PathHunter®*select* Cell Culture Kits (DiscoveRx, Cat. #92-0018G Series) • Preserve™ Freezing Reagent (DiscoveRx, Cat. #92-0017FR Series) • Cell Detachment Reagent (DiscoveRx, Cat. #92-0009) • PathHunter® Cell Plating (CP) Reagent (DiscoveRx, Cat. #93-0563R Series) • Control compound

NOTE:
Refer target specific data sheet for additional details.

STORING & REMOVING CRYOVIALS FROM LIQUID NITROGEN

Cryovials are shipped in 2 vials on dry ice and contain 1.2 - 2.0 x 10^6 cells/vial in 1 mL of freezing medium. Upon receipt, the vials can be stored for up to 2 weeks at -80°C or transferred to the vapor phase of liquid nitrogen. **DO NOT** store at –80°C for extended periods as this could result in significant loss in cell viability. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage. A face shield, gloves, and a lab coat should be worn during these procedures.

1. InCELL Hunter™ cells must arrive in a frozen state on dry ice. If cells arrive thawed, do not proceed, contact technical support.
2. Frozen cells must be transferred to either liquid nitrogen storage or a –80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at –80°C. For longer storage, place vials in the *vapor phase* of liquid nitrogen storage.

   NOTE:
   # CRYOVIALS ARE NOT RATED FOR STORAGE IN THE LIQUID PHASE OF LIQUID NITROGEN. CRYOVIALS SHOULD BE STORED IN THE VAPOR PHASE.

3. When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid nitrogen inside the vial to evaporate.
4. Proceed with the thawing protocol in the following section.

CELL THAWING AND PROPAGATION METHODS

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

1. Pre-warm 5-10 mL Revive™ Medium (RM) in a 37°C water bath.
2. Place the frozen cell vials briefly in a 37°C water bath under sterile conditions until only small ice crystals remain and the cell pellet is almost completely thawed (30 sec - 1 min). **Caution: Longer incubation times may result in cell death**.
3. Transfer thawed cells to a sterile 15 mL conical tube containing the 5-10 mL of pre-warmed Revive medium. Centrifuge at 300 x g for 4 minutes to pellet cells. Remove media.
4. Resuspend cell pellet in 5 mLs of pre-warmed RM. Transfer cells to a T25 flask and incubate for 24 hours at 37°C/5% CO_2.
5. After 24 hours, gently remove media (being careful not to disturb the cell monolayer) and replace with 5 mLs of Cell Culture Media (refer to target specific data sheet for specific Cell Culture Media requirements).
6. Once the cells become >70% confluent in the T25 flask, trypsinize using Cell detachment reagent and transfer the cells to a T75 flask containing 10-12 mLs of cell culture media.

   NOTE:
   To maintain the logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.
7. Passage the cells every 2–3 days, based on the doubling time of the cell line, using the Cell Detachment Reagent. For routine passaging, prepare a 1:2 dilution of cells in a total volume of 10 mL of cell culture media. Transfer 5 mL of the diluted cells to each of two new T75 flasks containing 10-12 mLs of cell culture media.
8. The clone has been found to be stable for at least 10 passages with no significant drop in assay window and EC_{50}.

CELL FREEZING PROTOCOL

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

1. Remove T225 flasks from incubator and place in tissue culture hood. Aspirate the media from the flasks.
2. Add 10 mL PBS into each T225 flask and swirl to rinse the cells. Aspirate PBS from flask.
3. Add 5 mL of Cell Detachment Reagent to the flask. Rock the flask back and forth gently to ensure surface of the flask is covered. Return flask to the incubator for 5 minutes, or until cells have detached.
4. Remove the flask from the incubator and view under a microscope to confirm that the cells are detached. Tap the edge of the flask to detach cells from the surface, if necessary.
5. Add 8-10 mL of Cell Plating Reagent (refer target specific data sheet) to each T225 flask. Rinse the cells from the surface of the flask using the added media. Remove the cells from the flask and transfer to a 50 mL conical tube. (If necessary, add an additional 5 mL of reagent 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 1500 x g for 5 minutes.
7. After centrifugation, discard the supernatant. Resuspend the cell pellet in Preserve™ Freezing Reagent. Based on the cell number obtained from Step 5, dilute the resuspended cells to a concentration of 1.2– 2.0 x 10^6 cells/mL.
8. Transfer 1 mL cells to each 2 mL cryogenic tube. (Keep cells on ice during this process and transfer to a cryogenic container pre-chilled at 4°C).
9. Transfer tubes to –80°C and store overnight. Transfer tubes into the vapor phase of a liquid nitrogen tank for long-term storage.

TIPS FOR OPTIMAL PERFORMANCE:

- Cells must be maintained exactly as mentioned to maintain expression of fusion protein.
- Ideally cells should be maintained at approximately 70% confluence. Cells should not be allowed to grow at confluence for more than 24 hours.
- Allowing the cells to adhere and grow overnight prior to any assay is recommended.

ASSAY PROCEDURE - COMPOUND DOSE RESPONSE CURVE

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

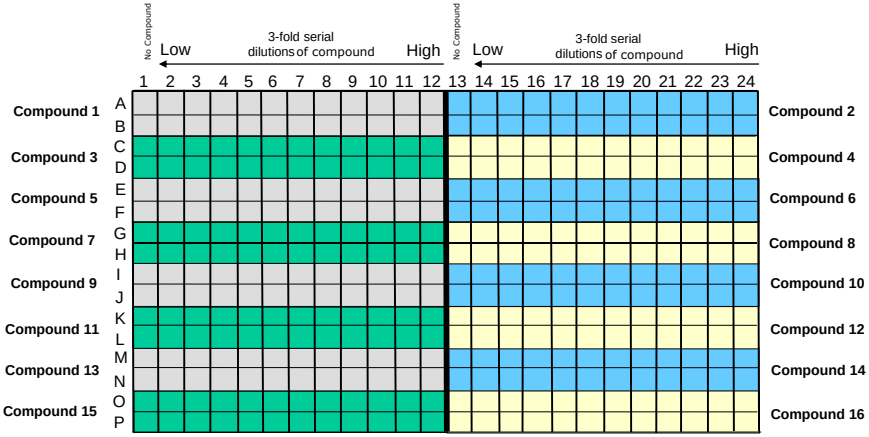


Figure 1. This plate map shows 12-point dose curves with 2 data points at each concentration for 16 compounds per plate for a total of 160 compound dilutions per 384-well plate.

PROTOCOL: COMPOUND DOSE RESPONSE

1. Harvest cells as follows from a confluent T25 or T75 flask using Cell Detachment Reagent (DiscoveRx, Cat. #92-0009).
   a) Remove medium.
   b) Wash cells with 5 mL PBS and aspirate.
   c) Add 0.5 mL Cell Detachment Reagent for a T25 flask, or 1 mL Cell Detachment Reagent for a T75 flask.
   d) Place flask in the incubator for 5 minutes, or until cells have detached.
   e) Add 3 mL of Cell Plating Reagent and transfer to a conical tube.
2. Determine cell density using a hemocytometer.
3. Using Cell Plating Reagent, adjust the volume of the suspension to achieve a cell concentration per well indicated in the target specific data sheet.
4. Transfer 20 µL of the cell suspension to each well of a 384-well white-walled microplate.
5) Incubate the plate overnight at 37°C/5% CO_2.

6. Remove InCELL Hunter™ cells from the incubator (previously plated on day 1).
7. Transfer 5 µL of the compound (**5X**) to the plate as shown in the quick start guide.
8. Incubate cells with the compounds for the indicated times and temperatures in the target specific data sheet.

DETECTION REAGENT PREPARATION AND ADDITION

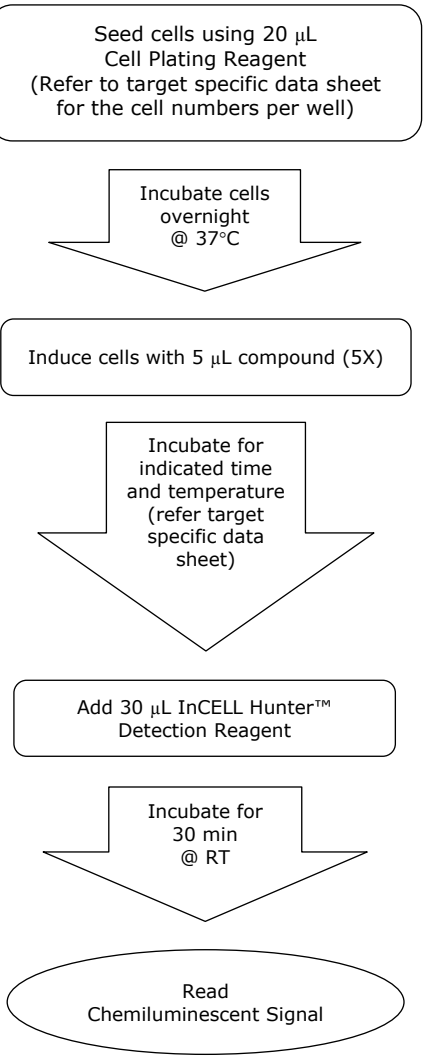
1. Prepare InCELL Hunter™ Detection Reagent as described in the InCELL Hunter™ Detection Kit Product Insert (Cat. #96-0002). Add 30 µL of prepared detection reagent to the appropriate wells. **DO NOT pipette up and down in the well to mix or vortex/shake plates.**
   a) **EA reagent:** Ready to use, no preparation necessary.
   b) **Working Solution:** Prepare Working Solution by mixing 1 part EA reagent with 1 part Lysis Buffer and 4 parts Substrate Reagent. Gently mix the components prior to use.

Component	Entire Plate (384 wells)
EA Reagent	2 mL
Lysis Buffer	2 mL
Substrate Reagent	8 mL

2. Incubate for 30 min at room temperature (23°C) in the dark before reading the plate on a chemiluminescent reader.
   NOTE:
   For a list of readers and settings, please visit the URL below. http://www.discoverx.com/instrument_chart.php
3. Read samples on any standard luminescence plate reader. [Compound potencies can be derived from a four-parameter nonlinear curve-fitting analysis.]
4) Use GraphPad Prism® or other comparable program to plot your compound dose response.

QUICK-START PROCEDURE: COMPOUND DOSE RESPONSE

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



NOTES:

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