DiscoverX

User Manual

InCELL Pulse™

Target Engagement Starter Kit

To Measure Compound Binding to a Cellular Target by Detecting Changes in Protein Thermal Stability

Catalog No. 94-4007 Series

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Please read entire booklet before proceeding with the assay.
For additional information or Technical Support see contact information below.

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Overview

The InCELL Pulse Target Engagement Starter Kit provides reagents and protocols for the design and development of InCELL Pulse assays for the measurement of inhibitor cellular potency against user-defined protein targets of interest. The assay applies DiscoverX's proprietary enzyme fragment complementation (EFC) technology to measure ligand-dependent thermal stabilization of target proteins in a cellular context. InCELL Pulse assays employ a simple protocol requiring minimal hands-on time and enable the measurement of quantitative inhibitor EC_{50} values. InCELL Pulse assays have been successfully developed for a number of enzymes from diverse classes, including protein kinases, methyltransferases, and hydrolases. For information on assay-ready target-specific InCELL Pulse kits and to view data for additional targets, please visit discoverx.com/incell.

Technology Principle

InCELL Pulse Cellular Target Engagement Assays measure compound binding to a cellular target by detecting changes in protein thermal stability. InCELL Pulse is a novel cellular application of the EFC technology utilizing β-galactosidase split into two inactive fragments, the enhanced ProLabel® (ePL) peptide and the enzyme acceptor (EA), that associate to form a fully active β-galactosidase enzyme. In this assay, the target is fused to ePL and expressed in the selected cell background. The fusion is detected with high sensitivity through the addition of EA and substrate in the presence of a cell lysis buffer.

The ePL tag is an ideal fusion partner for InCELL Pulse for the following reasons: 1) ePL is a small unstructured peptide that does not affect a protein's thermal denaturation profile; 2) ePL enables simple, sensitive, and precise fusion protein detection; and 3) the sensitivity of ePL detection depends on the macromolecular state of the fusion protein. Whereas native, properly folded ePL fusion proteins are detected with high sensitivity, denatured aggregated states of fusion proteins are poorly detected.

In this system, cells expressing a target protein-ePL fusion are subjected to a brief heating and cooling cycle in the presence or absence of a test compound. In the absence of compound binding, the target protein is sensitive to the heat pulse and forms denatured/aggregated structures that poorly complement with EA, resulting in reduced β-galactosidase activity. Compound binding, however, protects the target protein from denaturation/aggregation, which results in a dose-dependent gain of β-galactosidase activity that is quantitatively detected by using a chemiluminescent substrate. Overall, these assays provide a rapid, quantitative and direct readout of cellular compound permeability and target binding.

InCELL Pulse Assay Principle

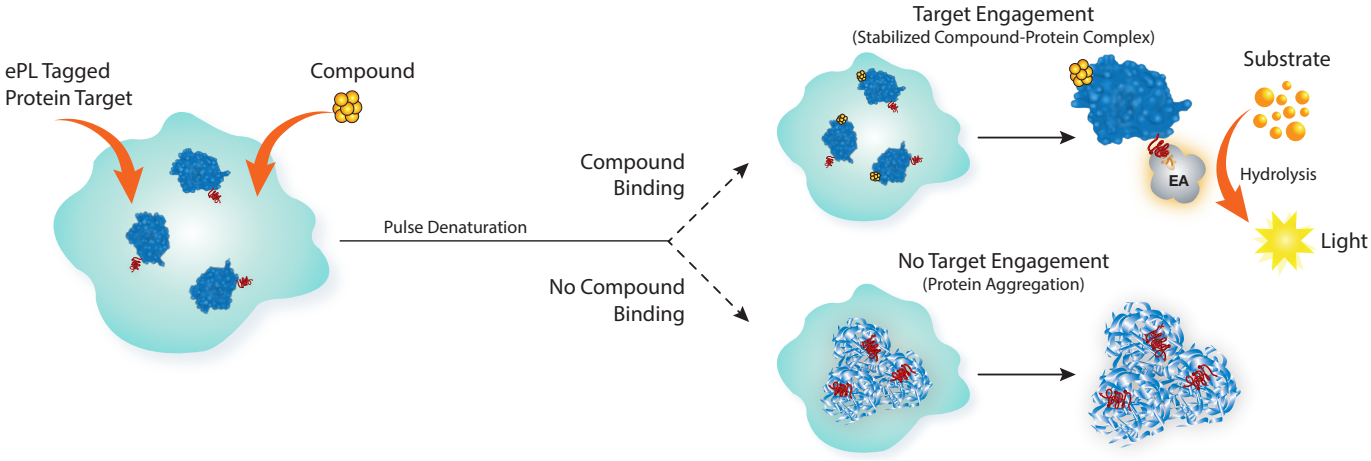


Figure 1. Cells expressing a protein of interest fused to the small ePL tag are treated with test compound and then subjected to elevated temperatures during a pulse denaturation step. Compound binding protects the target protein from thermal denaturation, which enhances complementation between EA and ePL and increases the chemiluminescent signal measured using the EFC-based detection system. In the absence of compound binding, the target protein forms denatured aggregates that poorly complement with EA, which results in a low chemiluminescent signal.

Intended Use

The InCELL Pulse Target Engagement Starter Kit provides a simple and easy-to-use method for building on-demand intracellular inhibitor binding assays. This unique platform allows rapid generation of cell-based assays that measure quantitative, dose-dependent changes in protein thermal stability upon compound binding. This technology is validated across diverse target classes including kinases, methyltransferases, hydrolases, and bromodomains and is an ideal option for targets from other protein classes as well. The complete set of vectors and assay development tools provided, combined with optimized assay development protocols and EFC-based chemiluminescent detection, makes it the ideal platform for measuring direct compound-target engagement in mammalian cells.

Once developed, InCELL Pulse assays address several key drug discovery challenges:

- Establish that compounds are cell-penetrant
- Validate cellular target engagement for screening hits identified in biochemical assays
- Rank order compound cellular potency by measuring quantitative EC_{50} values
- Monitor compound cell potency improvements during lead optimization
- De-risk off-targets identified in biochemical screens
- Measure inhibitor cellular EC_{50} values over a broad potency range (picomolar and higher)

The kit includes ePL cloning vectors, a positive control vector expressing the MTH1 hydrolase, anti-ePL antibody, optimized detection reagents, an EFC detection system control reagent (ePL positive control), and assay plates. The kit is designed for 96-well analysis and includes enough detection reagents for up to 400 data points. A positive control compound(s) (not provided) with known potent cellular or biochemical activity against the user-defined target is required for optimal assay development.

Materials Provided

Cat. No.	List of Components	Quantity
94-4008	**InCELL Pulse Target Engagement Vectors**	
	pICP-ePL-N Vector	10 µg
	pICP-ePL-C Vector	10 µg
	pICP-MTH1-ePL Vector*	10 µg
96-0079S**	**InCELL Detection Kit**	
	EA Reagent	5 mL
	Lysis Buffer	5 mL
	Substrate Reagent	24 mL
92-0010	PathHunter® Anti-PK/PL Antibody	50 µg
30-425	ePL Positive Control	100 µL
15-106**	Black 96-well Skirted PCR Assay Plates	5 plates

* The pICP-MTH1-ePL Vector encodes the MTH1 hydrolase InCELL Pulse positive control assay target.
** Multiple sizes available (Cat. No. 96-0079 series for InCELL Detection Kit, and Cat. No. 92-0031 for Black 96-well Skirted PCR Assay Plates).

Storage Conditions

InCELL Pulse Target Engagement Vectors
Store at -20°C.

InCELL Detection Kit
Store at -20°C.

PathHunter Anti-PK/PL Antibody
Store at -20°C.

ePL Positive Control
Store at -20°C.

Black 96-well Skirted PCR Assay Plates
Store in room temperature.

Kits are shipped on dry ice and should arrive in a frozen state. Upon receipt, store at -20°C immediately.

Thaw InCELL Detection Kit reagents at room temperature before use. After thawing, components can be stored for up to 7 days at 4°C. The reagents are stable for three freeze-thaw cycles with no impact on performance, but if you plan to perform multiple experiments on different days we recommend preparing aliquots of the InCELL Detection Kit components and freezing separately.

The working detection solution of InCELL Pulse Detection Reagents should be prepared immediately before use.

Additional Materials Required

Material	Ordering Information
TH588	92-1325
AssayComplete™ Cell Detachment Reagent	92-0009
AssayComplete Freezing Reagent*	92-51XXFR Series**
AssayComplete Thawing Reagent*	92-41XXTR Series**
AssayComplete G418*	92-0030
Non-Breathable Adhesive Film for PCR Plates	E&K Scientific, Cat. No. T79610
Lipofectamine® 2000 Transfection Reagent	Thermo Fisher Scientific, Cat. No. 11668-500
PBS (Dulbecco's Phosphate Buffered Saline, 1X without calcium and magnesium)	Corning, Cat. No. 21-031-CM
Green V-Bottom Ligand Dilution Plates	92-0011
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Test and Control Compounds	discoverx.com/ligands
Test and Control Compounds	
Mammalian Host Cells for Transfection	
Dimethyl Sulfoxide (DMSO)	
Tissue Culture Disposables and Plastic-ware	
Disposable Sterile Pipettes	
6-well Tissue Culture Plates	
10 cm Cell Culture Dishes	
Sterile Polypropylene Tubes (15 mL)	
Single and Multichannel Micro-pipettors and Pipette tips	
Hemocytometer or Cell Counter	
Cryogenic Vials for Freezing Cells	
Standard Cell Culture Equipment: Microscope, Cell Culture Incubator and Hood	
PCR Thermocycler (96-well Footprint Accommodating 50 µL Sample Volumes per Well)	
Multimode or Luminescence Plate Reader	discoverx.com/instrument-compatibility

* For optional stable cell line generation step.
** Series refer to the different sizes available for that reagent or kit.

Assay Complete Cell Detachment Reagent should not be substituted with trypsin, which can potentially interfere with assay performance. PBS used for washing cells prior to detachment should not include calcium and magnesium, both of which can inhibit activity of the cell detachment reagent.

TH588 is a potent MTH1 hydrolase inhibitor to be used as a tool compound for the positive control MTH1 assay. The recommended Non-Breathable Adhesive Film for PCR plates has been demonstrated to work optimally with the PCR plates provided in this kit. Lipofectamine 2000 Transfection Reagent has been used for internal InCELL Pulse development, but an equivalent product may be acceptable as well.

PCR Thermocycler: Instruments with a thermal gradient feature are required for efficient assay development (but not to run final assay). Ideal instruments will also have programmable temperature ramp rate settings, a programmable heated lid, and a "calculated" temperature control mode. For optimal assay performance use a thermocycler that provides uniform heating to eliminate minor but undesirable artifacts caused by poor temperature homogeneity across the heat block. Newer instruments and instruments with low prior use are recommended

Assay Development Process Overview

InCELL Pulse assays are based on the ability of a compound to stabilize ePL fusion proteins from thermal denaturation. This efficient assay development process occurs in discrete phases and has been optimized for measuring compound binding following transient transfection. Positive control inhibitors with known potent cellular and/or biochemical activity against the target of interest are required for assay development (Phases II & III).

InCELL Pulse Assay Development Overview

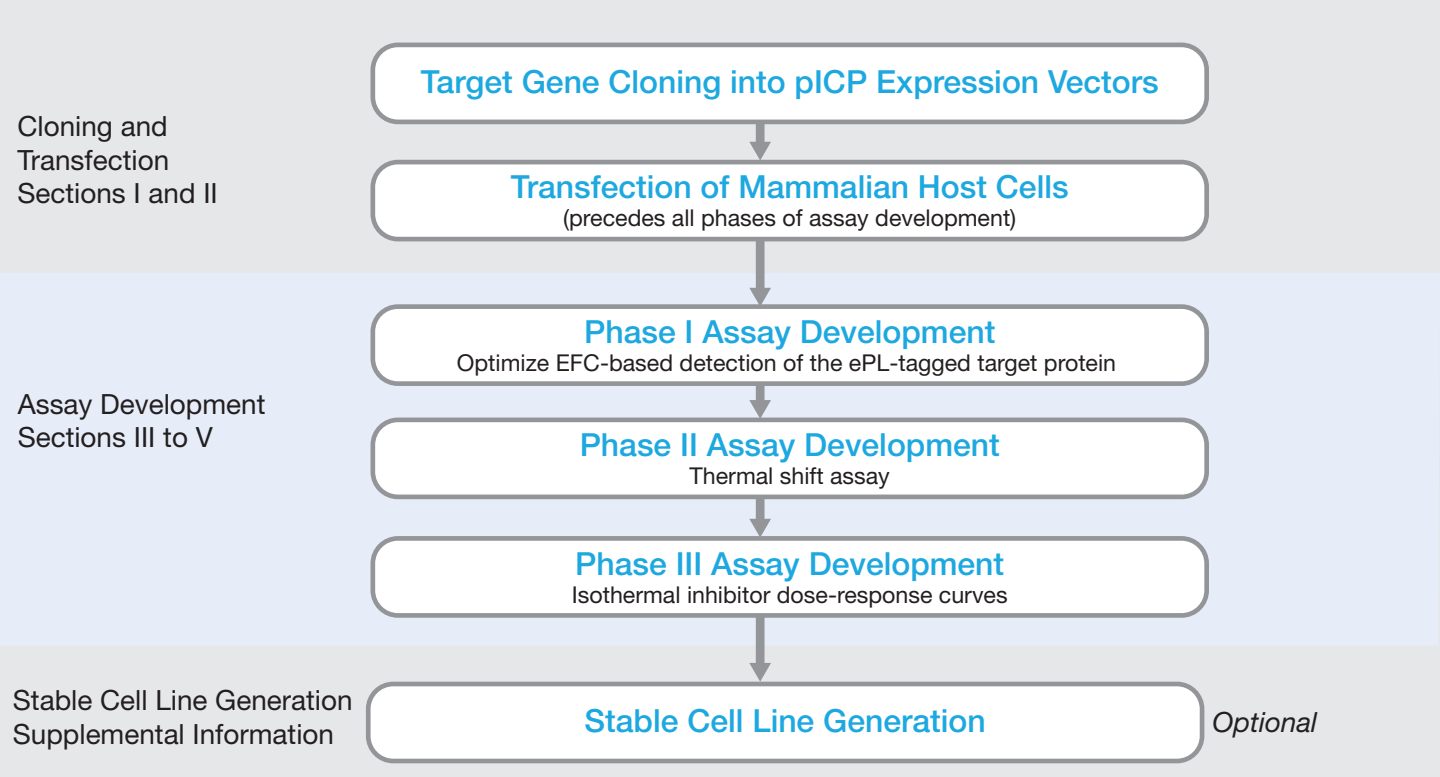


Figure 2. InCELL Pulse assay development comprises three assay development phases that follow cloning and transfection steps. This process has been optimized to maximize efficiency and assay development success rates.

Detailed Assay Protocol

Cloning and Transfection

I. Target Gene Cloning into pICP Expression Vectors

The InCELL Pulse Target Engagement Starter Kit provides plasmid-based vectors for adding the ePL tag, a small fragment of β-galactosidase, to the C-terminus or N-terminus of your protein of interest. An in-frame flexible linker (14 amino acids) containing a TEV protease recognition site serves as a short spacer between ePL and the expressed target protein. Each vector includes a CMV promoter for maximal expression in mammalian cells and a selectable marker for transfection.

I-A. Clone Design

Depending on the protein of interest, constructs can be designed to encode the full length protein and/or only the catalytic/compound binding domain (i.e. a construct based on a crystal structure of the relevant domain). We suggest designing full-length and partial-length target protein constructs and to generate both N- and C-terminal ePL-tagged versions of each.

When designing clones for InCELL Pulse, consider that full-length proteins can comprise multiple independently folding domains that are all subject to thermal denaturation. It is therefore possible that ligand binding to one domain will not protect the full-length protein from denaturation/aggregation during heating, which can result in false-negative compound binding results during assay development. For this reason, when evaluating multi-domain proteins, we suggest testing a construct comprising only the relevant ligand binding domain of interest in addition to the full-length protein. Previous studies measuring ligand binding to full-length native proteins using related thermal denaturation-based methods are valuable resources when designing InCELL Pulse assays [1-5].

I-B. General Cloning Guidelines and Resources

- The InCELL Pulse Target Engagement Starter Kit includes 10 µg of each pICP plasmid. To propagate, transform the plasmid DNA into *E. coli* using ampicillin or carbenicillin (50 µg/mL) as a selection agent.
- To generate stocks of pICP-ePL-N and pICP-ePL-C for cloning purposes, purify the DNA using a suitable DNA purification kit to achieve high yields of transfection-quality plasmid DNA. In parallel, also generate a stock of the positive control vector pICP-MTH1-ePL, which encodes the catalytic domain of the MTH1 hydrolase.
- Insert the gene of interest (GOI) into the multiple cloning site (MCS) of the vector in frame with the ePL tag. The EcoRI and XbaI sites in the MCS are convenient for cloning the GOI into pICP-ePL-N and pICP-ePL-C, respectively. Use of the EcoRI restriction site at the 5'-end of the MCS is recommended to maintain proper frame with the N-terminal ePL tag (pICP-ePL-N), and use of an XbaI site at the 3'-end of the MCS is recommended to maintain proper frame with the C-terminal ePL tag (pICP-ePL-C). Use of other restriction sites will likely require the addition of nucleotides in your PCR primer to maintain reading frame integrity. We suggest generating both N- and C-terminal tagged constructs and testing them in parallel.
    - pICP-ePL-C Cloning Vector. To create a C-terminal ePL fusion protein, a proper start sequence and Kozak sequence should be present in the GOI. We utilize the CACC Kozak sequence immediately prior to the ATG start codon to enhance expression. The GOI must be fused in frame with the ePL peptide and must not contain a stop codon at the end of the coding sequence.

- pICP-ePL-N Cloning Vector. To create an N-terminal ePL fusion protein, the GOI should contain a stop codon at the end of the coding sequence.
- Suggested Forward Sequencing Primer (not included with kit). pICP-F: 5'-TGTCCACTCCCAGTTCAATT-3'. Anneals ~60 bp before the ePL coding sequence in pICP-ePL-N and ~60 bp before the NheI site in pICP-ePL-C.
- Suggested Reverse Sequencing Primer (not included with kit). pICP-R: 5'-GTGGTTTGTCCAAACTCATC-3'. Anneals ~30 bp after the NotI site in pICP-ePL-N and ~30 bp after the ePL coding sequence in pICP-ePL-C.

Refer to the InCELL Pulse Cloning Vector Maps section in the Supplemental Information for complete plasmid maps including the multiple cloning site (MCS). To receive the entire text sequence for any of the InCELL Pulse pICP-ePL vectors, see contact information below.

II. Transfection of Mammalian Host Cells

All assay development phases (Figure 2) require a transfection step. The following transfection protocol has been developed for HEK 293 cells using the Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Cat. No. 11668-500). Although any standard transfection method can be used to transiently transfect plasmids into mammalian cells, we have found that the overall health of the cells is better following transfection with lipid-based DNA transfection reagents.

Because the transfection efficiency for different cell lines can vary dramatically, when using cell lines other than HEK 293 it may also be necessary to optimize parameters such as the amount/purity of DNA, cell density, media conditions, and transfection time for ideal InCELL Pulse assay performance.

Refer to the manufacturer's recommended protocol for detailed instructions on the handling and use of the preferred DNA transfection reagent.

II-A. General Considerations

1. InCELL Pulse Target Engagement Assays are intended to be performed using transiently transfected cells. If required, stable transfectants of all pICP-ePL fusion vectors can be selected using G418. Refer to the Stable Cell Line Generation section in the Supplemental Information for details on how to generate stable cell lines.
2. The control plasmid pICP-MTH1-ePL is a useful positive control for all phases of assay development (Figure 2) and can be transfected in parallel with the vector(s) expressing the GOI.
3. Transfections should be performed 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 prior to plating.

II-B. Transfection Protocol

This protocol was developed for transfection into one well of a 6-well plate and yields enough cells for >300 assay wells in 96-well plates, which is more than sufficient for each phase of assay development.

Day 1: Plating HEK 293 Cells

1. Approximately 16 to 24 hours before transfection, plate 1 x 10^6 exponentially growing HEK 293 cells (ATCC CRL-1573) per well of a 6-well plate in 2 mL of Complete Growth Medium (DMEM plus 10% FBS for HEK 293 cells). Cells should be 70-90% confluent on the day of transfection. One well is required for each plasmid to be transfected.

2. Incubate the cells overnight at 37°C and 5% CO_2.

Day 2: Transfection of HEK 293 Cells

1. Prepare the DNA transfection mix:
   a. Dilute 2 µg of purified plasmid DNA in 250 µL of serum-free medium. Mix gently.
   b. Mix 250 µL of serum-free medium with 10 µL Lipofectamine 2000 Transfection Reagent. Incubate for 5 minutes at room temperature.
   c. After the 5-minute incubation, combine the diluted DNA with diluted Lipofectamine 2000 Transfection Reagent. Mix gently and incubate for 20 minutes at room temperature.
2. Remove the 6-well plate containing the adherent HEK 293 cells from the incubator (from Day 1). Add each sample from Step 1.c. to one well of the 6-well plate.
3. Return the plate to the incubator and incubate for 24 hours at 37°C and 5% CO_2.

Day 3: Harvest Transfected Cells and Perform InCELL Pulse Experiment

II-C. Harvesting Transfected Cells

The following protocol is for harvesting transfected cells from one well of a 6-well tissue culture plate and preparing them for plating in the InCELL Pulse assay plate at the recommended cell density.

1. Pre-warm cell culture medium to 37°C in a water bath. This medium serves as the “Assay Medium” for InCELL Pulse experiments. For HEK 293 cells, DMEM plus 1% FBS is a common Assay Medium for InCELL Pulse experiments, but FBS concentrations up to 10% are acceptable as well.
2. Thaw AssayComplete™ Cell Detachment Reagent (Cat. No. 92-0009) at room temperature or remove from 4°C storage if already thawed.

Do not thaw or pre-warm AssayComplete Cell Detachment Reagent at 37°C. This reagent can lose activity at 37°C.

Do not substitute trypsin in place of AssayComplete Cell Detachment Reagent for the cell detachment step. Trypsin may interfere with assay performance.

3. Remove cells transfected the previous day (Section II-B, Day 2) from the incubator.
4. Gently aspirate medium from the well(s) of the 6-well plate.
5. Add 2 mL of PBS into each well, swirl to rinse the cells, and gently aspirate.

Add PBS gently to side of well to avoid disrupting the cell monolayer.

6. Add 0.2 mL of AssayComplete™ Cell Detachment Reagent per well.
7. Gently rock the plate back and forth to ensure the surface of the well is thoroughly covered with Cell Detachment Reagent.
8. Incubate the plate at room temperature for 2 to 3 minutes.
9. Verify under the microscope that cells are detached. If necessary, gently tap the edge of the plate 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.
10. Add 1 mL of pre-warmed Assay Medium to each well.
11. Using a pipette, gently rinse the cells from the surface of the plate with the added medium. Pipette up and down gently to break up clumps and inspect under a microscope to verify a single-cell suspension.
12. Count cells using a hemocytometer or a cell counter and calculate the cell concentration.
13. Dilute the cell suspension to a final working concentration of 1.25 x 10^5 cells/mL using Assay Medium. This cell density delivers 5,000 cells per well, which is recommended for HEK 293 cells.

For transfections using HEK 293 cells one well of a 6-well plate generally yields sufficient cells for ≥ 300 assay wells at 5,000 cells per well. Adjust the scale of the transfection accordingly if additional assay wells are required.

III. Assay Development Phase I

Optimize EFC-based detection of the ePL-tagged target protein.

III-A. Overview and General Considerations

The goal of Phase I Assay Development is to optimize EFC-based detection of the target protein-ePL fusion to ensure a high signal to background ratio (S/B). Cell lines produce some amount of background luminescence when tested using InCELL detection reagents, and this background is cell-line-dependent. HEK 293 cells produce a low background luminescence and are well suited for EFC detection. CHO-K1 and U2OS cells are preferred backgrounds for EFC-based assays, as well.

- The EFC signal for cells expressing the target protein-ePL fusion is defined as the luminescence measured using a working detection solution that includes all three components: Enzyme Acceptor (EA) Reagent, Substrate Reagent, and Lysis Buffer. EFC signal will generally increase in proportion to the concentration of EA Reagent present in the working detection solution. Longer incubation times can increase the EFC signal as well.
- The EFC background for cells expressing the target protein-ePL fusion is defined as the luminescence measured using a working detection solution that lacks EA Reagent and includes only Substrate Reagent and Lysis Buffer. In the absence of EA reagent, the target protein-ePL fusion is silent, and only background luminescence generated by the cellular host is detected. The cellular background is constant and does not depend on either the EA reagent concentration or the incubation time.
- The EFC signal to background (S/B) ratio is calculated by dividing the EFC signal by the EFC background.

- The Phase I Assay Development experiment described below measures the EFC signal for cells expressing the target protein-ePL fusion(s) of interest at increasing EA Reagent concentrations. The EFC background is measured for these cells in parallel (no EA Reagent present). The goal is to identify an optimal EA Reagent concentration for assay development.
- Perform western blots using the provided PathHunter® Anti-PK/PL Antibody to confirm that the target protein-ePL fusion is expressed at the expected size. Antibody dilutions of 1:100 to 1:500 are recommended for this application. Proteins that form insoluble aggregates during expression are poorly detected by EFC but may give strong bands on western blots. Poor EFC detection of proteins giving strong bands on western blots may indicate that expression construct optimization is required. Refer to the Troubleshooting Guide for solutions to this problem.

III-B. Plate Layout for Phase I Assay Development

Example Plate Layout for a Phase I Assay Development Experiment

		1	2	3	4	5	6	7	8	9	10	11	12
Transfection Construct 1	A												
	B												
Transfection Construct 2	C												
	D												
EFC Negative Control	E												
	F												
EFC Positive Control	G												
	H												
Working Detection Solution:		EA-0	EA-1	EA-3	EA-10								

Figure 3. In this example, cells transfected with Constructs 1 or 2 are tested in duplicate against four different working detection solutions that either lack EA Reagent (EA-0) or include EA Reagent present at increasing concentrations (EA-1, EA-3, EA-10). Columns 1-4 each specify an individual working detection solution. The EA-0 condition is included to measure the EFC background for the cell line being tested. The EFC Positive and Negative Controls are included to confirm optimum performance of the EFC detection system.

- For each transfection the S/B ratio is calculated by dividing the luminescence intensity measured using working detection solutions that contain EA (EA-1, EA-3, EA-10) by the value measured for EA-0. The EA-0 condition measures the EFC background for the cell line being tested.
- The EFC Positive Control is included to confirm the expected activity of the EFC detection system when tested with the provided ePL Positive Control peptide. The EFC Positive Control should be inactive when tested using the EA-0 working detection solution (Column 1).
- The EFC Negative Control is included to measure background luminescence for the EFC detection system in the absence of cells and ePL.
- This example layout accommodates the testing of up to ten independent transfections per plate, which can include multiple versions of the same construct (i.e. N- and C-terminal ePL tag), different constructs for the same GOI (i.e. full-length and domain-only), different GOIs, and the positive control vector pICP-MTH1-ePL.

III-C. Phase I Assay Development Detailed Protocol

Materials Required

- Transfected cells harvested just prior to use from Section II-C, Step 13
- Assay Medium (see Section II-C, Step 1 for description)
- InCell Detection Kit (EA Reagent, Lysis Buffer, Substrate Reagent)
- ePL Positive Control
- 96-Well Black PCR Plate(s)

Step 1: Prepare and Harvest Transfected Cells

Follow procedures described in Section II.

Step 2: Assay Assembly

Prepare reagents and dispense into assay wells. This protocol is designed to support the layout shown in Figure. 3. Adjust volumes as necessary to support custom experiments.

a. Preparation and dispensing of ePL Positive Control and Negative Control Samples

1. Add 50 µL of the ePL Positive Control to 450 µL of Assay Medium and mix.
2. Dispense 50 µL of the ePL Positive Control stock diluted in Assay Medium into the 8 EFC Positive Control wells of a 96-Well Black PCR Plate.
3. Dispense 50 µL of Assay Medium into the 8 EFC Negative Control wells.
4. Dispense 10 µL of Assay Medium into the 16 Transfection Construct wells.

b. Prepare working detection solutions

1. Prepare master mixes of the four working detection solutions as shown in Table 1. Volumes support the plate layout shown in Figure 3.
2. Adjust the volumes accordingly if additional plate columns are being tested.

Working detection solutions must be prepared fresh before use. Discard any excess.

Table 1: Working Detection Solution Recipes

	EA-0		EA-1		EA-3		EA-10	
	Volume (µL) per Well or Column*							
Component	**Per Well**	**Per Column**	**Per Well**	**Per Column**	**Per Well**	**Per Column**	**Per Well**	**Per Column**
EA Reagent	0	0	1	10	3	30	10	100
Lysis Buffer	10	100	10	100	10	100	10	100
Substrate Reagent	50	500	49	490	47	470	40	400
Working Detection Solution (Total Volume)	**60**	**600**	**60**	**600**	**60**	**600**	**60**	**600**

*Volumes account for overages and dead volumes

c. Dispense 40 µL of transfected cells (5,000 cells) from Section II-C, Step 13 into the designated Transfection Construct wells.

Step 3: InCELL Pulse Detection Reaction

a. Add 60 µL per well of the corresponding working detection solution to each assay plate column and gently mix by pipetting up and down.

b. Incubate at room temperature in the dark for 60 minutes.

Step 4: Assay Plate Readout

Read samples on a standard luminescence plate reader at 0.5 or 1.0 seconds/well. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readouts.

Consult your instrument manual for proper labware definition to ensure optimal reading of the 96-well black PCR plates provided.

It is important that the supplied black skirted PCR plates are used with this kit, please do not substitute. InCELL Pulse assays can produce a high luminescent signal and the black skirted PCR plates are important for mitigating any well-to-well bleed-over of luminescence during the readout. The plate skirt ensures seamless compatibility with standard luminometers. Additional black skirted PCR plates can be ordered from DiscoverX (Cat. No. 92-0031).

III-D. Phase I Assay Development Data Analysis

1. Confirm optimum performance of the EFC detection system (Rows E-H). For each working detection solution, divide the average EFC Positive Control value by the average EFC Negative Control value.

Table 2: Typical Results for EFC Positive and Negative Controls

Working Detection Solution	EA-0	EA-1	EA-3	EA-10
EFC Positive/Negative Control Ratio	1	180	460	1500

Typical EFC Positive/Negative Control ratios are shown for the EA-0, EA-1, EA-3, and EA-10 working detection solutions. If the EFC Positive Controls fail, please see the Troubleshooting Guide or contact Technical Support.

2. Calculate Signal/Background (S/B) ratios for Transfection Constructs (Rows A-D). For each Transfection Construct, divide the average value for EA-1, EA-3, and EA-10 by the average value for EA-0. Typical results for the positive control pICP-MTH1-ePL Vector and an additional Transfection Construct are shown below.

Table 3: Typical Signal to Background Results for the Positive Control ICP-MTH1-ePL Vector

Working Detection Solution	pICP-MTH1-ePL	Transfection Construct 2
EA-1	50	2
EA-3	160	6
EA-10	520	21

Typical Signal to Background (S/B) results for the positive control ICP-MTH1-ePL Vector. S/B data for pICP-MTH1-ePL and a second target protein construct (Transfection Construct 2) are shown for the three working detection solutions.

III-E. Select an EFC working detection solution for Assay Development

Selection Guidelines

- The minimum S/B ratio required for InCELL Pulse assay development is 10-fold and ratios ≥50-fold but <300-fold are ideal.
- Choose a working detection solution that gives a S/B ratio of ≥50.
- If multiple working detection solutions give a S/B ratio ≥50, choose the one containing the lowest EA concentration

Example: For the pICP-MTH1-ePL construct shown in Table 3, all working detection solutions give S/B ratios ≥50, and EA-1 was chosen for assay development.

Conditions that give extraordinarily high signal (i.e. S/B ratios of over 300) can result in depletion of the luminescent substrate and should be avoided. When the guidelines given above are followed, InCELL Pulse assays should be stable for at least 2 hours after the addition of detection reagents.

- If S/B ratios ≥50 are not measured, then choose the working detection solution giving the highest value, keeping mind that this value must be ≥10.

Example: For Transfection Construct 2 shown in Table 3, all working detection solutions give S/B ratios <50, and EA-10 was chosen for assay development.

- If all S/B ratios are <10, do not proceed with assay development. Refer to the Troubleshooting Guide or contact Technical Support for assistance.

IV. Phase II Assay Development

Thermal shift assay

IV-A. Overview and General Considerations

The goal of Phase II Assay Development is to identify thermal denaturation temperatures for the sensitive detection of cellular target engagement. In this phase, thermal melting curves for the target protein are generated in the presence or absence of a potent positive control inhibitor. Potent inhibitors that engage the target protein right-shift its apparent melting temperature (T_m), and comparison of the melting curves identifies temperatures that produce optimal assay windows, as shown in Figure. 4 below. The identified temperatures are then tested in isothermal dose-response studies during Phase III Assay Development. Efficient execution of the thermal shift assay requires a thermocycler with a thermal gradient feature (see Instrument Compatibility Chart in the Supplemental Information).

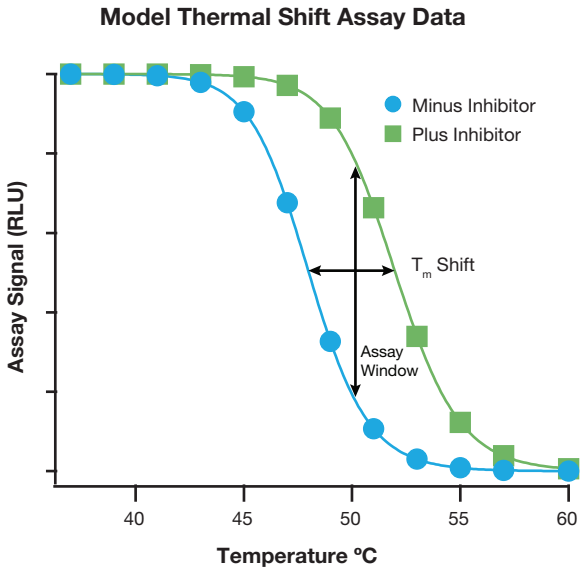


Figure 4. In this model experiment, cells expressing an ePL-tagged target protein are heated to temperatures ranging from 37°C to 60°C in the presence or absence of a potent positive control inhibitor. The positive control inhibitor protects the protein from thermal denaturation and right-shifts the melting curve (T_m shift, horizontal arrow). The assay window (vertical arrow) at a specified temperature is the signal ratio between the Plus and Minus Inhibitor samples.

- Temperature sensitivity of cells: HEK 293 cells are >90% viable after heating to 55°C for up to 5 minutes. High viability (>80%) is measured at 60°C for heating times up to 3 minutes. Temperature sensitivity should be measured when using cell lines other than HEK 293.
- Positive control inhibitor concentration. We suggest testing an inhibitor concentration(s) that is at least 10X the known cellular IC_{50}. If only biochemical data are available, then test at 100X the IC_{50} or higher. Keep in mind that compound solubility and non-specific effects on cells and on the EFC detection reagents can become problematic at extremely high inhibitor concentrations. A 10 µM positive control inhibitor concentration often meets these requirements.

- For first-time users we strongly recommend testing cells transfected with the included pICP-MTH1-ePL Vector, which expresses the MTH1 hydrolase, with its positive control inhibitor TH588 (Cat. No. 92-1325) when performing Phase II Assay Development.

IV-B. Plate Layouts for Phase II Assay Development

Example Plate Layouts for Phase II Thermal Shift Assays

A Thermocycler's thermal gradient is by column (12 temperatures)

37°C Temperature Gradient 60°C

Transfection Construct	Compound		1	2	3	4	5	6	7	8	9	10	11	12
1	DMSO (%)	A	1	1	1	1	1	1	1	1	1	1	1	1
		B	1	1	1	1	1	1	1	1	1	1	1	1
	Pos. Control Inhibitor 1 (μM)	C	10	10	10	10	10	10	10	10	10	10	10	10
		D	10	10	10	10	10	10	10	10	10	10	10	10
2	DMSO (%)	E	1	1	1	1	1	1	1	1	1	1	1	1
		F	1	1	1	1	1	1	1	1	1	1	1	1
	Pos. Control Inhibitor 2 (μM)	G	10	10	10	10	10	10	10	10	10	10	10	10
		H	10	10	10	10	10	10	10	10	10	10	10	10

B Thermocycler's thermal gradient is by row (8 temperatures)

	Transfection Construct											
	1				2							
	DMSO (%)		Pos. Control Inhibitor 1 (μM)		DMSO (%)		Pos. Control Inhibitor 2 (μM)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	10	10	1	1	10	10				
B	1	1	10	10	1	1	10	10				
C	1	1	10	10	1	1	10	10				
D	1	1	10	10	1	1	10	10				
E	1	1	10	10	1	1	10	10				
F	1	1	10	10	1	1	10	10				
G	1	1	10	10	1	1	10	10				
H	1	1	10	10	1	1	10	10				

60°C Temperature Gradient 37°C

Figure 5. Cells transfected with Constructs 1 or 2 are tested in duplicate against a 37°C to 60°C temperature gradient in the presence or absence of a positive control inhibitor at 10 μM. DMSO at 1% serves as the no compound control. Layouts are shown for thermocyclers that generate thermal gradients A. by column or B. by row.

IV-C. Phase II Assay Development Detailed Protocol

Materials Required

- Transfected cells harvested just prior to use (Section II-C, Step 13)
- EFC working detection solution(s) identified as optimal for the transfection construct(s) being evaluated (Section III-E)
- Positive control inhibitor 100X stock(s) in DMSO
- Assay Medium (see Section II-C, Step 1 for description) warmed to 37°C
- Complete Growth Medium containing 10% FBS warmed to 37°C
- DMSO
- Disposable Reagent Reservoirs
- 96-Well Black PCR Plates

Step 1: Prepare Positive Control Inhibitor Working Stocks

a. Prepare positive control inhibitor(s)

1. Prepare a 100X DMSO stock of the positive control inhibitor(s) (i.e. 1 mM if testing 10 µM in thermal shift assay). For the MTH1 positive control we suggest using 10 µM TH588 (Cat. No. 92-1325) in the thermal shift assay.
2. Prepare a 5X positive control inhibitor working stock by diluting the 100X DMSO stock 20-fold in Complete Growth Medium containing 10% FBS.
    - Each assay well requires 10 µL of the 5X positive control inhibitor working stock. See Figure 5 to determine the total volume required for an experiment.
3. For the DMSO control wells, prepare a 5% DMSO stock in Complete Growth Medium containing 10% FBS.

The final DMSO concentration for all InCELL Pulse assays should be ≤1% once the cells have been added.

10% FBS in the Complete Growth Medium used for compound dilutions is recommended to help ensure compound solubility.

Step 2: Dispense Compounds to a 96-Well Black PCR Plate

Dispense 10 µL of the 5X positive control inhibitor and 5% DMSO working stocks from Step 1.a. above to the assay plate as specified in Figure 5A or 5B. Choose the plate layout that is compatible with the directionality of the thermal gradient generated by your thermocycler.

Step 3: Harvest Transfected Cells

Follow procedures described in Section IIC to prepare transfected cells at the recommended working concentration in Assay Medium.

Step 4: Assay Setup and Compound Incubation

a. Add 40 µL of the cell suspension(s) from Section IIC, Step 13 (5,000 cells) to the plate prepared in Step 2.a. above as specified in Figure 5A or 5B.

b. Incubate plate for 1 hour in a 37°C and 5% CO_2 humidified incubator.

Gently mix cells in the reagent reservoir during dispensing to ensure that significant settling does not occur.

Step 5: Pulse Denaturation

The pulse denaturation step requires a thermocycler with a thermal gradient feature.

a. Remove thermal shift assay plate from the 37°C incubator and seal with non-breathable adhesive film.

b. Transfer plate to a thermocycler and execute the following denaturation protocol:

   1. Set the gradient from 37°C to 60°C by row or column depending on instrument.
   2. Set gradient heating time to 3 minutes followed by cooling at room temperature for 3 minutes.
   3. Additional settings:
      - Heated lid set 5°C higher than highest temperature tested (65°C).
      - Temperature ramp rates set to 1°C per second.
      - Temperature control mode set to “calculated” specifying a volume of 50 µL.

Step 6: Detection Reaction

The EFC working detection solution(s) required for this step should be prepared while the assay plate is being incubated at 37°C during Step 4 above.

a. Prepare the optimized EFC working detection solution(s) identified in Section III-E. Table 1 provides guidelines for preparing the required volumes of working detection solution.

b. Once the pulse denaturation step is complete, add 60 µL of the corresponding working detection solution to all rows of the assay plate.

c. Incubate the plate for 1 hour at room temperature in the dark.

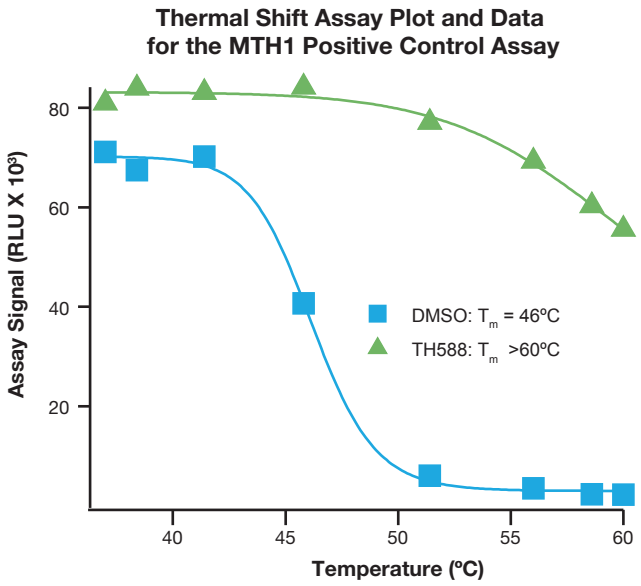
Step 7: Assay Plate Readout

See Section III-C, Step 4.

IV-D. Phase II Assay Development Data Analysis

1. Plot the thermal shift data as shown in Figure 4 and fit to the Hill equation to calculate the melting transition midpoints (T_m). Typical results for the MTH1 positive control assay tested using the potent inhibitor TH588 are shown in Figure 6.

2. Calculate assay windows at each temperature by dividing positive control inhibitor value by the DMSO control value. MTH1 assay window data are shown in Figure 6.



Temperature (°C)	37	38.4	41.4	45.8	51.4	56	58.6	60
Assay Window	1.1	1.2	1.2	2.1	13	20	25	24

Figure 6. Thermal shift assay plot and assay window data (table above) for the MTH1 positive control assay tested using the potent inhibitor TH588 at 10 µM. TH588 gives a large T_m shift (>14°C) to produce assay windows >2 and up to 25 at temperatures above 45°C. T_m shifts measured during typical assay development are often smaller (2°C-5°C) and yield acceptable assay windows at specific temperatures.

3. Evaluate thermal shift assay data according to the following guidelines.

   a. Ideal thermal melting curves for the DMSO control sample will have the following properties:

      - Assay signal declines in a single steep transition, with a majority of the signal loss occurring over a narrow temperature range (5-10°C).
      - Assay signal drops by at least 10-fold over the melting transition.

   b. Ideal thermal melting curves for the positive control inhibitor sample will have the following properties:

      - The T_m is right-shifted to higher temperatures compared to the minus-inhibitor control. Only relatively small changes in T_m effected by the inhibitor (≥2°C) are generally required to build and to validate InCELL Pulse assays.

- Assay windows of at least 2-fold are measured at one or more temperatures. Even assay windows below 2-fold can be acceptable as they can be optimized during Phase III Assay Development.

c. If significant assay windows are observed at 37°C and other non-denaturing temperatures, additional control experiments are required. Please refer to the Protein Abundance and Detection Reagent Control Experiments section in the Supplemental Information.

When significant assay windows are measured at non-denaturing temperatures, it is critical to perform the Protein Abundance and Detection Reaction Control Experiments described in the Supplemental Information. These control experiments are designed to rule out artifacts that can compromise data integrity but can also demonstrate feasibility of an alternative target engagement assay strategy based on protein abundance changes.

d. Refer to the Troubleshooting Guide or contact Technical Support if the following results are observed:

- Poor denaturation of the target protein (DMSO control). Shallow denaturation curve with only a small reduction (<5-fold) in luminescence across the temperature gradient. The shape of the MTH1 DMSO control denaturation curve shown in Figure 6 is ideal.
- Little or no T_m shift measured for the positive control inhibitor and little or no assay window measured at any temperature.

IV-E. Select Temperatures for Phase III Assay Development Isothermal Dose-Response Curves

Selection Guidelines

- Select the lowest temperature giving an assay window of at least two (i.e. 45.8°C for the MTH1 positive control in Figure 6). Assay windows can be improved during Phase III Assay Development (Section V), so proceed even if the best assay window measured is under 2.
- Select one or more additional temperatures based on the assay window data. Depending on the exact data, these temperatures may be higher and/or lower than the lowest temperature giving an assay window of at least two. For the MTH1 example in Figure 6, temperatures between 45.8°C and 51.4°C were selected.

V. Phase III Assay Development

Isothermal inhibitor dose-response curves

V-A. Overview and General Considerations

The goal of Phase III Assay Development is to optimize and validate InCELL Pulse assays by measuring isothermal inhibitor dose-response curves using various pulse denaturation times and temperatures. The resulting inhibitor EC_{50} and assay window values guide selection of the final InCELL Pulse assay conditions. Successful InCELL Pulse assays have the following characteristics:

- Assay windows >2 and preferably >3 for potent positive control inhibitors
- Correct rank-order potency values (EC_{50}) for known inhibitors

InCELL Pulse assays use elevated temperatures that can increase (right-shift) inhibitor EC_{50} values above those measured at 37°C using other methods. The degree of this increase often depends on both the denaturation time and temperature. Higher temperatures and longer denaturation times generally increase the EC_{50} values but can also improve the assay window. The goal is to identify pulse denaturation conditions that minimize EC_{50} values while giving acceptable assay windows.

V-B. Design of Phase III Assay Development Experiments and Plate Layout

- Each pulse denaturation time and temperature combination requires an individual black 96-well PCR plate.
- We suggest first testing the two temperatures selected based on Phase II Assay Development data. Use a 3-minute denaturation time for each temperature. This experiment requires two plates.
- Based on these data the temperature and denaturation times can be further fine-tuned in a second experiment. Denaturation times between 2-5 minutes are often optimal and must be co-optimized with temperature.
- For rigorous assay validation, test compounds of different known potency to confirm potency rank order. We also suggest testing a negative control compound (i.e. a known inhibitor of a different protein target).
- For the dose-response curves we suggest testing a top inhibitor concentration equal to that tested in the thermal shift assay in Phase II Assay Development. Higher concentrations can be tested in subsequent experiments if the dose-response curves do not show an upper plateau.

Example Plate Layout for Isothermal Inhibitor Dose-response Curves

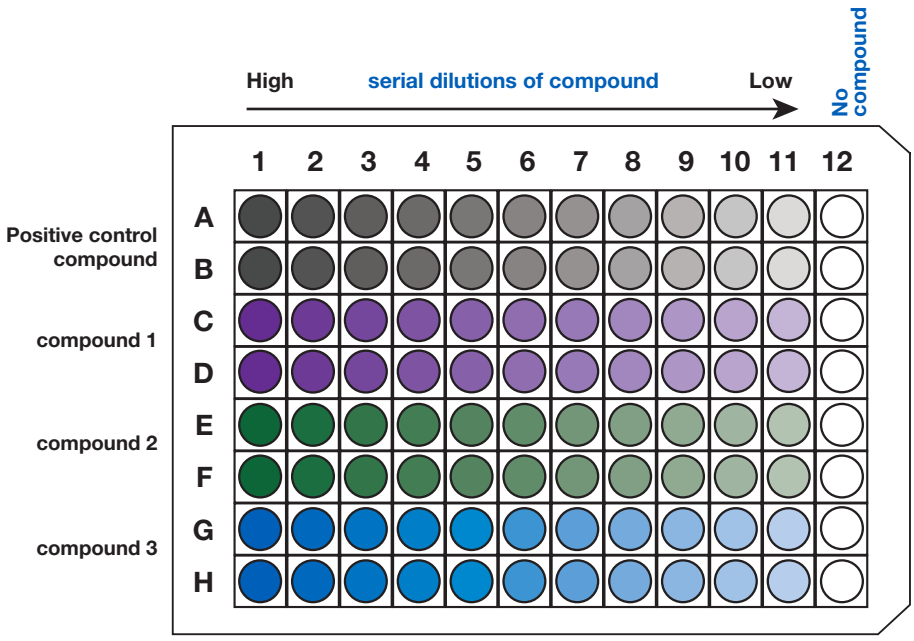


Figure 7. In a black PCR plate, create 12-point dose-response curves for up to four compounds in duplicate. Individual plates are required for each pulse denaturation temperature and time combination tested.

V-C. Phase III Assay Development Detailed Protocol

Materials Required

- Transfected cells harvested just prior to use (Section II-C, Step 13)
- EFC working detection solution(s) identified as optimal for the transfection construct(s) being evaluated (Section III-E)
- Positive control inhibitor 100X stock(s) in DMSO
- Assay Medium (see Section II-C, Step 1 for description) warmed to 37°C
- Complete Growth Medium containing 10% FBS warmed to 37°C
- DMSO
- Disposable Reagent Reservoirs
- Strip tubes (12-well) or 96-well plates for compound serial dilutions
- 96-Well Black PCR Plates

Step 1: Prepare Positive Control Inhibitor Serial Dilutions

a. Prepare serial dilutions of positive control inhibitors.

1. In a 96-well plate or a strip tube, prepare three-fold serial dilutions of the 100X DMSO stocks using DMSO as the diluent. The top concentration tested should be equal to the concentration being tested in the thermal shift assay.

   i. Add 20 µL of DMSO to wells 2-12 of a plate row or strip tube.

   ii. Add 30 µL of 100X test compound to well 1 of a plate row or strip tube.

   iii. Transfer 10 µL of compound stock from well 1 to well 2, mix by pipetting up and down, and continue serial dilution process through well 11 (well 12 serves as the DMSO control).

2. Prepare 5X positive control inhibitor serial dilution working stocks

   i. Dispense 5 µL of 100X compound serial dilution stocks into a 96-well plate or a strip tube with a multichannel pipette.

   ii. Add 95 µL of pre-warmed Complete Growth Medium containing 10% FBS into each well and mix to prepare 5X serial dilution stocks.

It is critical that the compound dilutions in DMSO are added to the dry plate first followed by the Complete Growth Medium containing 10% FBS to ensure rapid compound mixing and reduced precipitation of insoluble compounds in the aqueous medium.

The final DMSO concentration for all InCELL Pulse assays should be ≤1% once the cells have been added.

10% FBS in the Complete Growth Medium used for compound dilutions is recommended to help ensure compound solubility.

Step 2: Dispense Compounds to 96-Well Black PCR Plates

Dispense 10 µL of the 5X inhibitor serial dilution stocks from Step 1 above to the assay plate using a multi-channel pipette.

Step 3: Harvest Transfected Cells

Follow procedures described in Section II-C to prepare transfected cells at the recommended working concentration in Assay Medium.

Step 4: Assay Setup and Compound Incubation

a. Add 40 µL of the cell suspension from Section II-C Step 13 (5,000 cells) to the plate from Step 2 above.

b. Incubate plate for 1 hour in a 37°C and 5% CO_2 humidified incubator.

Gently mix cells in the reagent reservoir during dispensing to ensure that significant settling does not occur.

Step 5: Pulse Denaturation

a. Remove assay plate from the 37°C incubator and seal with non-breathable adhesive film.

b. Transfer plate to a thermocycler and execute the pulse denaturation protocol.

1. Set the specified denaturation temperature and time (i.e. 48°C for 3 minutes) followed by a cooling step (room temperature for 3 minutes).
2. Additional thermocycler settings
   - Heated lid set 5°C higher than the denaturation temperature.
   - Temperature ramp rates set to 1°C per second.
   - Temperature control mode set to “calculated” specifying a volume of 50 µL.

Step 6: Detection Reaction

The EFC working detection solutions required for this step should be prepared while the assay plate is incubated at 37°C during Step 4.

a. Prepare the optimized EFC working detection solution identified in Section III-E. Table 1 provides guidelines for preparing the required volumes of working detection solution.

b. Add 60 µL of working detection solution to the assay plates and gently mix by pipetting up and down.

c. Incubate the plates for 1 hour at room temperature in the dark.

Step 7: Assay Plate Readout

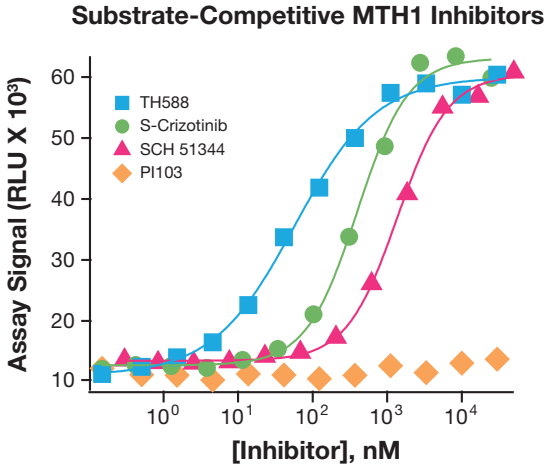
See Section III-C, Step 4.

Some applications may require that the assay windows and inhibitor EC_{50} values are stable for at least two hours or more (i.e. applications requiring batch processing of plates). It is therefore suggested that the assay plates are read-out after 1 and 2 hours (or longer) in Step 6 above. If the assay windows and/or inhibitor EC_{50} values change significantly at the incubation time required for your process, please refer to the Troubleshooting Guide.

V-D. Phase III Assay Development Data Analysis

1. Plot the isothermal inhibitor dose-response curve data as shown in Figure 8 below for the MTH1 positive control assay and fit to the Hill equation to calculate EC_{50} and assay window values.

Inhibitor Dose-response Curves for the Optimized MTH1 Positive Control Assay



Inhibitor	EC_{50} (nM)	Assay Window	Hill Slope
TH588	44	5.4	0.9
S-Crizotinib	400	5.1	1.4
SCH51344	1,600	4.6	1.1
PI103	>10,000	N/A	N/A

Figure 8. MTH1 InCELL Pulse dose-response curves for the diverse substrate-competitive inhibitors TH588, S-crizotinib, and SCH 51344 show the correct rank-order potencies and give assay windows >4. The lipid kinase inhibitor PI103 was included as a negative control. Pulse denaturation conditions: 2.5 minutes at 48°C. Working detection solution: EA-1.

2. Based on temperature-dependent trends in assay window and inhibitor EC_{50} values, determine if acceptable conditions have been identified. Inhibitor rank order potency is an important validation criterion as well.

- If necessary, test additional pulse denaturation time and temperature combinations to fine-tune the assay. It may also be necessary to test higher inhibitor concentrations (solubility permitting) for curves that do not reach an upper plateau.
- The effects of pulse denaturation temperatures and times on inhibitor EC_{50} and assay windows for an internally developed InCELL Pulse assay are shown in Table 4 below.

Table 4: Effects of Pulse Denaturation Temperature and Time on Assay Window and EC_{50} Values				
Denaturation Temperature (°C)	47	48	48	49
Denaturation Time (minutes)	3	3	4	3
Assay Window	1.7	3.5	5.3	5.9
Control Inhibitor EC_{50} (nM)	5.2	7.9	11	9.9

Effects of pulse denaturation conditions on key InCELL Pulse assay metrics. Results shown for an internally developed InCELL Pulse assay tested against a potent positive control inhibitor.

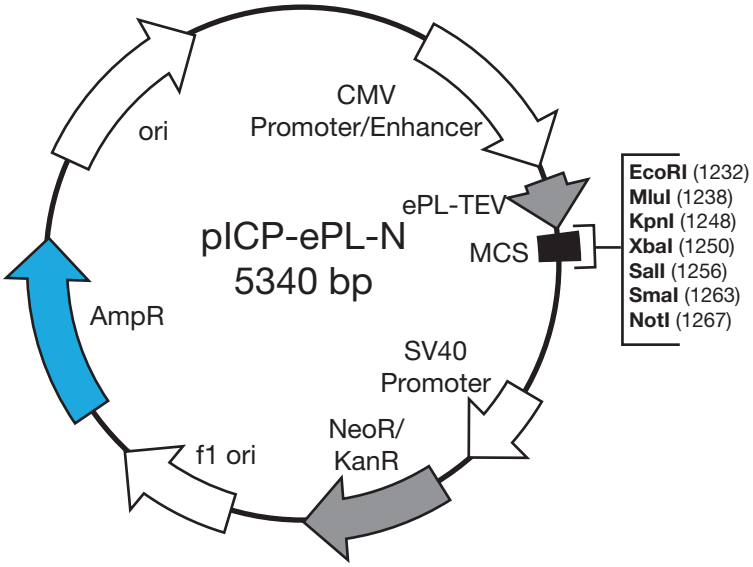
3. For successfully developed assays, refer to the Stable Cell Line Generation section of the Supplemental Information for details on how to generate stable cell lines (optional).

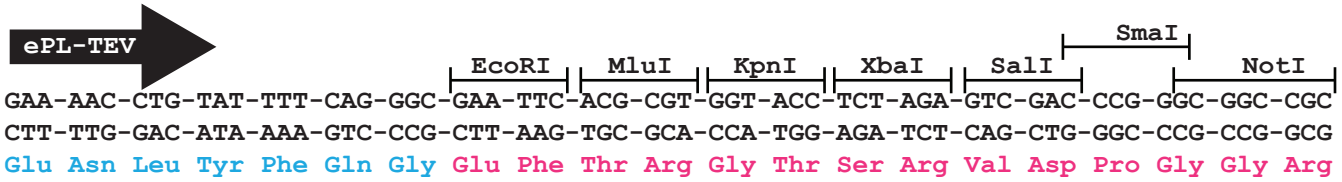
4. Refer to the Troubleshooting Guide or contact Technical Support if there are problems with the measured assay window or inhibitor EC_{50} values.

Supplemental Information

InCELL Pulse Cloning Vector Maps

pICP-ePL-N Vector Map and Multiple Cloning Site Sequence

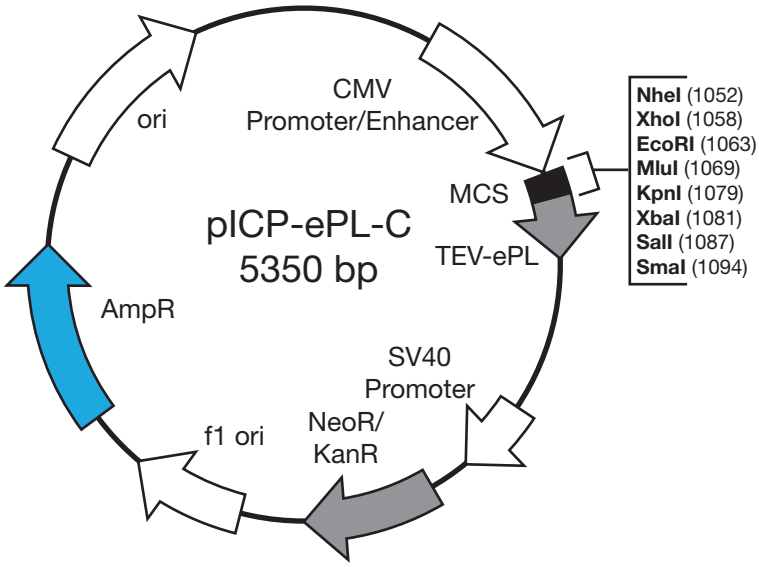


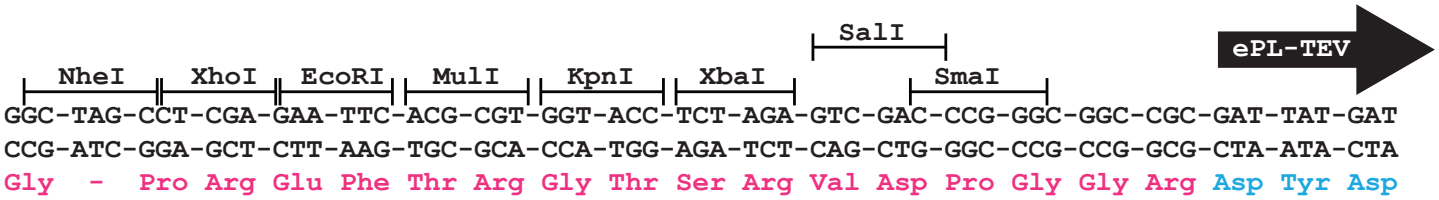


Blue = ePL-TEV Linker
Pink = MCS

Map of pICP-ePL-N Cloning Vector and MCS. Only unique restriction sites are shown. pICP-ePL-N is used to generate an N-terminal fusion of the ePL tag and a protein of interest, which are separated by a short linker (TEV). Transcription is initiated from the constitutive pCMV early promoter. Plasmids contain the ampicillin resistance gene (AmpR) for selection in *E. coli* and the neomycin/kanamycin resistance gene (NeoR/KanR) for selection in mammalian cells using G418.

pICP-ePL-C Vector Map and Multiple Cloning Site Sequence





Pink = MCS
Blue = TEV Linker

Map of pICP-ePL-C Cloning Vector and MCS. Only unique restriction sites are shown. pICP-ePL-C is used to generate a C-terminal fusion of the ePL tag and a protein of interest, which are separated by a short linker (TEV). Transcription is initiated from the constitutive pCMV early promoter. Plasmids contain the ampicillin resistance gene (AmpR) for selection in *E. coli* and the neomycin/kanamycin resistance gene (NeoR/KanR) for selection in mammalian cells using G418.

Protein Abundance and Detection Reagent Control Experiments

Perform control experiments evaluating inhibitor effects on steady state target protein levels and on the EFC detection reaction. These controls support Phase II Assay Development Data Analysis.

Overview and Plate Layout

1. The protein abundance control measures effects of the positive control inhibitor on steady state levels of the ePL-tagged target protein at 37°C in the absence of a heat denaturation step. Ligand binding can stabilize proteins from normal degradative processes to increase their steady state abundance, and this concept has been exploited to build EFC-based cellular target engagement assays[5-8]. This additional protein stabilization mechanism can augment the assay window produced by thermal stabilization. When the protein abundance changes are sufficiently large, it can be possible to build cellular target engagement assays that do not require a thermal denaturation step, and this application is supported by the InCELL Detection Kit (Cat. No. 96-0079) and assay development documentation (discoverx.com/um-incell-hunter-tea).

2. The detection reaction control is included to ensure that the measured target engagement has taken place on live intact cells and does not result from target engagement occurring post-cell lysis or from detection reaction artifacts. For this control, the positive control inhibitor is added as a component of the EFC working detection solution and is NOT present during any steps with live intact cells.

Plate Layout for the Protein Abundance and Detection Reaction Controls

			10 µM ←Inhibitor Serial Dilutions→ 0.2 nM DMSO											
Transfection Construct	**Control**		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
1	Protein Abundance	**A**													Positive Control Inhibitor 1
		B													
	Detection Reaction	**C**													
		D													
2	Protein Abundance	**E**													Positive Control Inhibitor 2
		F													
	Detection Reaction	**G**													
		H													

Figure 9. Cells transfected with Constructs 1 or 2 are tested in duplicate under two sets of conditions in dose-response experiments using positive control inhibitors. For the protein abundance controls, the positive control inhibitors are included in a 1-hour incubation at 37°C prior to the EFC detection reaction. For the detection reaction controls, the positive control inhibitors are omitted from the 1-hour 37°C incubation step and are added as part of the working detection solution used for the EFC detection reaction.

Detailed Protocol

Materials Required

- Transfected cells harvested just prior to use (Section II-C, Step 13)
- EFC working detection solution(s) identified as optimal for the transfection construct(s) being evaluated (Section III-E)
- Positive control inhibitor 100X stock(s) in DMSO
- Assay Medium (see Section II-C Step 1 for description) warmed to 37°C
- Complete Growth Medium containing 10% FBS warmed to 37°C
- DMSO
- Disposable Reagent Reservoirs
- Strip tubes (12-well) or 96-well plates for compound serial dilutions
- 96-Well Black PCR Plates

Step 1: Prepare Positive Control Inhibitor Serial Dilutions

Prepare serial dilutions of positive control inhibitors. The example volumes given below are for the plate layout shown in Figure 9. Adjust volumes accordingly for other layouts (i.e. if the same positive control inhibitor is used in all 8 rows, then double the volumes given below).

1. In a 96-well plate or a strip tube, prepare three-fold serial dilutions of the 100X DMSO stocks using DMSO as the diluent. The top concentration tested should be equal to the concentration being tested in the thermal shift assay.
    i. Add 20 µL of DMSO to wells 2 to 12 of a plate row or strip tube.
    ii. Add 30 µL of 100X test compound to well 1 of a plate row or strip tube.
    iii. Transfer 10 µL of compound stock from well 1 to well 2, mix by pipetting up and down, and continue serial dilution process through well 11 (well 12 serves as the DMSO control).
2. Prepare 5X positive control inhibitor serial dilution working stocks
    i. Dispense 5 µL of 100X compound serial dilution stocks into a 96-well plate or a strip tube with a multichannel pipette.
    ii. Add 95 µL of pre-warmed Complete Growth Medium containing 10% FBS into each well and mix to prepare 5X serial dilution stocks.

The final DMSO concentration for all InCELL Pulse assays should be ≤1% once the cells have been added.

10% FBS in the Complete Growth Medium used for compound dilutions is recommended to help ensure compound solubility.

Step 2: Dispense Compounds to 96-Well Black PCR Plates

Dispense 10 µL of the 5X positive control inhibitor serial dilution stocks from Step 1.a.2. above to the protein abundance control rows (A,B,E,F) as specified in Figure 9.

DO NOT add compounds to the detection reaction control rows (C,D,G,H)

The remaining 5X positive control serial dilution stocks are required for the detection reaction control rows (Step 4). **DO NOT DISCARD!**

Step 3: Harvest Transfected Cells

Follow procedures described in Section II-C to prepare transfected cells at the recommended working concentration in Assay Medium.

Step 4: Assay Setup and Compound Incubation

a. Add 40 µL of the cell suspension(s) from Section II-C, Step 13 (5,000 cells) to all rows of the plate prepared in Step 2 above, as specified in Figure 9.

b. Incubate plate for 1 hour in a 37°C and 5% CO_2 humidified incubator.

Gently mix cells in the reagent reservoir during dispensing to ensure that significant settling does not occur.

Step 5: Detection Reaction

The EFC working detection solutions required for this step should be prepared while the assay plate is incubated at 37°C during Step 4 above.

a. Prepare the optimized EFC working detection solution(s) identified in Section III-E. Table 1 provides guidelines for preparing the required volumes of working detection solution.

b. Prepare working detection solutions containing the positive control inhibitor. This reagent mixture is used for readout of the detection reaction controls (Figure 9, rows C,D,G,H).

    1. In a fresh 96-well ligand dilution plate, add 12 µL of the corresponding 5X positive control inhibitor serial dilution working stocks from Step 1 to rows C,D,G,H, as specified in Figure 9.

    2. Add 72 µL of the corresponding EFC working detection solution to these rows.

c. Once the assay plate incubation is complete (Step 4 above):

    1. Add 70 µL of the corresponding working detection solution containing dilutions of the positive control inhibitor to the detection reaction control rows (C,D,G,H) of the assay plate and gently mix by pipetting up and down.

    2. Add 60 µL of the corresponding working detection solution (lacking positive control inhibitor) to the protein abundance control rows (A,B,E,F) of the assay plate and gently mix by pipetting up and down.

d. Incubate the plates for 1 hour at room temperature in the dark.

Step 6: Assay Plate Readout

Refer to Section III-C, Step 4.

Data Analysis

1. Plot the inhibitor dose-response curve data as shown in Figure 10 below for the MTH1 positive control assay. Fit data to the Hill equation to calculate assay window and inhibitor EC_{50} values when significant dose-dependent effects are observed.

- For the MTH1 positive control assay, as anticipated from the thermal shift data (Figure 6), the positive control inhibitor TH588 has no effect in the detection reaction and protein abundance controls.
- When the thermal shift assay for your target shows a significant assay window at 37°C, this activity will likely be observed in the protein abundance control and maybe in the detection reaction control as well. Case-specific recommendations for how to proceed are given in Table 5 below.

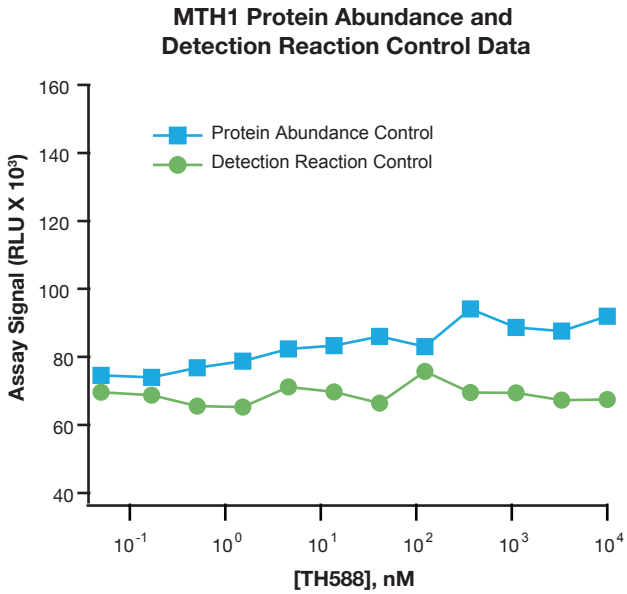


Figure 10. Protein abundance and detection reaction control data for the MTH1 positive control assay tested against the positive control inhibitor TH588. No effects of TH588 are observed for the detection reaction control and only minor, if any, effects on protein abundance are measured. These control data are consistent with the thermal shift results (Figure 6), where assay windows were measured only at denaturing temperatures (>45°C).

Table 5. Decision-Making Recommendations Based on Control Assay Data

Control Assay Result	Recommendation
Large assay window* measured for the protein abundance control and little or no assay window measured for the detection reagent control	Proceed with assay development. If the protein abundance assay window is sufficiently large, a pulse denaturation step may not be required. Visit (discoverx.com/um-incell-hunter-tea) for information on how to develop a protein abundance-based target engagement assay.
Large assay window* measured for the detection reaction control	DO NOT proceed. Post-cell lysis artifacts are significantly contributing to the assay window. See the Troubleshooting Guide.

*Large assay windows for these controls are defined as those that would be significant relative to the windows generated by the pulse denaturation step alone. The type of analysis shown in Figure 6 is helpful for guiding decision making.

Stable Cell Line Generation

InCELL Pulse pICP-ePL-C and pICP-ePL-N vectors carry a neomycin resistance gene for selection of mammalian cells using G418. Once an InCELL Pulse assay has been developed and validated, it can be beneficial to generate a stable cell line expressing the ePL-tagged target protein. Stable cell lines provide a convenient renewable and consistent source of InCELL Pulse cells to support ongoing screening and lead optimization campaigns.

Step 1: Establishing Optimal G418 Concentration

Prior to transfection, we recommend setting up a G418 kill curve with your cell line of choice to determine the lowest dose of G418 required to kill the cells. The normal concentration used for G418 is between 100 µg/mL to 1000 µg/mL so use at least 3 concentrations within this range.

a. Split your cells 1:10 the day before selection.

b. Change the medium with the different concentrations of G418 every 2 to 3 days.

c. Check the viability of your cells every day, and after 1 week find the lowest dose that kills 100% of the cells. This is the G418 concentration you should use to select your stable transfected clones.

Step 2: Transfection

Refer to Transfection of Mammalian Host Cells Section II. Transfect one well of a six-well plate.

Step 3: Generating Stable Transfectants

a. 24-hours post-transfection:

   1. Aspirate medium, rinse once in PBS, and detach using 0.2 mL AssayComplete™ Cell Detachment Reagent (Cat. No. 92-0009).

   2. Cells usually detach in 2-5 minutes. Verify that cells are detached using a microscope.

   3. Add 2 mL of Complete Growth Medium and transfer to a 15 mL conical tube.

b. Transfer cells to a 10 cm dish containing 8 mL of Complete Growth Medium containing the appropriate concentration of G418 determined from Step 1.

Be sure to include mock transfected cells as a control.

c. Return cells to incubator at 37°C and 5% CO_2. Exchange medium containing G418 every 3-4 days.

d. After 1-2 weeks, the majority of cells should be dead. The cells that remain growing in the selective medium have retained the plasmid which has integrated into the genome of the targeted cells. Be sure to monitor the mock control cells to ensure the cells are dead. No cells should be attached after 1-2 weeks.

e. After approximately 3 weeks, transfected cells should reach ~70% confluency and can be tested in InCELL Pulse assays. Measure assay windows and EC_{50} values for a compound(s) of interest as outlined in the Phase III Assay Development section.

f. If the desired response is achieved (i.e. assay window >2 and EC_{50} in expected range), the cell pool is a good candidate for clonal dilution. We recommend freezing down a vial of the original cell pool (at least 1 x 10^6 cells per vial) for use in future studies. For isolation of individual colonies, there are many commercially available tools and protocols to assist in this process. One approach is to do a serial dilution of your stably transfected cells. Briefly:

   1. Plate 0.5, 1, and 2 cells/well in 384-well tissue culture plates with the goal of obtaining wells with single colonies.
   2. After ~4 to 7 days (HEK 293) identify at least 20 wells with single colonies and allow to grow to confluence.
   3. Once the cells reach confluency, transfer into a 96-well plate. Repeat this process until the cells reach confluency in a 48-well and again in a 24-well plate. At this time, each well contains a clonal population of stably transfected cells that can be maintained in normal growth medium (without G418) or under conditions of low G418 to maintain selective pressure.

Step 4: Identify Clones with Optimal EFC Signal to Background Ratios

Measure EFC signal to background (S/B) ratios for at least 20 clones following the procedures outlined in the Phase I Assay Development section. Select at least 5 clones giving S/B ratios of >10 and ideally >50 for further analysis.

Step 5: Identify Clones with Optimal InCELL Pulse Assay Performance

For the clones identified in Step 4 above, measure assay windows and EC_{50} values for a compound(s) of interest as outlined in the Phase III Assay Development section. Choose the clone(s) giving the best assay performance.

Step 6: Prepare Frozen Stocks of Clonal Cell Lines

Freeze down 3-4 vials of each clone (at least 1 x 10^6 cells per vial) in AssayComplete™ Freezing Reagent and store in the vapor phase of liquid nitrogen for future use.

References

1. Martinez MD. *et al.* Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science*. 2013. 341(6141):84-7.

2. Savitski MM. *et al.* Tracking cancer drugs in living cells by thermal profiling of the proteome. *Science*. 2014. 346(6205):1255784.

3. Huber KV. *et al.* Proteome-wide drug and metabolite interaction mapping by thermal-stability profiling. *Nat Methods*. 2015. 12(11):1055-7.

4. Reinhard FB. *et al.* Thermal proteome profiling monitors ligand interactions with cellular membrane proteins. *Nat Methods*. 2015. 12(12):1129-31.

5. Schürmann M. *et al.* Small-Molecule Target Engagement in Cells. *Cell Chem Biol.* 2016. 23(4):435-41.

6. Auld DS. *et al.* Examining Ligand-Based Stabilization of Proteins in Cells with MEK1 Kinase Inhibitors. *Assay Drug Dev Technol*. 2015. 5:266-76.

7. Schulze J. *et al.* Cell-based protein stabilization assays for the detection of interactions between small-molecule inhibitors and BRD4. *J Biomol Screen*. 2015. 2:180-9.

8. Kaniskan HÜ. *et al.* A potent, selective and cell-active allosteric inhibitor of protein arginine methyltransferase 3 (PRMT3). *Angew Chem Int Ed Engl.* 2015. 54(17):5166-70.

Frequently Asked Questions

Can InCELL Pulse be used for high-throughput screening of compound collections?

- InCELL Pulse is compatible with high throughput screening in higher density plate formats, and this application is under development.

Can InCELL Pulse be used to detect compound interactions with membrane proteins?

- Thermal stability-based methods have been applied successfully to membrane proteins[3,4]. InCELL Pulse applications for membrane proteins are under development.

Can I order additional InCELL Pulse reagents and plates?

- Yes, please refer to the Additional Materials Required section for product names and catalog numbers.

How do I reduce expression of the ePL-tagged protein to more closely approximate normal cellular levels?

- For transfection, combine your target protein expression plasmid at various ratios with the provided parental pICP-ePL-C Vector. The Transfection Protocol (Day 2) requires mixing 2 µg of purified plasmid DNA in 250 µL of serum-free medium. To reduce expression, the delivered plasmid DNA (still 2 µg) should be a mixture of both plasmids at a specified ratio. For example, to reduce expression by 10-fold, this mixture would include 0.2 µg of the target protein expression plasmid and 1.8 µg of pICP-ePL-C. DO NOT mix the parental pICP-ePL-N Vector with the target protein expression vector for this application since pICP-ePL-N will express the ePL peptide and mask the target protein signal.

What should I do if my target protein has an associated partner protein required for inhibitor binding?

- The partner protein (no ePL tag) can be co-expressed with the ePL-tagged target protein. Alternatively, expression of the ePL-tagged target protein can be reduced (see Frequently Asked Questions above) to more closely approximate normal cellular levels required for stoichiometric interaction with endogenous partner proteins.

Does InCELL Pulse work for full-length proteins or just for the ligand binding domains?

- InCELL Pulse assays for both full-length and ligand binding domain-only constructs have been successfully developed. As for any thermal denaturation-based approach, proteins with multiple independently folding domains are potentially problematic because ligand binding to one domain may not protect the other domains from denaturation/aggregation. Please see the Clone Design recommendations given in the Cloning and Transfection section.

There are no potent positive control inhibitors available for my target. Can I still build an assay?

- It may be possible to build an assay without the benefit of a positive control inhibitor, and this assay could then in principle be used to screen for inhibitors. In this case, start with constructs that include only the ligand binding domain and make N-terminal and C-terminal ePL fusions. Choose a construct that gives ideal thermal melting curves, such as those shown in Figure 6 for the MTH1 positive control assay. Then select two temperatures at the lower end of the melting transition for compound screening (i.e. temperatures between 45°C and 50°C for MTH1 in Figure 6).

What targets have been successful using the InCELL Pulse approach, and are assay validation data available?

- InCELL Pulse assays have been successfully developed for a number of enzymes from diverse classes, including protein kinases, methyltransferases, and hydrolases. For information on assay-ready target-specific InCELL Pulse kits and to view data for additional targets, please visit discoverx.com/incell.

How do I confirm that the data measured for my inhibitor are due to cellular target engagement and not from any effects on the detection reagents or interactions with the target that occur post-lysis?

- Please refer to the Protein Abundance and Detection Reagent Control section of the Supplemental Information for a description of the relevant control experiments.

For additional information or technical support, please see contact below.

Troubleshooting Guide

The MTH1 positive control assay should be used to help troubleshoot all Assay Development Phases.

Problem	Cause	Solution(s)
Assay Development Phase I		
Low or no signal for EFC Positive Control	Improper preparation of EFC detection reagents	EFC detection reagents should be prepared just prior to use and are sensitive to light.
	Improper storage of EFC detection reagents	1. Do not freeze/thaw the InCELL Pulse Detection Reagent components more than 3 times. Aliquot and freeze for multiple experiments. 2. Use the Working Detection Solution of detection reagents immediately after preparation.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.5 to 1.0 seconds/well.
Signal to Background ratio (S/B) for transfected cells is less than 10 for all Working Detection Solutions	Low protein expression	1. Extend the incubation time for the detection reaction from 60 to 120 minutes. 2. Perform a western blot using the provided PathHunter® Anti-PK/PL Antibody to confirm target protein expression. 3. Test additional expression constructs or host cell lines.
	Problem with transfection and/or EFC detection reaction	Perform Phase I Assay Development using the supplied MTH1 positive control vector to diagnose problem.
	Protein expresses as an insoluble aggregate	Test additional expression constructs (i.e. full-length vs. domain) and/or positioning of the ePL tag.
	Problem with expression vector	Verify that ePL tag is in-frame with target gene.
	Host cell line gives high luminescent background	HEK 293, CHO-K1 and U2OS cells are preferred backgrounds for EFC assays.
	Trypsin used to detach cells	Use Assay Complete™ Cell Detachment Reagent (Cat. No. 92-0009).
Assay Development Phase II		
Poor denaturation of target protein (DMSO control)	Low signal to background (S/B) ratio	Ensure that Phase I Assay Development guidelines have been carefully followed
	Expressed protein is aggregated	1. Test additional constructs expressing longer or shorter versions of the target protein. Test N- and C- terminal positioning of ePL tag. 2. Reduce expression levels (see FAQs) 3. Change host cells

Problem	Cause	Solution(s)
No T_m shift for positive control inhibitor; no assay window	Positive control inhibitor lacks sufficient potency or is not cell permeable	1. Test higher inhibitor concentration 2. Test additional inhibitor(s)
	Positive control inhibitor toxic or insoluble at high concentrations	
	Multi-domain protein: compound binding to the ligand-binding domain does not protect protein from thermal denaturation	Test construct expressing only the ligand binding domain (i.e. from a crystal structure)
	Target protein requires a partner protein for ligand binding	1. Co-express partner protein 2. Reduce expression to make stoichiometric with endogenous partner protein (see Frequently Asked Questions) 3. Use host cell line with high endogenous levels of partner protein
	Expression construct requires optimization	1. Test ePL tag on both N- and C-terminus 2. Explore constructs with different lengths of unstructured protein/linker sequence between the folded domain and the ePL tag
Assay Development Phase III		
Small assay window	Compound binding curve not complete	1. Solubility permitting, use a top inhibitor concentration that completes the upper plateau of the dose response curve 2. Test more potent inhibitors to achieve upper plateau of dose-response curve
	Thermal denaturation conditions too gentle	Increase the denaturation temperature in 1°C increments and/or increase the denaturation time in 30 second increments. Keep in mind that inhibitor EC_{50} values generally rise as the denaturation time and temperatures are increased.
	Expression construct requires optimization	See Solutions provided above for Assay Development Phase II
High inhibitor EC_{50} values	Compound solubility problem; low compound concentrations delivered to assay wells	1. Be sure to prepare the compound serial dilutions in DMSO and not in aqueous medium. Follow the compound serial dilution protocol in the Phase III Assay Development section. 2. For particularly insoluble compounds, it may not be possible to prepare the specified 5X stocks of the serial dilution in Complete Growth Medium containing 10% FBS. In this case add DMSO stocks directly to the dry assay plate before adding cells, keeping in mind that the final DMSO concentration once the cells have been added should not exceed 1%.
	Thermal denaturation conditions too harsh	Decrease the denaturation temperature in 1°C increments and/or decrease the denaturation time in 1 minute increments. Keep in mind that assay windows generally decrease as the denaturation conditions become more gentle.

Problem	Cause	Solution(s)
Assay window and EC_{50} not stable for detection reaction incubation times >60 minutes	High β-galactosidase activity is depleting the EFC substrate reagent	1. Reduce concentration of EA Reagent in EFC Working Detection Solution 2. Reduce expression of ePL-tagged protein (see Frequently Asked Questions for how to do this)
Detection Reaction Control Experiment		
Significant assay window measured for the Detection Reaction Control	Compound binding to expressed protein post-cell lysis allosterically enhances EFC detection of ePL tag	1. Change position of ePL tag to N- or C-terminus of construct 2. Change the linker length between the structured portion of the protein and the ePL tag 3. Design longer and shorter versions of the protein construct
	Inhibitor interacts with detection reagents to increase luminescence	Test inhibitor in a detection reaction using the ePL Positive Control as described in the Phase I Assay Development section. If this is the cause, then other compounds from the same chemical series should be tested for this artifact as well.

For additional information or technical support, please use the contact information below.

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