DiscoverX

User Manual

InCELL Hunter™

Target Engagement Cell Lines

For Assessment of Compound Cellular Penetration and Chemiluminescent Detection of Intracellular Compound-Target Engagement

Table of Contents

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

w discoverx.com usa 1.866.448.4864 e SupportUS@discoverx.com eu +44.121.260.6142 e SupportEurope@discoverx.com

© 2017 DiscoverX Corporation. All Rights Reserved. DiscoverX logo and DiscoverX are registered trademarks of DiscoverX Corporation. 20772 103117

Overview

Monitoring cellular drug penetration and drug-target interaction enables assessment of compound efficacy and confirmation of mode of action. InCELL Hunter cellular compound-target engagement assays provide the ability to confirm membrane permeability of compounds, and target binding. They are based on the proprietary Enzyme Fragment Complementation (EFC) technology and allow for the determination of compound engagement to an intracellular protein target in a native cellular environment. In addition, these assays are engineered and validated for the screening and profiling of inhibitor compounds to confirm hits identified in biochemical assays, measuring their cellular EC_{50} values, and ranking their potency. These simple binding assays require no custom chemical tracer or antibody reagents and provide a convenient solution when functional assays are difficult or unavailable.

This user manual covers the storage and handling of InCELL Hunter cell lines and includes a detailed assay protocol. Depending on the specific cell lines being studied, there are two different assay formats that are described below.

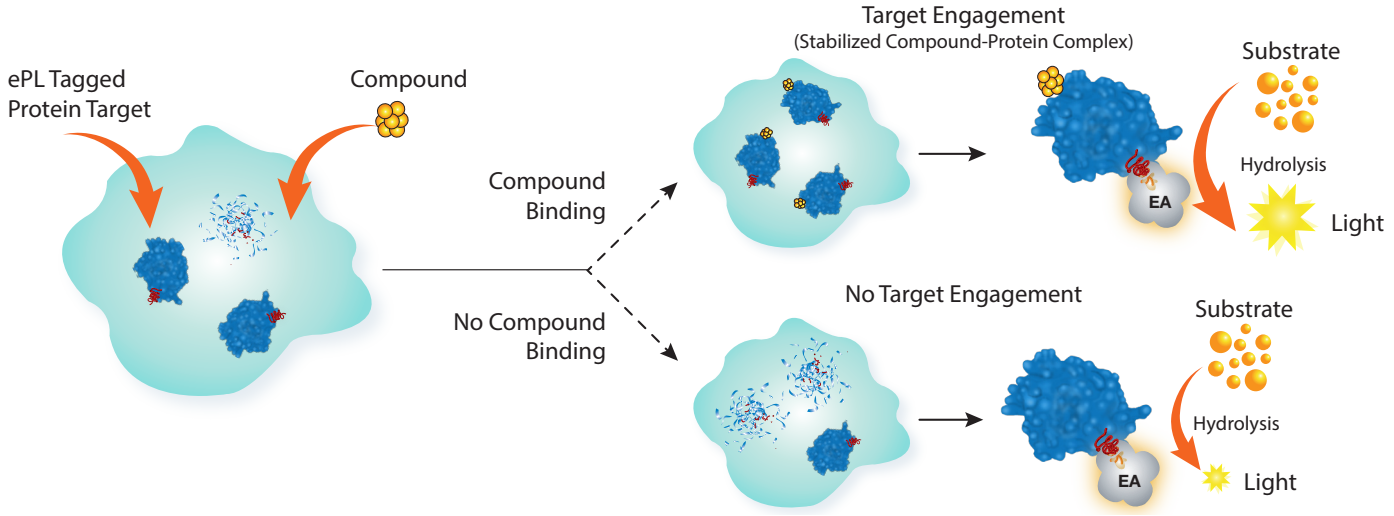
Technology Principle

The InCELL Hunter target engagement assay's EFC-based technology provides the ability to measure compound-target engagement in cells. EFC is based on two recombinant β-galactosidase (β-gal) fragments – a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately, the β-gal fragments are inactive, but when combined, they form an active β-gal enzyme that hydrolyzes substrate to produce a chemiluminescent signal.

There are two types of InCELL Hunter target engagement assay formats. Please refer to the stabilized compound-protein complex assay format for the cell lines that focus on target-compound engagement resulting in changes in protein stabilization, and refer to the destabilized partner protein assay format for the cell lines that include a partner protein stabilized by target protein interaction like BIM or BAX proteins.

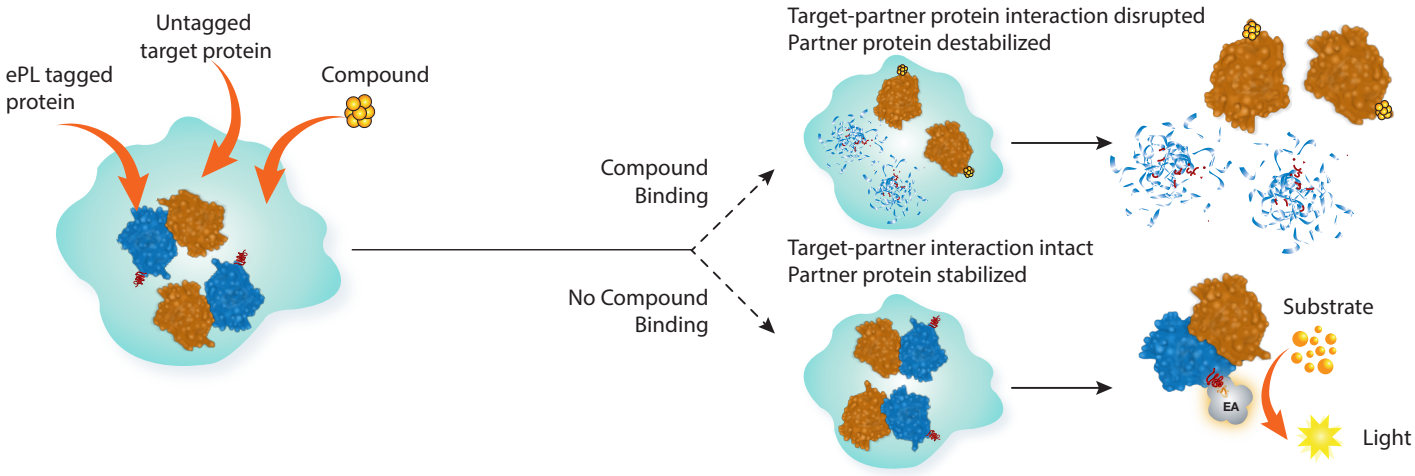
Stabilized Compound-Protein Complex Assay Format

The intracellular target protein in these InCELL Hunter target engagement assays is fused with the ED fragment (also called enhanced ProLabel® or ePL). Upon addition of a compound that binds the target, protein levels are stabilized or altered in the cell, and this change can be monitored by measuring target protein abundance using chemiluminescent detection. The detection reagents include a chemiluminescent substrate added with the EA fragment that naturally complements with the ePL tag on the target protein to create an active β-gal enzyme. The resulting active enzyme hydrolyzes the substrate to generate a chemiluminescent signal.



Destabilized Partner Protein Assay Format

In these assays, cell lines are engineered to co-express an untagged target protein and an interacting partner protein fused with ePL. In the absence of an inhibitor compound, the untagged target and ePL-tagged partner protein interact, resulting in stable steady-state levels of ePL-tagged partner protein. Addition of inhibitors that bind the target protein and disrupt the target-partner protein interaction results in ePL-tagged partner protein degradation. The abundance of ePL-tagged partner protein is measured by chemiluminescent detection. The detection reagents include a chemiluminescent substrate added with the EA fragment that naturally complements with the ePL-tagged partner protein to create an active β-gal enzyme. The resulting active enzyme hydrolyzes the substrate to generate a chemiluminescent signal.



Materials Provided

Number of Components	Configuration
2 Vials	Refer to cell line-specific datasheet

Storage Conditions

Cells must arrive in a frozen state on dry ice and should be transferred to the **vapor phase** of liquid nitrogen storage **immediately upon arrival**.

If the cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For storage longer than 24 hours, place the cryovials in the **vapor phase** of liquid nitrogen storage.

Additional Materials Required

Refer to the cell line datasheet to determine catalog numbers for the media and reagent requirements for the specific InCELL Hunter cell line you are testing. Each InCELL Hunter cell line has been validated for optimal assay performance using the recommended AssayComplete™ Cell Plating (CP) Reagent and its control inhibitor indicated on the cell line-specific datasheet.

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

Material	Ordering Information
InCELL Hunter Detection Kit	96-0079 Series*
AssayComplete Thawing Reagent	Refer to cell line-specific datasheet
AssayComplete Freezing Reagent	Refer to cell line-specific datasheet
AssayComplete Cell Culture Kit	Refer to cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Cell Plating Reagent	Refer to cell line-specific datasheet
Selection Antibiotics	Refer to cell line-specific datasheet
Compound Dilution Buffer (0.1% BSA/PBS)	
Trypsin-EDTA, 0.25%	
Serial Dilution and Assay Plates	discoverx.com/microplates
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094
Tissue Culture Disposable Pipettes (1 mL – 25 mL) and Tissue Culture Flasks (T25 and T75 Flasks, etc.)	
15 mL Polypropylene Tubes and 1.5 mL Microtubes	
Single and Multichannel Micro-pipettors and Pipette Tips (10 µL to 100 µL)	
Cryogenic Vials for Freezing Cells	
Hemocytometer	
Multimode or Luminescence Plate Reader	discoverx.com/instrument-compatibility
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	

*Series refer to the different sizes available for that reagent or kit.

Recommended Materials	Ordering Information
Control Inhibitors	Refer to cell line-specific datasheet

Unpacking Cell Cryovials

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells 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.

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.

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.

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 the Cell Propagation instructions below. 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.
2. Gently aspirate the media from the flasks.
3. Add 5 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.
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.

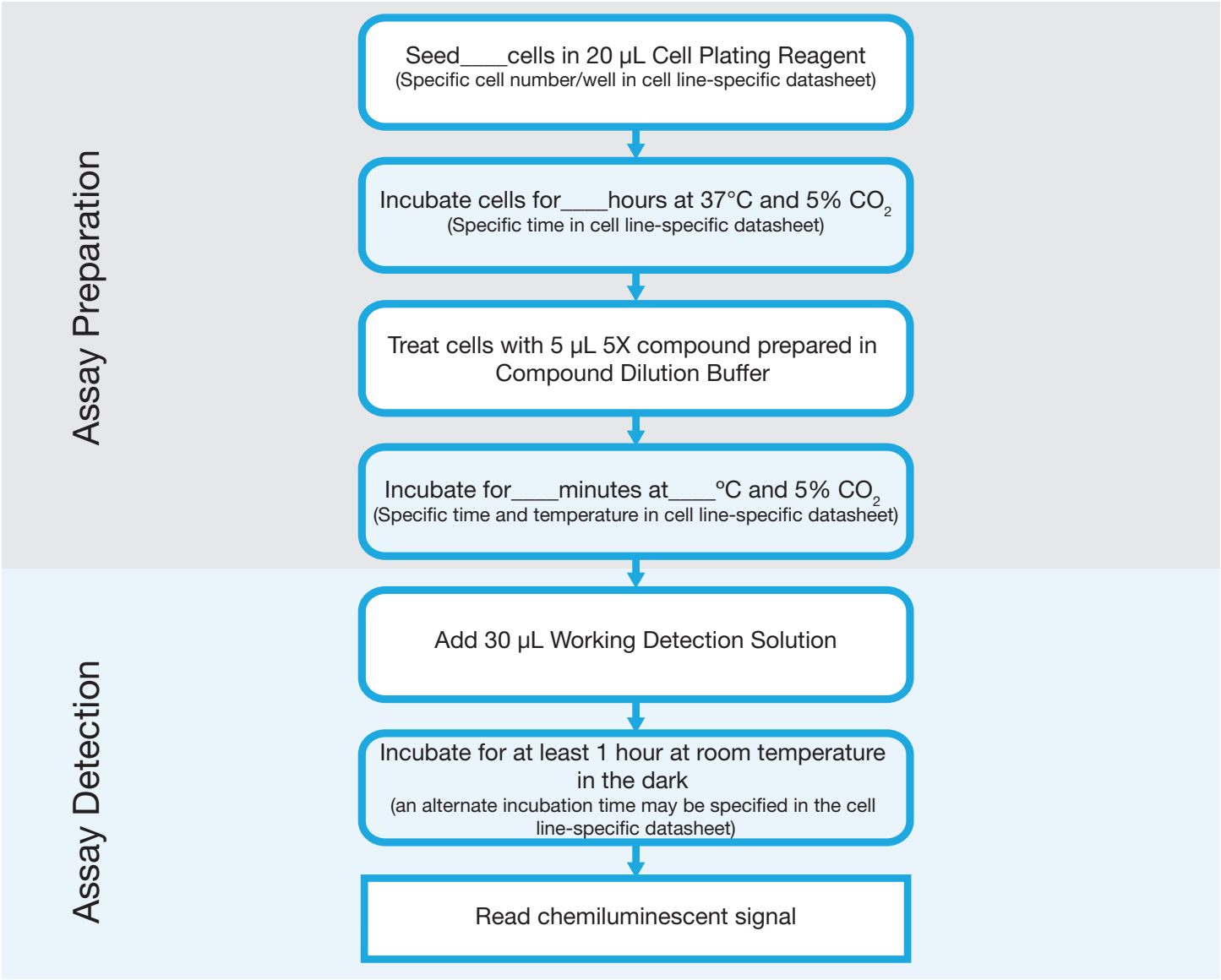
Protocol Schematic

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

Assay Name:_____ Date: _____

Product Details: _____

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



Detailed Assay Protocol

The following procedure is for determining the dose-dependent compound response in a 384-well tissue culture plate.

Section I: Preparation and Plating for Adherent Cells

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

For suspension cells protocol, refer to the Supplemental Information section.

For preparing and plating suspension cells, see protocols in the Supplemental Information section.

1. Aspirate the media from the flask.
2. Add 2 mL AssayComplete Cell Detachment Reagent into each T75 flask (or 3 mL to T225 flasks). Swirl to rinse the cells.
3. Gently aspirate AssayComplete Cell Detachment Reagent from flask.
4. Add 1 mL fresh AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL to the T225 flasks).
5. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with AssayComplete Cell Detachment Reagent.
6. Incubate at 37°C and 5% CO_2 for 2 to 5 minutes or until the cells have detached.
7. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
8. Add 4 mL AssayComplete Cell Plating Reagent to T75 flasks (or 7 mL to T225 flasks). Note: Refer to cell line-specific datasheet to determine the correct Cell Plating Reagent for this cell line.
9. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
10. Remove the entire volume of cells from the flask and transfer to a 15 mL conical centrifuge tube.
11. For the purpose of determining the concentration of cells in the suspension,

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

    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 other cell counting device.
    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.
12. Centrifuge the collected cells at 300 X g for 4 minutes.

13. After centrifugation, discard the supernatant, and re-suspend the cell pellet in AssayComplete Cell Plating Reagent. Based on the cell number obtained in Step 11 above, dilute the resuspended cells to the desired concentration (e.g. 250,000 cells/mL or 5,000 cells/20 µL).

14. Transfer 20 µL/well of the cell suspension to a 384-well assay plate.

15. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line datasheet for recommended cell incubation time.

16. Proceed to compound preparation and addition steps.

Section II: Compound Preparation

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

1. Prepare an 11-point series of 3-fold compound serial dilutions in DMSO, in a separate dilution plate or vials.

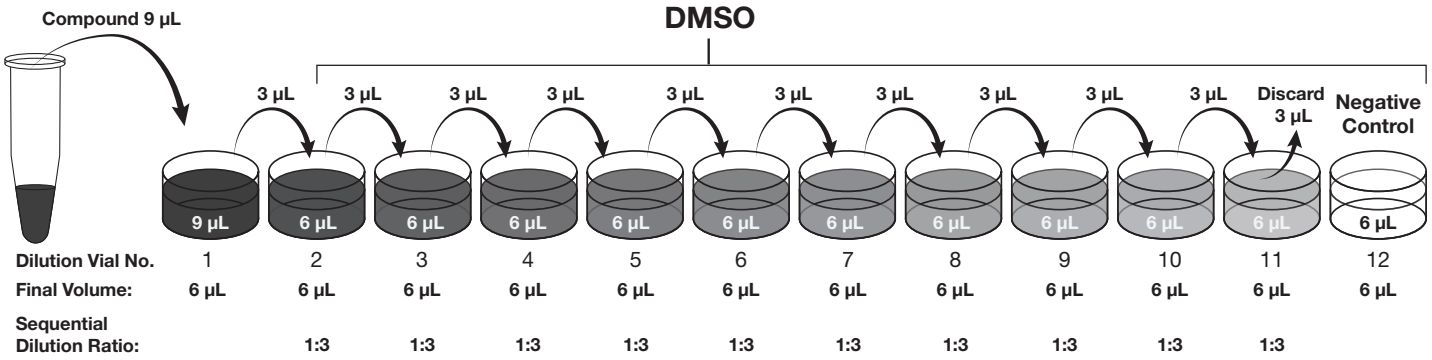
2. The concentration of each dilution should be prepared at 100X the final screening concentration.

   a. For each compound, label the wells of a dilution plate (or polypropylene vials) No. 1 through No. 12.

   b. Add 6 µL of DMSO to dilution wells No. 2 through No. 12. This is enough volume required for ten rows of wells for each concentration. The volume may be adjusted according to the number of wells desired.

   c. Prepare the highest concentration of compound in DMSO. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the compound. Therefore, prepare a working concentration that is 25,000X the expected EC_{50} per well to get a 100X working compound concentration. For example, for an expected EC_{50} of 1 nM, prepare the highest working concentration at 25 µM. This is 100X the screening or final top concentration of 250 nM, and the expected EC_{50} will lie near the center of the dose response curve.

   d. Add 9 µL of the highest concentration of compound to well No. 1 (see figure: Compound Serial Dilutions).



Compound Serial Dilutions: Make eleven 3-fold serial dilutions of the compound in the dilution plate or vial.

   e. Remove 3 µL from well No. 1 and add it to well No. 2. Mix gently.

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

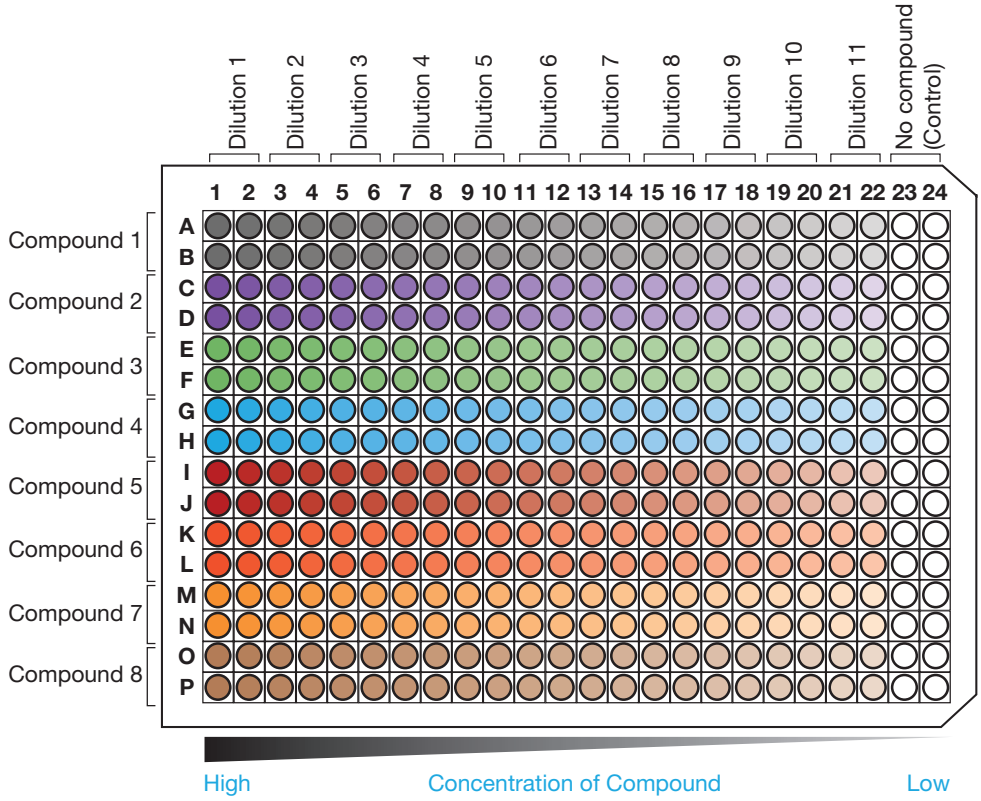
   g. Repeat this process until well No. 11 is reached. Discard 3 µL from well No. 11. Do not add compound to well No. 12 since this is the negative control well. By this point, all twelve wells should contain 6 µL.

   h. Dilute the 100X compound dilution series to a 5X concentration by adding 114 µL of Compound Dilution Buffer to the twelve wells. Gently mix all twelve wells with using a multichannel pipettor. The resulting 5X compound dilution series should also contain 5% DMSO.

3. Set up serial dilutions for any additional compounds in a similar manner.

Section III: Compound Addition

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



Compound Assay Plate Map: Create 11-point dilution series for eight different compounds in quadruplicate.

1. Add 5 µL of each 5X compound serial dilution in quadruplicate to the designated compound rows (e.g. compound 1 in Rows A and B, Columns 1 and 2; compound 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated in the figure: Compound Assay Plate Map.

2. Incubate assay plate at the indicated time and temperature for the specific cell line (refer to the specific cell line datasheet for conditions). For the best results, the optimal incubation time should be empirically determined.

Section IV: Detection Reagent Addition and Plate Reading

At this point, the compound treatment step has been completed. The following section contains procedures for adding the InCELL Detection Kit (Cat. No. 96-0079 Series) and reading the assay plate on a luminometer.

Detection reagents must be prepared as a working solution prior to use. Dilution of EA Reagent may or may not be required during preparation of this working solution; this requirement is specific for each InCELL Hunter cell line and is indicated on the cell line-specific datasheet.

1. If the InCELL Hunter cell line in use does not require dilution of the EA Reagent, then skip this step and proceed to Step 2. If EA dilution is necessary, then dilute the EA reagent by mixing 4-parts of EA Dilution Buffer with 1-part of EA Reagent in a separate tube.

Refer to specific InCELL Hunter Cell Line-specific datasheet for appropriate EA Reagent dilution requirement before proceeding.

2. Prepare a stock of working detection solution in a 15 mL polypropylene tube or reagent reservoir by mixing 1-part of EA Reagent (or diluted EA reagent), 1-part of Lysis Buffer, and 4-parts of Substrate Reagent according to the table below.

Do not store pre-mixed working reagent for more than 24 hours.

Do not substitute Lysis Buffer with alternative buffers. It is formulated for optimal assay performance.

The working detection solution should be prepared immediately before use (for assay detection).

Working Detection Solution for 384-Well Format		
Component	**Volume Ratio**	**Volume per Plate (mL)**
EA Reagent*	1	2
Lysis Buffer	1	2
Substrate Reagent	4	8
Total Volume		**12**

* Dilution of EA Reagent with EA Dilution Buffer may be required. Refer to Step 1 for instructions

3. Add 30 µL of working detection solution to all wells of the assay plate.

4. Incubate the assay plate at room temperature, in the dark. The standard incubation period is 1 hour. Some cell lines may specify a different incubation period that could be as long as 16 hours (i.e. overnight). Please refer to the Functional Information section (i.e. the bottom of the first page) on the cell line-specific datasheet. Follow any alternative instructions that may be indicated for the cell line in use.

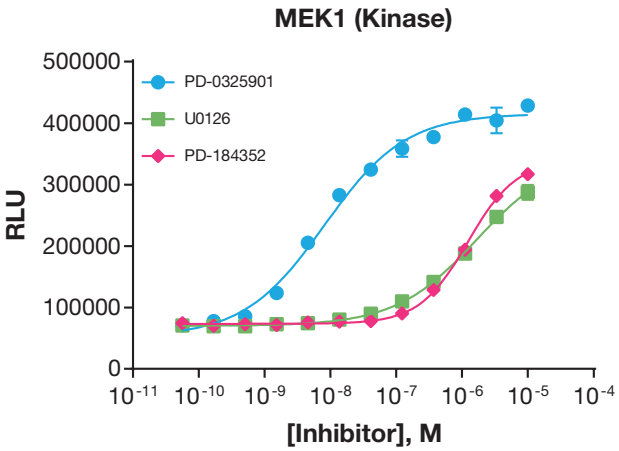
5. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The actual signal characteristics over time are affected by lab conditions such as temperature. The user should establish an optimal read time accordingly. Luminescence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

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

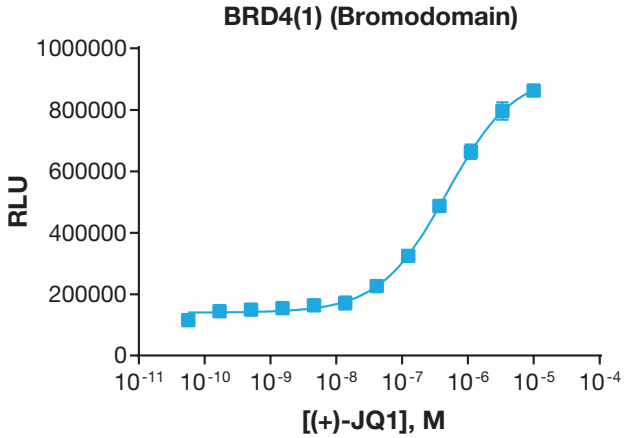
Typical Results

Shown below are typical results from several InCELL Hunter cell lines treated with compound inhibitors. MEK1 (kinase) and BRD4(1) (bromodomain) examples follow the stabilized compound-protein assay format, while the BCL2-BIM example follows the destabilization partner protein assay format.

Stabilized Compound-Protein Complex Assay Format

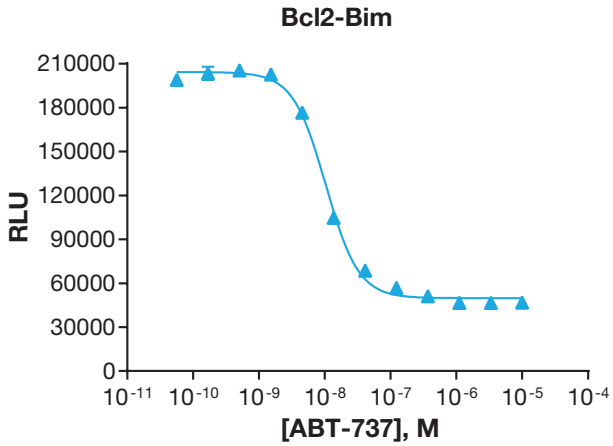


Inhibitor	EC_{50}	S/B
PD-0325901	8.12 nM	5.8
U0126	1.67 µM	4.1
PD-184352	1.23 µM	4.3



EC_{50}	S/B
0.48 µM	7.4

Destabilized Partner Protein Assay Format



EC_{50}	S/B
10.4 nM	4.4

Supplemental Information

Cell Culture Protocol for Suspension Cells

The following procedures are for thawing suspension 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.

Refer to the cell line product datasheet for the specific AssayComplete™ products listed in the protocol below.

Cell Thawing

The following is a protocol for thawing cells in a T25 flask.

1. Place 9 mL of AssayComplete Thawing reagent in T25 flask and pre-warm in a humidified 37˚C and 5% CO_2 incubator for 15 minutes to equilibrate pH and temperature.
2. Remove the cell cryovials from -80°C or liquid nitrogen vapor storage and immediately place them in dry ice.

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. Place the cryovials 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.

Prolonged thawing at 37°C may result in cell death.

4. Decontaminate the surface of the cryovial by spraying and wiping it with 70% ethanol.
5. With a pipette, gently transfer the thawed cells to the pre-filled T25 flask and incubate at 37°C and 5% CO_2.
6. Maintain the cells in the same flask for 48 hours. Then proceed to the Cell Propagation instructions below.

Cell Propagation

The following is a protocol for propagating cells once they have reached an appropriate density in a T25 flask.

1. Passage the suspension cells in the T25 flask when they have reached a density of 1 x 10^6 cells/mL by taking an aliquot of cells to determine density.

To maintain logarithmic growth of the cells, cultures should not exceed a density of 3 x 10^6 cells/mL.

   a. Remove from the flask and transfer to a conical tube (if necessary, add an additional 5 mL of medium to the flask, and rinse to collect the remaining cells. Transfer the additional volume to the conical tube).

   b. Centrifuge at 180 X g for 3 minutes at room temperature to pellet cells.

   c. Decant supernatant or carefully remove medium with a 10 mL pipette without disturbing the cell pellet.

2. For routine passaging, resuspend cells at a concentration of 1 x 10^5 cells/mL by adding the appropriate volume of pre-warmed AssayComplete Cell Culture Kit reagent supplemented with selection antibiotics for the given cell line. Refer to the recommendations in the table below for final culture volumes.

Working Detection Solution for 384-Well Format			
Flask Size	**T25**	**T75**	**10 cm Dish**
AssayComplete™ Cell Culture Kit Reagent (mL)	10	20	10

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 flasks. If smaller flasks are used, adjust the volumes accordingly. This protocol assumes that cells have reached the desired cell density in an adequate number of flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 2 x 10^6 per vial).

It is recommended to freeze cells at a low passage number (2 to 3 passages). For optimal assay performance, ensure cells are in log-phase growth at time of freeze down.

1. Remove the cell suspension from the flask and transfer to a conical tube (if necessary, add an additional 5 mL of media to the flask, and rinse to collect the remaining cells. Transfer the additional volume to the conical tube).
2. Take an aliquot of cells to determine cell number.
3. Centrifuge at 180 X g for 3 minutes at room temperature to pellet cells.
4. Decant supernatant, or carefully remove media with a 10 mL pipette without disturbing cell pellet.
5. Immediately resuspend the cells in ice-cold AssayComplete Freezing Reagent to a concentration of 2 x 10^6 cells/mL.

Keep cells on ice during this process to maintain cell viability.

6. Make aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.
7. 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 days.

Preparation and Plating

The following is a protocol for harvesting cells from a T75 flask (with cell density of 1 x 10^6 cells/mL) and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that cells have been cultured in their specified cell culture media. The cell culture media comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic.

1. Warm the AssayComplete Cell Plating reagent in a clean 37°C water bath for 15 minutes.
2. Take an aliquot of cells (in suspension) from the flask, and determine the cell density by first counting cells, then calculating the cell concentration per mL. The calculated concentration can be used to determine the total number of cells in the tissue culture flask.
3. Remove the cells from the flask and transfer to a conical tube (if necessary, add an additional 5 mL of media to the flask and rinse to collect the remaining cells. Transfer the additional volume to the conical tube).
4. Centrifuge at 180 X g for 3 minutes at room temperature to pellet cells.

5. Decant supernatant or carefully remove media with a 10 mL pipette without disturbing cell pellet.
6. Resuspend the cell pellet at the desired concentration (e.g. to achieve 5,000 cells/well in 20 µL resuspend cells at 2.5 x 10^5 cells/mL) in pre-warmed AssayComplete Cell Plating reagent.
7. Using a multichannel pipettor and reagent reservoir, seed 20 µL of the cell suspension into one 384-well assay plate.
8. Incubate the assay plate in a 37°C and 5% CO_2 humidified tissue culture incubator. Refer to the cell line datasheet for recommended cell incubation time.
9. Follow Compound Preparation (Section II), Compound Addition (Section III), and Detection Reagent Addition and Plate Reading (Section IV) in the Detailed Assay Protocol to complete the assay.

Frequently Asked Questions

What is the passage number of the frozen cells in the vials I receive when I purchase the cell line?

- The cells are shipped at passages 2 or 3.

What if there is no or low signal?

- Visually inspect the cells before and after compound incubation in a clear bottom plates to ensure that they are healthy. Make sure proper cell plating procedure is followed to ensure assay performance.
- Make sure that the correct detection kit was used, and whether stored and prepared as indicated in the datasheet.
- Make sure the plate reader is setup properly to read luminescence.
- White-walled assay plates should be used, since black-walled plates may decrease signal.

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

- Make sure that the compound is prepared correctly, and ensure it is completely dissolved before use.
- Make sure DMSO and other solvent concentrations are not too high (not more than 1% final concentration).
- When testing compounds at high concentrations, make sure the vehicle is compatible. Also, visually inspect cells incubated with higher compound concentrations for compound cytotoxicity when plated on clear-bottom plates.
- Make sure compound is incubated for the specified time and at the specific temperature.
- Make sure plates are protected from light during incubation.

What if the variability between replicates is high?

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

Do I need to use clear-bottom plates?

- We recommend using clear-bottom plates to visualize cells after plating and prior to detection reagent addition. However, other plate formats can be used with some assay optimization.

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

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

For additional information or Technical Support see contact information at the bottom of this page.

Limited Use License Agreement

These products may be covered by issued US and/or foreign patents, patent application and subject to Limited Use Label License.

Please visit discoverx.com/license for limited use label license and trademark information for this product.

w discoverx.com usa 1.866.448.4864 e SupportUS@discoverx.com eu +44.121.260.6142 e SupportEurope@discoverx.com