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Customer Notification: Protocol Change and Harmonization

Catalog Number/s	Several
Product Name/s	InCELL Hunter™ eXpress Kits (Several)
Effective Date	February 2, 2021
Update/s	1) Protocol Changes 2) User Manual Consolidation
Affected Document/s	**Discontinued User Manuals:** 1) InCELL Hunter eXpress Bromodomain Assays (Document Number: 70-242) 2) InCELL Hunter eXpress Histone Methyltransferase Assays (Document Number: 70-287) 3) InCELL Hunter eXpress Protein Binding Assays (Document Number: 70-292) 4) InCELL Hunter eXpress Kinase Binding Assays (Document Number: 70-288) **Replaced with New User Manual:** InCELL Hunter eXpress Assays User Manual (Document Number: 70-429)

Dear Customer,

This letter has been issued to inform you about the updates to the InCELL Hunter eXpress kit configuration and protocol. We have simplified the eXpress kit configuration to include the standard Cell Plating Reagent size of 100 mL bottle in the 2-plate Kit. The 10-plate kit configurations remains unchanged. There is no change in the reagents' formulation and manufacturing, or the ordering process.

All InCELL Hunter eXpress assay protocols have been harmonized to use a single generic protocol, and the four user manuals have been consolidated into the new user manual, InCELL Hunter eXpress Assays User Manual (Document No. 70-429). Specific protocol changes have been made to the detection reagent incubation time for InCELL Hunter eXpress Protein Binding Assays, increasing the incubation time from 30 minutes to 60 minutes. The ligand dilution scheme used for all InCELL eXpress Assay has been changed from 1:4 to 1:3 fold series, which delivers a better dose-response curve. These updates are also summarized in the table below.

Update	Affected eXpress Kits	Express Kit Component/Protocol Step	Old	New
Configuration	2-plate kits InCELL Hunter eXpress Kits ONLY	Assay Complete™ Cell Plating Reagent	2 x 20 mL bottle	1 x 100 mL bottle
Configuration	Kinase Binding eXpress Kits	Detection Reagent Kit	96-0065CP series	96-0079 series

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Update	Affected eXpress Kits	Express Kit Component/Protocol Step	Old	New
Configuration	Bromodomain, Protein Binding, Histone Methyltransferase eXpress Kits	Detection Reagent Kit	96-0007CP series	96-0079 series
Protocol	All InCELL Hunter eXpress Kits	Ligand Dilution Series	1:4	1:3
Protocol	InCELL Hunter eXpress Protein Binding Assays (Cat. No.: 96-0008E3 & 96-0009E3)	Detection Reagent Ligand Incubation Time	30 minutes	60 minutes

The updated user manual is attached with this letter.

If you have any questions, please contact our technical support team at DRX_SupportUS@EurofinsUS.com.

Sincerely,

Alpana Prasad, Ph.D.

Senior Strategic Portfolio Manager

Attachment: *InCELL Hunter eXpress Assays User Manual (Document Number: 70-429)*

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User Manual

InCELL Hunter™ eXpress Assays

For Chemiluminescent Detection of Intracellular Compound-Target Engagement

Document Number 70-429 Revision 0A

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Table of Contents

Important: Please read this entire user manual before proceeding with the assay.

Table of Figures

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

Overview

InCELL Hunter™ eXpress Assays provide a cell-based approach to evaluate changes in protein stability upon compound binding. These assay kits have been validated for use in 96-well and 384-well microplate formats. The eXpress kits contain all the materials needed for a complete assay including cells, cell plating reagents, detection reagents, and plates. The pre-validated, cryopreserved eXpress cells have been manufactured for short-term use and are provided in a ready-to-use format for faster implementation.

Assay Principle

InCELL Hunter eXpress Assays are based on the Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme has been split into two inactive fragments—a smaller fragment called ProLabel (ePL) and the larger fragment, enzyme acceptor (EA). Independently, these fragments have no β-gal activity; however, when they bind and complement each other, they form an active β-gal enzyme.

The intracellular target protein in InCELL Hunter eXpress cells is tagged with the ePL fragment. 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, which provides a measurement of the cellular amount of tagged protein. 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.

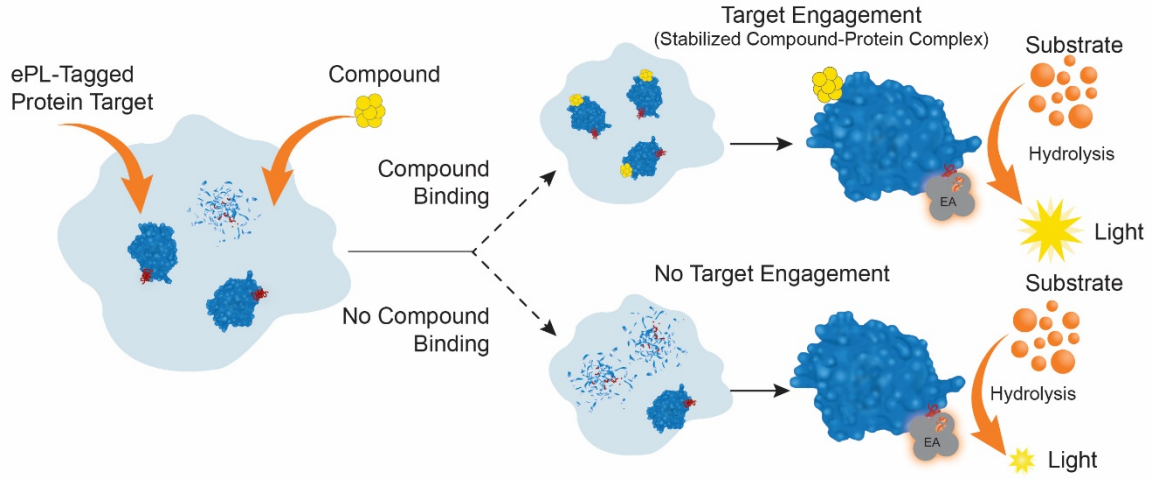


Figure 1. Assay Principle: The intracellular target protein in these assays is fused with the ePL fragment of β-gal. Upon addition of a compound that binds the target, protein levels are stabilized or altered in the cell, and this change can be monitored by measuring target protein abundance using chemiluminescent detection. The detection reagents include a chemiluminescent substrate added with a large enzyme acceptor (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. A larger signal corresponds to a greater amount of compound-target engagement in the cell.

Materials Provided

List of Components	2-Plate Kit	10-Plate Kit
InCELL Hunter eXpress Cells (1.2 x 10^6 cells in 1 mL per vial)	2	10
AssayComplete™ Cell Plating Reagent* (100 mL per bottle)	1	2
InCELL Detection Kit		
InCELL EA Reagent	5 mL	25 mL
InCELL Lysis Buffer	5 mL	25 mL
InCELL Substrate Reagent	24 mL	120 mL
InCELL EA Dilution Buffer**	20 mL	100 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

*Cell Plating Reagent is recommended for thawing, plating the cells and for compound dilution. Please refer to the target-specific datasheet for additional details.

**The InCELL EA Dilution Buffer is needed for specific targets only, as indicated on the target-specific datasheet. If it is not required for this target, it may be discarded.

Storage Conditions

InCELL Hunter eXpress Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

- Short-term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

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

AssayComplete™ Cell Plating Reagent

Upon receipt, store at -20°C. Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 20 mL of reagent per aliquot can be dispensed and frozen down.

InCELL Hunter Detection Kit

Upon receipt, store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For long-term storage (up to the expiration date listed on the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

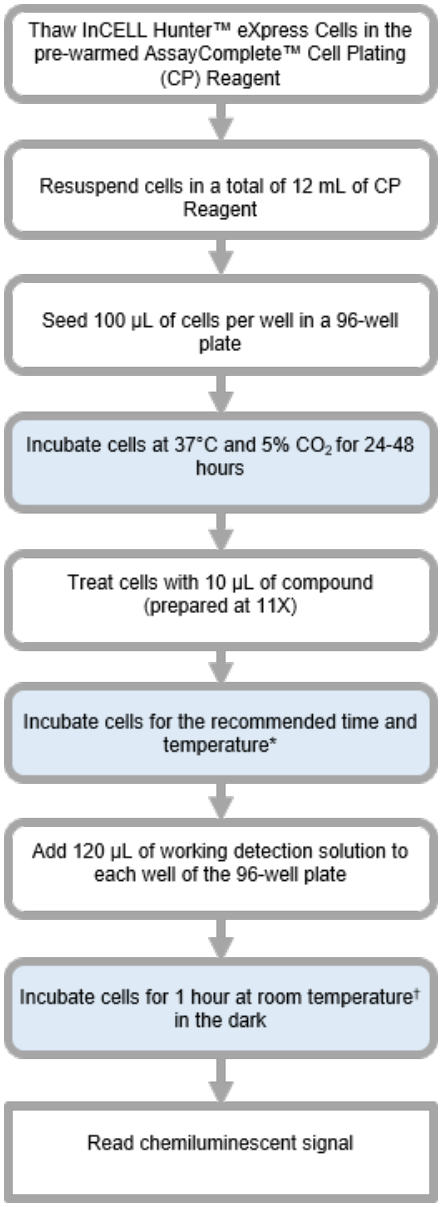
Additional Materials Required

The following equipment and additional materials are required to perform these assays:

Material	Ordering Information
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Multimode or luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micropipettes and pipette tips (10 µL-1,000 µL)	
Polypropylene tubes (50 mL and 15 mL)	
Microcentrifuge tubes (1.5 mL)	
Tissue culture disposable pipettes (1 mL-25 mL) and tissue culture flasks (T25 and T75 flasks, etc.)	

Protocol Schematic

Quick-start Procedure: In a 96-well plate, perform the following steps.



*Refer to the target-specific datasheet for specific recommendations.

†Room temperature refers to a range of 23-25°C.

Detailed Protocol

Day 1: Cell Thawing and Plating

The following protocol is for thawing and plating cryopreserved InCELL Hunter eXpress cells from cryovials.

1. Pre-warm the AssayComplete™ Cell Plating Reagent in a 37°C water bath.
2. Dispense 12 mL of the pre-warmed CP reagent into a 15 mL conical tube.
3. Remove the cryovials from the liquid nitrogen vapor and immediately place it in dry ice.

DO NOT use a heated water bath to thaw the vial. Hold the cryovials by the cap; **DO NOT** touch the sides or bottom of the vial to avoid thawing of the cell pellet. Wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood.

4. Add 0.5 mL of pre-warmed CP reagent from the 15 mL conical tube into the cryovial to thaw the cells. Slowly pipet the cell suspension up and down several times to uniformly resuspend the cells.
5. Transfer the cell suspension to the conical tube containing the remaining 11.5 mL of CP reagent. Remove any remaining suspension form the cryovial to ensure maximum recovery of all the cells.
6. Replace the cap on the conical tube and gently invert it several times to ensure that the cells are properly resuspended in the CP reagent, without creating any froth in the suspension. Immediately pour the suspension into the sterile 25 mL reagent reservoir.
7. If running the assay in a 96-well plate, transfer 100 µL of the cell suspension to each well (10,000 cells/well) of the plate, using a multichannel pipet. For recommendations on running the assay in a 384-well plate, refer to the Supplemental Information section.
8. Incubate the assay plate according to the time and conditions indicated in the target-specific datasheet. Typically, plate incubation is done at 37°C and 5% CO_2, but the incubation time is specific for each kit.

Refer to the target-specific datasheet for any variation in assay conditions.

Day 2: Compound Preparation and Addition

1. If required, prepare a compound stock solution by dissolving the dry compound in an appropriate reconstitution solution.
2. Generate an intermediate concentration of the compound (referred to as intermediate stock compound solution) that is 11X the final screening or testing concentration by diluting the stock compound solution with the supplied CP Reagent.
3. The steps below are for preparing 3-fold serial dilutions of the compound in an 11-point dose curve using a dilution plate (e.g. 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, Cat. No. 92-0011). These dilutions require using the supplied CP Reagent. For running the assay in a 96-well plate, the concentration of each dilution should be prepared at 11X the final screening concentration. For recommendations on running the assay in a 384-well plate, refer to the Supplemental Information section.

    3.1. For each compound, label the wells of a dilution plate as Well 1 to Well 12.

    3.2. Add 100 µL of the intermediate stock compound solution that was prepared in Step 2, to Well 12.

3.3. Add 60 µL of the CP Reagent to Well 11 through Well 1.

3.4. Using a clean pipet tip, transfer 30 µL of the diluted compound from Well 12 to Well 11. Mix thoroughly by pipetting up and down several times.

3.5. Replace the pipet tip with a clean one and transfer 30 µL of the diluted compound from Well 11 to Well 10. Mix thoroughly by pipetting up and down several times. Repeat this process until Well 2 is reached, resulting in an 11-point 1:3 dilution series. No sample is transferred to Well 1 as this is the negative control well.

3.6. Prepare additional test samples in a similar manner.

4. Remove the assay plate from the incubator and place it in the tissue culture hood.

5. If using a 96-well assay plate, transfer 10 µL of the compound dilution series from each row of the dilution plate to the corresponding wells of the assay plate, as shown in Figure 2. Representative Assay Plate Map. For recommendations on running the assay in a 384-well plate, refer to the Supplemental Information section.

6. Incubate the assay plate at the specific temperature and time indicated on the target-specific datasheet.

Representative Assay Plate Map

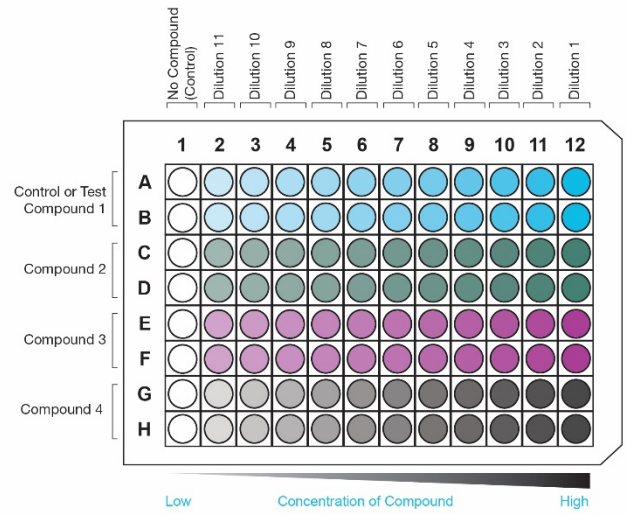


Figure 2. Representative Assay Plate Map. The 96-well plate map shows an 11-point dose curve with 2 data points at each concentration, for each test compound with a 1:3 serial dilution scheme.

Day 2: Detection

The following protocol is for adding the InCELL Detection Kit reagents to the assay plate. The detection reagents must be prepared as a working detection solution prior to use. Dilution of EA Reagent with the EA dilution buffer may or may not be required during preparation of this working solution; this requirement is specific for each InCELL Hunter eXpress Assay and is indicated on the target-specific datasheet.

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

   Refer to the target-specific datasheet for appropriate EA Reagent dilution requirement before proceeding.

2. Prepare a stock of the 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.

Working Detection Solution for a 96-well Format		
Components	Volume Ratio	Volume per Plate (mL)
InCELL EA Reagent*	1	2.5
InCELL Lysis Buffer	1	2.5
InCELL Substrate Reagent	4	10
Total Volume		**15**

   *Dilution of the EA Reagent with EA Dilution Buffer may be required. Refer to the target-specific datasheet for requirements.

   The working detection solution should be used immediately after preparation.

3. Add 120 µL of the working detection solution to each well of the assay plate.

4. Incubate for 60 minutes at room temperature (23-25°C) in the dark before reading the plate on a chemiluminescent reader.

   The working detection solution is light sensitive, thus incubation in the dark is necessary.

5. Read samples on any standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers, or 5-10 seconds for imager.

The actual signal characteristics are affected by lab conditions such as temperature. The user should establish an optimal read time accordingly. Luminescent readout of the InCELL Detection Kit collects signals from all wavelengths. Some instrument manufacturers may include a cutoff filter at long wavelengths, but usually no wavelength setting is needed for luminescence readout. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

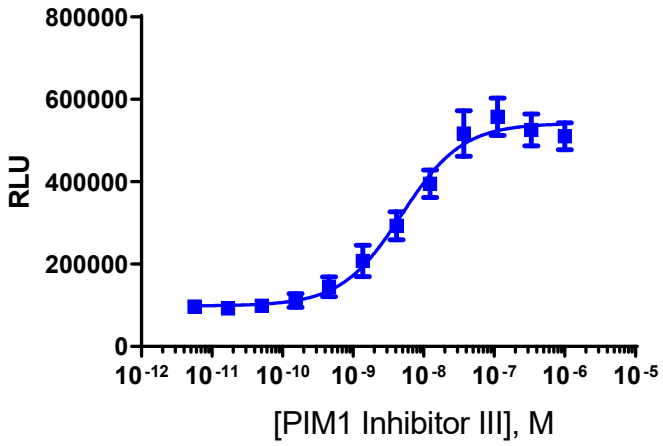
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel.

Typical Results

The following graph is an example of a typical dose-response curve for the InCELL Hunter eXpress Assays generated using the protocol outlined in this manual. The data shows potent, dose-dependent cellular compound binding when cells expressing PIM1 were treated with PIM1 Inhibitor III.

The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.

A.



B.

EC_{50} (nM)	4.9
S/B	5.3

Figure 3. Typical Results: Representative **A**, dose-response curve and **B**, the EC_{50} and assay window for cellular PIM1 and PIM1 Inhibitor III engagement.

Supplemental Information

Running the Assay in a 384-well Plate

The eXpress kits are configured to run assays in a 96-well plate. The assay can be easily modified to run these in a 384-well plate by adjusting the volumes using the following guidelines:

1. Suspend the cells in Cell Plating (CP) Reagent to a final volume of 10 mL.

2. Adjust volumes for the assay reagents for each protocol as indicated in the table below. The compound dilutions should be prepared at 5X the final screening concentration.

Assay Reagents	Volume per Well (µL)
Cell Suspension in CP Reagent	20
Compound Dilutions	5
Working Detection Solution	30
Total Assay Volume	**55**

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
Decreased or no response	Incorrect thawing procedure	Refer to the thawing instructions in the Cell Thawing and Plating section of this user manual.
	Incorrect compound used or incorrect compound incubation time	Refer to the Certificate of Analysis for recommended ligand and assay conditions.
	Incorrect preparation of the compound	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of the compound.
	Cells are rounded up and possibly dead	Thaw and plate another vial cells. But poor cell viability is a sign that the frozen vials were mishandled and exposed to room temperature, thus compromising the cells. Be extremely careful to avoid exposing cell vials to room temperature when handling and transporting vials before use.
Low or no signal	High DMSO/solvent concentration	Maintain DMSO or solvent concentrations at low concentrations (≤1%).
	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should ideally be prepared just prior to use.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 second/well.
Experimental S/B does not match the value noted in the Certificate of Analysis provided	Incorrect incubation temperature	Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Incorrect preparation of compound	Some compounds are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper compound handling or storage	Ensure that the compounds are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Certificate of Analysis.
	Problems with dynamic range or dilutions	Changing tips during dilution can help in avoiding carryover.
High variability between replicates	Instrument calibration	Ensure that the dispensing equipment is properly calibrated, and proper pipetting technique is used.

For questions on using this product, please contact Technical Support at 1.866.448.4864 or DRX_SupportUS@eurofinsUS.com

Document Revision History

Revision Number	Date Released	Revision Details
0	January 2021	• New document This document replaces the following User Manuals: 1. InCELL Hunter eXpress Bromodomain Assays (70-242) 2. InCELL Hunter eXpress Histone Methyltransferase Assays (70-287) 3. InCELL Hunter eXpress Kinase Binding Assays (70-288) 4. InCELL Hunter eXpress Protein Binding Assays (70-292)

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InCELL Hunter™ eXpress Assays User Manual 70-429 Revision 0A