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User Manual

PathHunter® EA Parental Cell Lines

Parental Cell Lines of Various Cell Types to Be Used with ProLabel®/ ProLink™ Tagged Proteins to Create Subcellular Translocation, SH2 Recruitment, or β-Arrestin Recruitment Assays

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

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Overview

The PathHunter EA Parental Cell Lines are engineered cell lines that stably express a particular reporter protein fused to a large β–galactosidase (β-gal) enzyme reporter fragment called EA (enzyme acceptor). The EA parental cell lines have various cell backgrounds and are intended to be used with ProLabel®/ ProLink™-tagged proteins to create assays to study subcellular translocation (e.g. including membrane receptor trafficking), SH2 recruitment for receptor kinases, or GPCR β-arrestin recruitment.

This user manual covers the storage and handling of EA parental cell lines as well as the introduction and selection for stable expression of a ProLabel/ ProLink-tagged target protein. For protein translocation and β-arrestin recruitment assay development protocols, please refer to DiscoverX application-specific user manuals as indicated in Detection and Assay section of this user manual.

Technology Principle

The PathHunter EA Parental Cell Lines of various cell types (e.g. CHO-K1, HEK 293, U2OS) include translocation, β-arrestin, and SH2 recruitment EA parental cell lines. All parental cell line types are used to develop assays that rely on the well-established enzyme fragment complementation (EFC) technology (see Figure 1). Their intended use is to interrogate cellular localization; analyze SH2 recruitment for receptor tyrosine kinases, cytokine receptors, or cytosolic tyrosine kinases; or study GPCR β-arrestin recruitment in the presence of a ligand of interest.

Enzyme Fragment Complementation

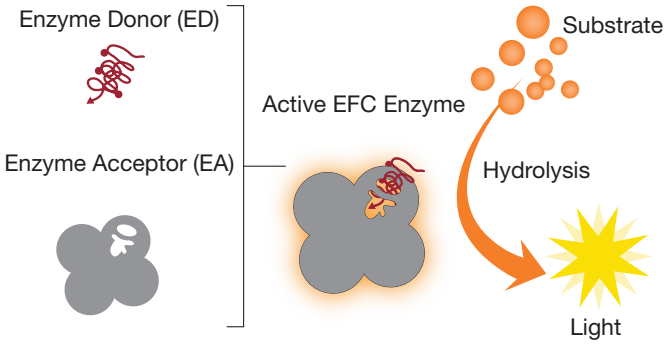


Figure 1. The EFC technology is based on two recombinant β-gal fragments – a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED; also known as enhanced ProLabel (ePL) or ProLink (PK). Separately, the β-gal fragments are inactive, but when combined, they form an active β-gal enzyme that hydrolyzes a substrate (detection reagent) to produce a chemiluminescent signal.

Easy-to-Follow Protocols for EFC Assay Generation

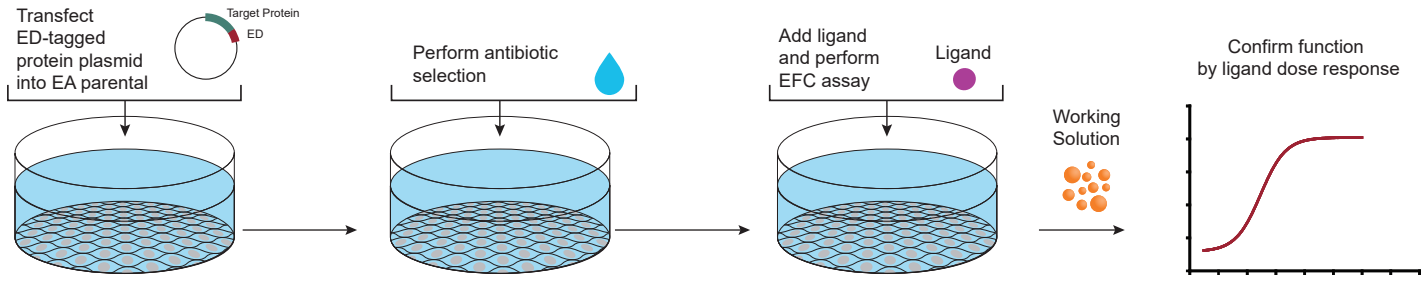


Figure 2. Generate PathHunter EFC assays by first creating an ED-tagged target protein plasmid followed by transfection of this plasmid into your PathHunter EA parental cell line of interest. Perform antibiotic selection, detect the level of expression of the two fusion proteins (optional), and perform an assay in the presence of a ligand.

PathHunter Translocation EA Parental Cell Lines

The PathHunter Translocation EA Parental Cell Lines are used to study the translocation of a target protein of interest (e.g. GPCRs, nuclear hormone receptors (NHR), receptor tyrosine kinases (RTK), ion channels, etc.) between subcellular compartments – membrane, endosome, or nucleus. To study the translocation, the target protein must first be cloned into an ePL/PK plasmid vector, which then must be transfected into a PathHunter parental cell line. After transfection, the parental cell line will express both the ePL/PK-tagged target fusion protein, and the subcellular compartment (reporter) EA fusion protein (ENDO-EA, MEM-EA, or NUC-EA). Any experimental condition (including agonist, pharmacochaperone, protein subunits, etc.) that induces co-compartmentalization of the ePL/PK and EA-tagged proteins by driving the fusion proteins into close proximity, will result in complementation of ePL/PK to EA. Addition of EFC detection reagent, which includes a substrate that is hydrolyzed by β-gal, results in a chemiluminescent signal that can be read by a luminometer.

Product Highlights

- Create simple, homogeneous assays in multiple cell types that reveals the membrane, nuclear, or endosomal translocation of the fusion protein of interest
- Monitor translocation in live cells without the requirement of expensive, low throughput antibody or microscopy methods
- Obtain quantitative pharmacological data in the presence of a ligand (dose response curves, compound potency, etc.)
- Create HTS assays that are biologically relevant using a small reporter tag (ePL/PK) that doesn't affect the natural protein-protein interaction
- Enables detection of translocation of mutant and ortholog proteins
- Discover pharmacological chaperones that rescue membrane proteins trapped in the endoplasmic reticulum (ER)

PathHunter ENDO-EA Parental Cell Lines

- Study membrane protein recycling patterns by creating internalization assays (see Figure 3)
- Detect total (basal and ligand-induced) internalization of transmembrane proteins to the early endosomes
- Discover compounds that have unique qualities (e.g. agonists that behave as functional antagonists with respect to receptor internalization)

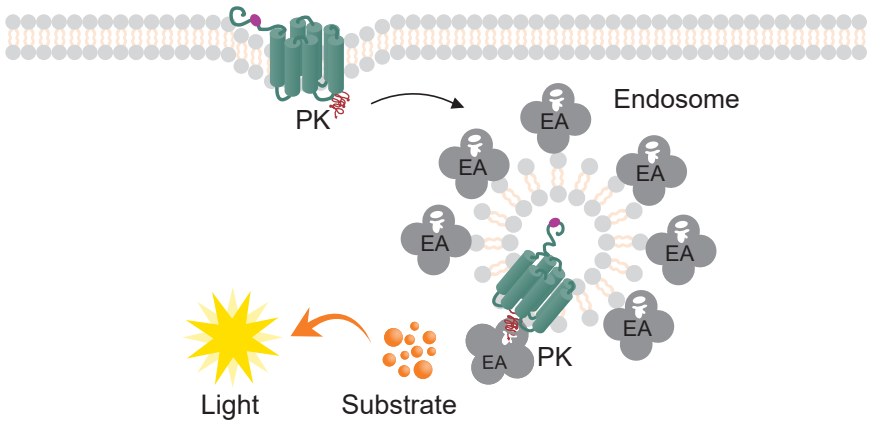


Figure 3. Study membrane protein recycling: Use a PK-tagged GPCR along with an ENDO-EA parental cell line to monitor internalization of GPCRs from the membrane to the endosome.

PathHunter MEM-EA Parental Cell Lines

- Generate trafficking assays to monitor the translocation of membrane proteins from the ER to the membrane
- Study multi-subunit ion channel assembly resulting in translocation to the plasma membrane (see Figure 4)
- Screen pharmacological chaperones that rescue mutant membrane proteins trapped in the ER (see Figure 5)

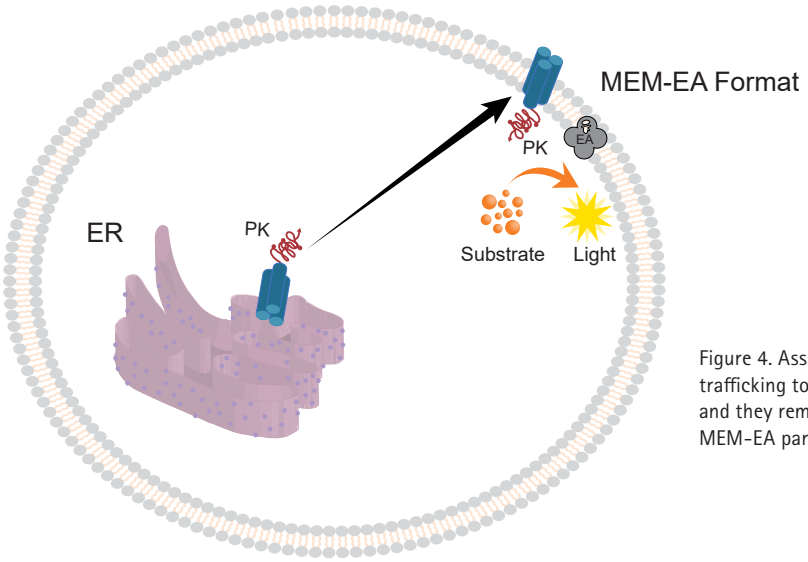


Figure 4. Assembly of ion channel subunits occur in the ER and is critical for proper trafficking to the membrane. If any of the subunits are missing, there is no trafficking and they remain trapped in the ER. Use a PK-tagged ion channel subunit along with a MEM-EA parental cell line to analyze proper ion channel subunit assembly.

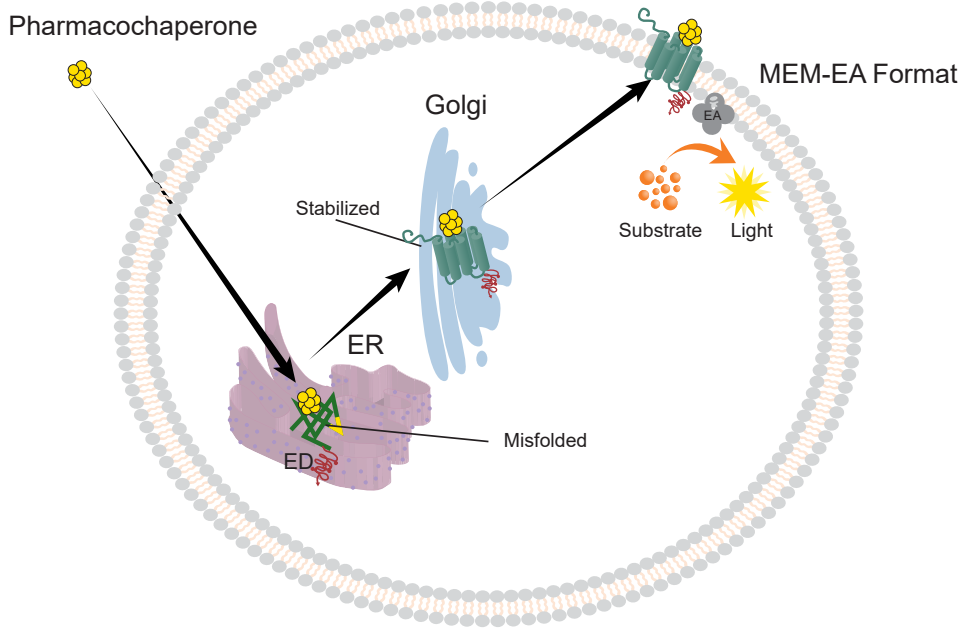


Figure 5. Mutations in GPCRs (or other membrane proteins) can lead to misfolding of the GPCR and improper trafficking to the membrane. Use a PK-tagged GPCR along with a MEM-EA parental cell line to screen small molecule pharmacological chaperones (pharmacochaperones) that bind and rescue mutant GPCRs trapped in the ER.

PathHunter NUC-EA Parental Cell Lines

- Generate assays to monitor the translocation of specific NHRs from the cytoplasm to the nucleus (see Figure 6)
- Discover compounds that activate NHR translocation in the cellular environment

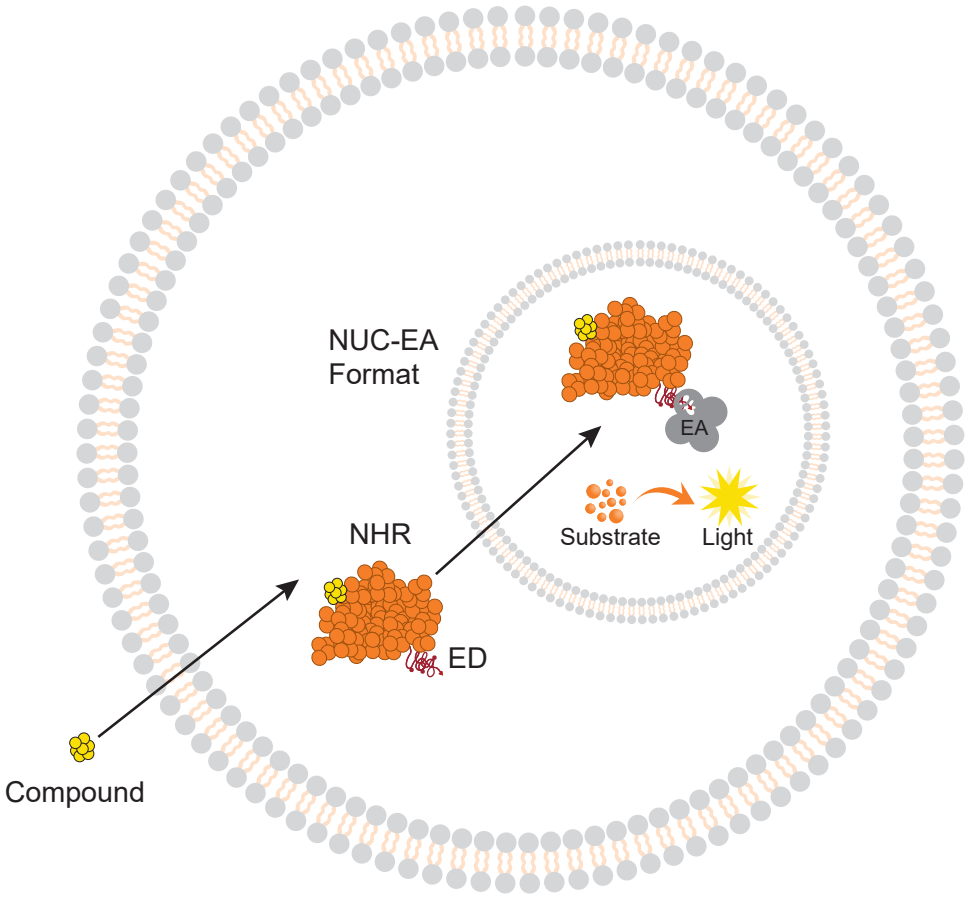


Figure 6. PathHunter nuclear translocation assays detect binding of a compound to an NHR of interest tagged with ED (e.g. ePL) leading to the translocation of the NHR to the nucleus. Complementation occurs when the two β-gal components — the ED tag fused to the NHR and the EA localized in the nuclear compartment (NUC-EA). These components interact once they are colocalized in the nuclear compartment, forming the active β-gal enzyme that converts substrate to a detectable chemiluminescent signal.

PathHunter β-Arrestin-EA Parental Cell Lines

The PathHunter β-Arrestin-EA Parental Cell Lines express EA fused to the scaffold protein β-arrestin1 or 2. Arrestin proteins participate in agonist-mediated desensitization of G-protein-coupled receptors (GPCRs) and also act as scaffolds for other signaling proteins including certain kinases, metabolic enzymes, nucleic binding proteins, and more. To study β-arrestin recruitment to a target protein (e.g. GPCR), the target protein must first be cloned into a ProLink™ (PK) plasmid vector, which then must be transfected into a PathHunter β-arrestin-EA Parental Cell Line (refer to the Supplemental Information section for guidance on GPCR applications). After transfection, the parental cell line will express both the PK-tagged target fusion protein and the β-arrestin EA (reporter) fusion protein. The cell line is then ready for evaluation of ligand-induced β-arrestin recruitment. Addition of EFC detection reagent, which includes a substrate that is hydrolyzed by β-gal, results in a chemiluminescent signal that can be read by a luminometer (see Figure 7).

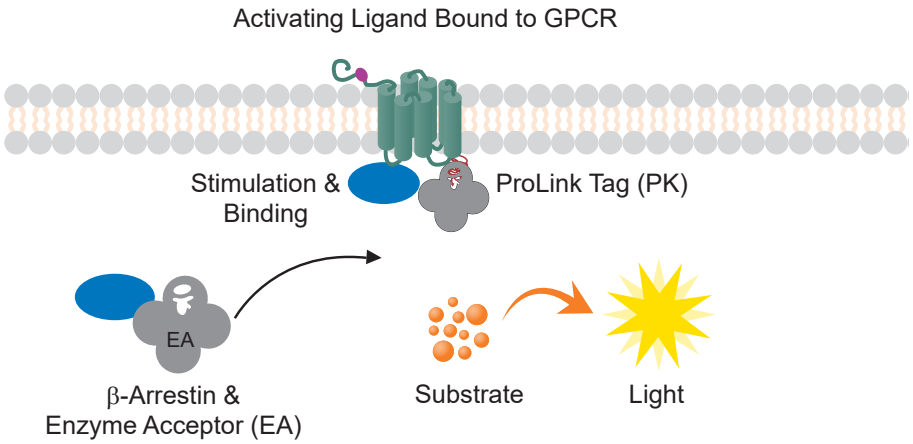


Figure 7. β-Arrestin recruitment to GPCRs occurs upon ligand-stimulation and subsequent phosphorylation by G-protein receptor kinases. This is followed by endocytosis of the GPCR and inactivation of G-protein dependent pathways. Use a PK-tagged GPCR along with a β-Arrestin-EA Parental Cell Line to analyze ligands that activate β-arrestin recruitment.

Create β-Arrestin Recruitment Cell-Based Assays

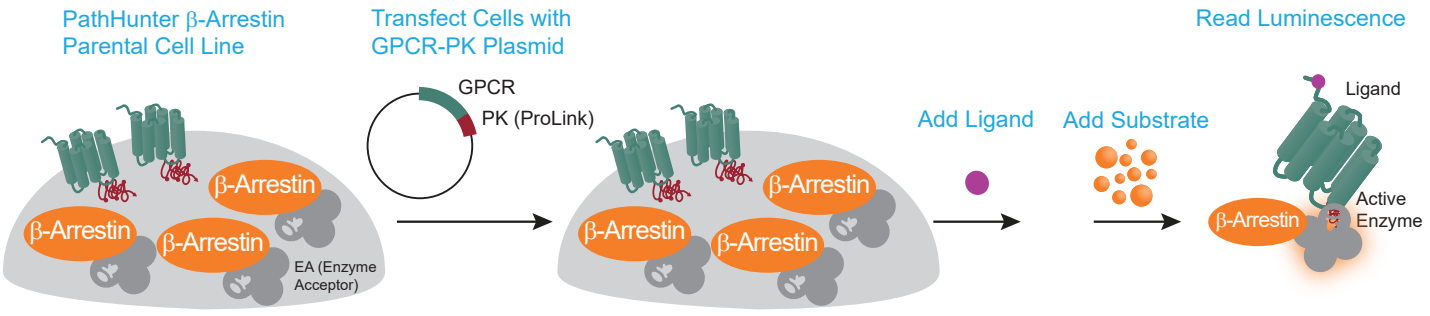


Figure 8. Generate your own GPCR (or other β-arrestin binding protein) β-arrestin recruitment assays. Use a PK-tagged GPCR along with a β-arrestin-EA parental cell line. Simply transfect the parental cell line with a plasmid containing a PK-tagged GPCR, generate a stable pool, and run the β-arrestin recruitment assay by adding your ligands of interest and EFC detection substrate.

PathHunter SH2 Recruitment EA Parental Cell Lines

The PathHunter SH2 Recruitment EA Parental Cell Lines express EA fused to the SH2 (Src Homology 2) domains of adaptor proteins, SHC1 and PLCG1. These cell lines can be used to build your own receptor tyrosine kinase (RTK), cytokine receptor kinase, or cytosolic tyrosine kinase (CTK) functional assay cell lines to monitor activation of these receptors or tyrosine kinases of interest in your research. To develop a functional assay cell line, a researcher will introduce the PK-tagged receptor, and, in some cases, an untagged co-receptor, into one of these parental cell lines. Activation of the receptor-PK induces receptor dimerization and tyrosine phosphorylation, leading to SH2-EA recruitment, and forcing complementation of the two β-galactosidase enzyme fragments (EA and PK). The resulting functional enzyme hydrolyzes substrate to generate a chemiluminescent signal (see Figure 9).

- Study activation and signaling of receptor tyrosine kinases
- Investigate cytokine receptor activation and receptor phosphorylation by cytosolic tyrosine kinases
- Build cell-based, functional assays to study anti-ligand and anti-receptor antibodies
- Discover and study compounds that interfere with ligand binding, receptor dimerization, receptor phosphorylation, and SH2 adaptor protein recruitment

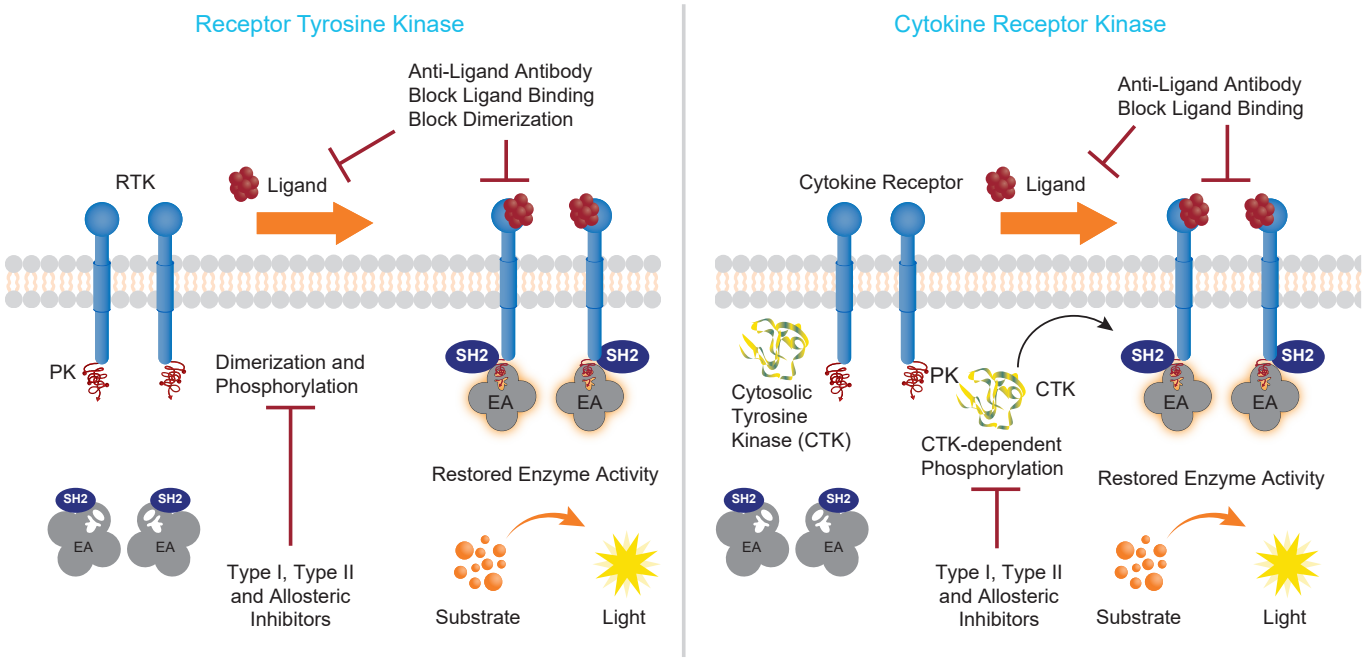


Figure 9. Study SH2 recruitment: Use a PK-tagged receptor along with an SH2 recruitment EA parental cell line to analyze the function of receptor tyrosine kinases, cytokine receptors, or cytosolic tyrosine kinases. In this PathHunter assay approach, the receptor of interest is PK-tagged at the C-terminus and recombinantly expressed in the PathHunter SH2 Recruitment EA Parental Cell Line that expresses a SH2 domain adapter protein. Ligand induced activation of the receptor causes either homo- or hetero-dimerization of the receptor resulting in phosphorylation. The SH2-EA fusion protein then binds the phosphorylated receptor forcing complementation of the PK and EA fragment. This interaction generates an active β-galactosidase enzyme, which is detected using a chemiluminescent substrate.

Materials Provided

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

Storage Conditions

Cells must arrive in a frozen state on dry ice and should be transferred to either liquid nitrogen storage or a –80°C freezer **immediately upon arrival.** If cells will be thawed and used within 24 hours, they can be stored temporarily at –80°C. For longer storage, place vials in the **vapor phase** of liquid nitrogen storage.

Additional Materials Needed

Refer to the cell line-specific datasheet to identify catalog numbers for required media and reagents for the specific PathHunter EA parental cell line being used.

Required Materials	
Description	**Ordering Information**
Only one Detection Kit* is required: PathHunter Detection Kit PathHunter Flash Detection Kit	 93-0001 Series**** 93-0247 Series
AssayComplete™ Thawing Reagent	Refer to cell line-specific datasheet
AssayComplete Cell Culture Kit	Refer to cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Freezing Reagent	Refer to cell line-specific datasheet
At least one ProLabel® or ProLink™ Vector ** is required:	
• pCMV-ProLabel N Vector	93-0009
• pCMV-ProLabel C Vector	93-0012
• pCMV-ProLink 1 Vector	93-0167
• pCMV-ProLink 2 Vector	93-0171
• pCMV-ARMS1-ProLink 2 Vector***	93-0489
• pCMV-ARMS2-ProLink 2 Vector ***	93-0490
Optional bundles:	
• ProLink Cloning Vector Bundle (4 Pack)	93-0491
• ProLabel Cloning Vector Bundle (2 Pack)	93-1109

* A detection kit is required for PathHunter EFC cell-based assays. Refer to the cell line-specific datasheet for the required detection kit. In general, we recommend using the PathHunter Detection Kit (Cat. No. 93-0001 Series) for initial experiments. If testing with this kit does not reveal a satisfactory response, the PathHunter Flash Detection Kit (Cat. No. 93-0247 Series) should be evaluated – it may lead to more robust results

** An enhanced ProLabel (ePL) or ProLink (PK) vector, also called the enzyme donor (ED), is required to build a plasmid containing the ePL/PK-tagged target protein (e.g. GPCR-PK or NHR-ePL, etc.) of interest for engineering the desired cell-based assay. Refer to the Supplemental Information section and the ProLabel/ProLink Vectors User Manual (Document No. 70-385) and datasheets for guidance.

*** The ARMS-ProLink2 vectors are specific for β-arrestin recruitment assays only and are required for GPCRs that do not naturally recruit β-arrestin. Additional information for these vectors is available in the ProLink vector datasheet.

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

Material	Ordering Information
AssayComplete™ Hygromycin B	92-0029
AssayComplete G418	92-0030
PathHunter Anti-EA Antibody	93-0246
PathHunter PL/PK Detection Kit (For Optional Total EFC Detection of PK-tagged Target Protein)	93-0812S
PathHunter EA Cell Line Confirmation kit (For Optional Total EFC Detection of EA-tagged Reporter Protein)	93-1096S
pCMV-CCR5-PK Expression Vector as a Control Vector	93-0224V
Recombinant Human CCL3 as a Control Ligand	92-1002
Serial Dilution and Assay Plates	discoverx.com/microplates
Ligands	discoverx.com/ligands
Tissue Culture Disposables and Plastic-Ware (10 cm and 6-well Cell Culture Dishes, Polypropylene Conical Tubes [15 or 50 mL], T75 Flasks)	
Cryogenic Vials for Freezing Cells	
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	
Single and Multichannel Micro-Pipettors and Pipette Tips (10 µL to 100 µL)	
Multimode or Luminescence Plate Reader	discoverx.com/instrument-compatibility
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094
Hemocytometer or Other Cell Counting Device	
PBS (Dulbecco's Phosphate Buffered Saline, 1X without Calcium and Magnesium	Corning Cat. No. 21-031-CM or similar

Unpacking Cell Cryovials

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

1. Cells must arrive in a frozen state on dry ice.

Contact technical support immediately, if cells are thawed upon arrival.

2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.

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

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

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

4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line-specific datasheet for appropriate AssayComplete products mentioned in the protocol below.

Cell Culture Protocol

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

Care should be taken while handling cells to avoid contamination.

Cell Thawing

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

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.
2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask in a sterile tissue culture hood. Set aside for Step 6 below. DO NOT add selection antibiotics to the thawing reagent.
3. Remove the cell cryovials from -80°C or liquid nitrogen storage, and immediately place them on dry ice prior to thawing.

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

4. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.
5. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.

6. With a pipette, gently transfer the thawed cells to the pre-filled T75 flask and incubate at 37°C and 5% CO_2.

7. Maintain the cells in culture until they are >70-80% confluent. Then proceed to "Cell Propagation" instructions. Do not split if cells are below this confluency, or growth issues may occur.

Cell Propagation

The following is a protocol for propagating cells once they become >70-80% confluent in a T75 flask.

1. Pre-warm AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.

2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.

3. Gently aspirate media from the T75 flask.

4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire surface of the flask to rinse the cells.

5. Gently aspirate PBS from flask.

6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.

7. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with trypsin.

8. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.

9. Remove the flask from the incubator and confirm that the cells have detached by viewing them under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells have not detached, return the flask to the incubator for an additional 1 to 2 minutes, and repeat this step until all cells are in suspension.

Prolonged treatment with Trypsin- EDTA may compromise cell viability.

10. Add 4 mL of AssayComplete Thawing Reagent to the T75 flask.

11. Using a pipette, gently rinse the cells from the surface of the flask with the added media.

12. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK 293	1:3
U2OS	1:2

For example, for CHO-K1 cells, add 4 mL of AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5th of the total reagent in the flask) into each new tissue culture flask.

13. Fill a new T75 or T225 flask with AssayComplete Thawing Reagent until the total volume equals 12 mL for T75 flasks (or 45 mL for T225 flasks), and add cell suspension (added volume determined at Step 12) to the media in the flask. Transfer flask to a tissue culture incubator and incubate cells for 24 hours at 37°C and 5% CO_2. To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

14. After 24 hours, examine the cells under a microscope. If the cells appear to be healthy (adhering to the surface of the flask with only a small number of cells remaining in suspension) exchange the AssayComplete™ Thawing Reagent with 12 mL for T75 flasks (or 45 mL for T225 flasks) cell culture media supplemented with the recommended concentration of selection antibiotic. Refer to cell line-specific datasheet to determine the correct Cell Culture Kit and the recommended antibiotic and antibiotic concentration for your cell line.

The cell culture media comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic.

15. Return the flask to a tissue culture incubator. If the cells do not appear to be healthy or if confluency is <25%, incubate for additional 24 to 48 hours to allow for additional cell recovery before splitting cells.

16. Once the cells become ≥70% confluent in the flask, split the cells every 2 to 3 days, based on the doubling time of the cell line, using cell culture media containing appropriate selection antibiotics. Typical split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:10
HEK 293	1:5
U2OS	1:3

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 1 x 10^6 per vial).

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.
2. Gently aspirate the media from the flasks.
3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).
6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.
7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
9. Add 5 mL cell culture media containing appropriate selection antibiotic, to each T75 flask (or 15 mL to each T225 flask).

Care should be taken in handling to avoid contamination.

Keep cells on ice during this process and transfer to a cryogenic container.

10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.

11. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional cell culture media containing appropriate selection antibiotic, to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.

12. For the purpose of determining the concentration of cells in the suspension:

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.

    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.

13. Centrifuge the collected cells at 300 X g for 4 minutes.

14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.

15. Based on the total cell number calculated in Step 12 above, re-suspend cells to the desired concentration (e.g. 1 x 10^6 to 2 x 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent.

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

17. Freeze cells in a –80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

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

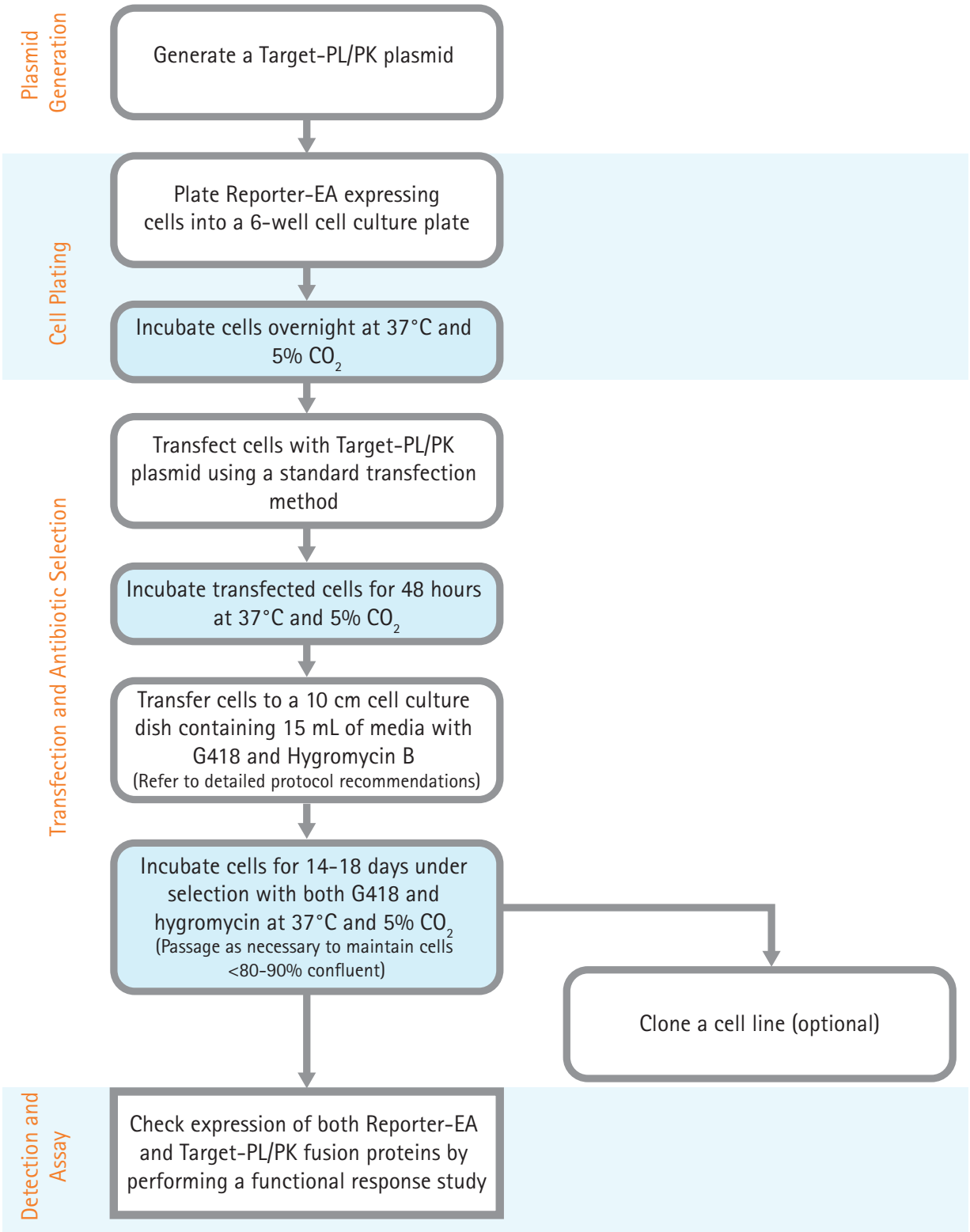
Protocol Schematic

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

Assay Name: _____ Date: _____

Product Details: _____

Quick-Start Procedure:



Detailed Protocol Generating an EFC Cell-Based Assay

The following section provides detailed steps for the generation of an EFC cell-based assay using an EA parental cell line containing a reporter protein (e.g. β-Arrestin-EA, SH2-EA, ENDO-EA, NUC-EA, and MEM-EA). The steps include guidelines for generating a PL/PK-tagged target protein (Target-PL/PK) plasmid, and transfecting that plasmid into an EA reporter protein (Reporter-EA) parental cell line, with the ultimate goal of generating a double stable cell line co-expressing both the Target-PL/PK and Reporter-EA fusion proteins. These double stable cells may be used for translocation, SH2 recruitment, or β-arrestin recruitment assays.

Section I: Plasmid Generation

For your EFC cell-based assay, you will ultimately need to create a stable cell line expressing Target-PK and Reporter EA fusion proteins. Thus, for the first section you will need to build a Target-PL/PK plasmid using a ProLink™ (PK) or enhanced ProLabel® (ePL) cloning vector.

For receptor kinase or GPCR applications, please refer to the tables in the Supplemental Information section for recommendations on which PK expression vector to use for many liganded and ortholog GPCR targets or receptor kinases. Alternatively, you may choose to test all 4 available PK tags indicated in the table shown below to empirically determine which provides the best S:B ratio. Note that PK1 is the most commonly used ProLink tag for DiscoverX β-arrestin2 cell lines, as seen in the Supplemental Information section tables. If your GPCR of interest is not indicated, please contact our support team for advice.

Vector Cloning Information

pCMV-ProLink and ProLabel vectors contain the enzyme donor (ED) PK or PL tag, a small 4-kDa fragment of β-gal for complementing the EA fragment. All vectors utilize a CMV promoter for maximal expression in mammalian cells, and result in fusion proteins that contain the ED tag. Several variations of the ED tag are available which provide additional options for the user and extend the system's versatility for assay development. The binding affinity of PK1, PK2 and ePL tags for the EA fragment increase in the order written. PK1 and PK2 are typically used for strong and weak protein-protein interactions, respectively, while ePL (or sometimes PK2) is typically used for assays of nuclear translocation. ePL is also used for assays of protein stability and turnover. For SH2 recruitment mediated by RTKs or CTKs, we recommend using the PK1 vector. For GPCR β-arrestin2 recruitment assays, the addition of Arrestin Recruitment Modulating Sequences, ARMS1 and ARMS2, may improve interaction with β-Arrestin2 thus improving S:B in the PathHunter assays. For intracellular proteins, the location of the ED tag fusion in relation to the folded target protein or membrane (e.g. amino-versus carboxyl-terminal fusion) can affect the performance of the assay. Please refer to the ProLabel and ProLink Mammalian Cloning Vectors User Manual (Document No. 70-385) for a comprehensive discussion on the choice and placement of ED tags and considerations for cloning. For SH2 recruitment

ProLink and ProLabel Vectors			
Vector Name	**Cat. No.**	**Vector Features**	**Application**
pCMV-ProLink 1	93-0167	Low affinity PK1 tag	Standard protein:protein interaction assays
pCMV-ProLink 2	93-0171	High affinity PK2 tag	Weak protein:protein interactions
pCMV-ARMS1-ProLink 2	93-0489	High affinity PK2 tag; ARMS1* sequence	Improve signal-to-background (S:B) ratios by addition of an ARMS1 sequence
pCMV-ARMS2-ProLink 2	93-0490	High affinity PK2 tag; ARMS2 sequence	Improve S:B ratios by addition of ARMS2
pCMV-ProLabel	93-0009 93-0012	High affinity enhanced PL tag	Nuclear translocation

* ARMS (Arrestin Recruitment Modulating Sequence) is an 18-21 amino acid sequence between the GPCR and the PK tag that has been shown to enhance β-Arrestin recruitment thus improving S:B in the PathHunter assays. ARMS1 and ARMS2 are different variants of the 18-21 amino acid sequence.

assays mediated by CTK or RTK heterodimers, the CTK or one of the RTKs, respectively, may be (and in most cases should be) expressed without a PK tag using a standard mammalian expression vector of your choice (with a selection marker other than hygromycin or G418 which are already used by EA and ED proteins). It will be important to confirm overexpression of the non-PK tagged protein (by flow cytometry, immunoblot, activity, or other means), and it may be useful to epitope-tag this protein (in a manner that does not compromise its function) for this purpose. Where needed, introduction of this untagged co-receptor or CTK may be done simultaneously, before, or after introducing the PK-tagged protein.

Using the pCMV-CCR5-PK Vector (Optional for β-Arrestin2 Assays)

The pCMV-CCR5-PK Expression Vector (Cat. No. 93-0224V) is recommended to be used as a positive control plasmid for transfecting and assaying your engineered β-arrestin2-EA parental cell line. The plasmid encodes human Chemokine (C-C motif) Receptor 5 (CCR5; BC038398.1) with the PK sequence fused to the C-terminus of the protein. The product comes with 10 µg of pCMV-CCR5-PK plasmid DNA and the datasheet includes vector map. To propagate, transform plasmid DNA into *E. coli* cells using 50 mg/mL Kanamycin as a selection marker.

If transfecting pCMV-CCR5-PK as a control ligand for functionally testing the co-expression of β-arrestin2-EA and pCMV-CCR5-PK, we recommend using Recombinant Human CCL3 (MIP-1α) (Cat. No. 92-1002). Refer to the ligand datasheet for additional details regarding this control agonist.

Section II: Cell Plating

For adherent cells, prepare the EA parental cells for transfection with a Target-PL/PK plasmid. For suspension cells, proceed to Section III.

1. Plate 100,000 to 400,000 EA parental cells into a 6-well cell culture plate.
2. Incubate cells overnight at 37°C and 5% CO_2.

Section III: Transfection and Antibiotic Selection

This section goes over the process for introducing your Target-PL/PK plasmid followed by antibiotic selection. Successful and expedient generation of a stable cell line with a pCMV-Target-PL/PK plasmid will require efficient delivery of the plasmid to user's cells of interest. Different methods of gene delivery may be considered including transfection and nucleofection*. Refer to the manufacturer's recommended protocol for detailed instructions on the handling and use of the preferred DNA transfection reagent or nucleofection protocol. In this section, we will assume the use of a transfection reagent. For GPCRs, we suggest optionally using pCMV-CCR5-PK Expression Vector (Cat. No. 93-0224V) as a positive control.

1. Prepare the transfection mix with the pCMV-Target-PL/PK plasmid and transfect cells in a 6-well plate according to transfection reagent manufacturer's recommended protocol.
2. Incubate transfected cells for 48 hours at 37°C and 5% CO_2.

3. Transfer cells to a 10 cm plate containing 15 mL (final volume) of medium used to propagate the EA parental cell line being used and supplemented with G418 (Cat. No. 92-0030), in order to select for cells expressing the Target-PL/PK construct while maintaining selection for the Reporter-EA protein. Refer to the table to the right for recommended antibiotic concentrations for the EA parental cell lines.

Recommended Final Antibiotic Concentrations		
Parental Cell Line	G418 (µg/mL)	Hygromycin B (µg/mL)
U2OS	500	250
HEK 293	800	200
CHO-K1	800	300

Hygromycin is required for maintaining the selection for the Reporter-EA protein.

4. Incubate cells for an additional 14 to 18 days under antibiotic selection with both G418 and Hygromycin B at 37°C and 5% CO_2. Cells should be split as needed to maintain below 80-90% confluency.

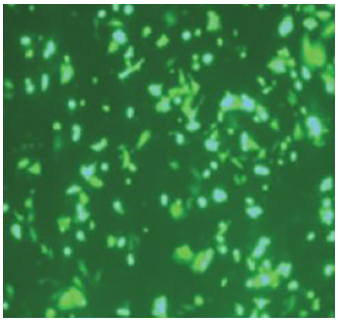
5. After at least 7-10 days and as soon as cells reach 80-90% confluency, cells should be expressing both the Reporter-EA and Target-PK fusion proteins in a stable manner.

6. At this point, we recommend to aliquot several vials of 1 x 10^6 to 2 x 10^6 cells of the transduced pool to freeze in liquid nitrogen for backup and/or later use (please refer to the Cell Freezing Protocol in this user manual).

7. This stable pool or a clonal cell line may be used for experiments. Refer to the Supplemental Information section for the protocol Generation of a Stable Clone from a Stable Cell Pool.

Determine Transfection Efficiency (Optional)

To determine the percentage of cells that are transfected with DNA, a reporter gene is often used. This step would typically be performed in parallel (in a separate well) with your PL/PK-tagged protein transfection. One convenient reporter for monitoring transfection efficiency is the Enhanced Green Fluorescent Protein (EGFP). When excited by UV light, the EGFP protein emits a green fluorescent signal that can be readily detected by microscopy or flow cytometry. The ratio of fluorescent versus non-fluorescent cells determines the overall efficiency of the transfection experiment and can be used to estimate the number of cells that would be expressing your protein of interest.



Example transfection using the PathHunter GPCR Explorer Kit (Cat. No. 93-0164E2V2). The highly transfectable PathHunter CHO-K1 EA-Parental Explorer cells were plated at 10,000 cells/well in CP2 reagent for 24 hours and transfected with 2 µg of pEGFP vector. 24 hours post-transfection, transfection efficiency was determined to be greater than 60% based on EGFP expression.

Section IV: Assay and Detection

There are several methods available for evaluating expression levels of the Target-PL/PK and Reporter-EA fusion proteins. These include functional assays (e.g. testing for functional activity using a relevant ligand), immunoassays (e.g. Western blots, ELISAs, or flow cytometry with anti-tag antibodies), and total EFC detection assays (e.g. with and without excess exogenous EA or ED). Functional assays are strongly recommended for validating that the cell line is capable of functionally responding to a validated ligand (e.g. an agonist). The other aforementioned assays are useful for characterizing expression of specific fusion protein components (refer to Confirmation of Fusion Proteins by Total EFC Detection Assay in the Supplemental Information section), but insufficient for functional characterization of the assay cell line.

Functional assays may be tested with a full dose response curve or with a single high concentration of ligand versus a buffer only control. For details on evaluating the expression levels of the Reporter-EA and Target-PL/PK fusion proteins by performing an application specific functional assay, please refer to separate User Manuals indicated in the table below that are available on the DiscoverX website.

*Nucleofection, also referred to as Nucleofector™ Technology, is trademarked by Lonza.

Application Specific Protocols for Translocation, SH2 Recruitment, and β-Arrestin Recruitment	
Application	**User Manual***
β-Arrestin recruitment	PathHunter β-Arrestin Assay for GPCR Cell Lines
Translocation (protein trafficking of GPCRs, Ion Channels, or other plasma membrane protein from ER to plasma membrane or from plasma membrane to early endosomes)	PathHunter Pharmacotrafficking Assays
Translocation (internalization of GPCRs)	PathHunter Total GPCR Internalization Assay
Translocation (internalization of RTKs)	PathHunter Receptor Tyrosine Kinase Internalization Assays
Translocation of nuclear hormone receptors	PathHunter NHR Assay
SH2 recruitment mediated by RTKs	PathHunter Receptor Tyrosine Kinase Functional Assay
SH2 recruitment mediated by CTKs	PathHunter Cytosolic Tyrosine Kinase Functional Assay

* User Manuals: discoverx.com/tools-resources/document-resource-library/#manuals-tab

A detection reagent is required for all EFC assays and indicated in the cell line-specific datasheets. The vast majority of our assays are performed using the PathHunter Detection Kit (Cat. No. 93-0001), so this is the detection technology recommended to start with for most β-arrestin and translocation assays. PathHunter Flash Detection Kit (Cat. No. 93-0247) allows for more sensitivity and may be used where the assay window is low (particularly where expression of the EA and PL/PK tagged proteins or their complementation is unusually low). Recommendations for detection kits for specific applications can be found in the above listed application specific protocols.

Assay Optimization (As Needed)

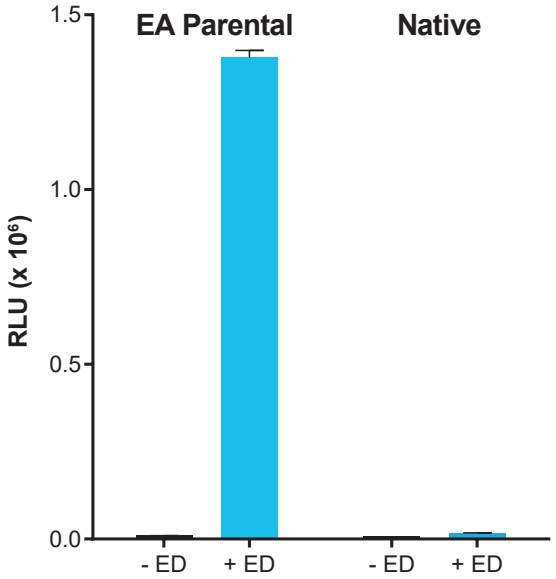
While the initial assay conditions attempted may result in an acceptable assay, often assay performance will improve due to experimentally optimizing one or more assay parameters. The table below indicates several suggested assay parameters and conditions to consider during assay optimization.

Assay Parameter	Starting Condition	Alternate Condition(s)
Cell Number	5000 cells/384-well (10K cells/96-well)	Titrate cell number for optimizing the assay window
Cell Plating Reagent	For CHO-K1, U2OS, and HEK 293 cells refer to a specific cell plating reagent below*	An appropriate base media with reduced concentrations of fetal bovine serum (or dialyzed serum, charcoal-dextran stripped serum, alternate serum species/sources, etc)
Ligand Incubation Time	90 minutes	Try a range of 2 to 6 hours
Ligand Incubation Temperature	37˚C	25˚C
Ligand Diluent	PBS and 0.1% BSA	An appropriate base media or buffer plus 1% FBS

* AssayComplete™ Cell Plating 2 Reagent (Cat. No. 93-0563R2) for CHO-K1; AssayComplete Cell Plating 5 Reagent (Cat. No. 93-0563R5) for U2OS; and AssayComplete Cell Plating 7 Reagent (Cat. No. 93-0563R7) for HEK 293 cells.

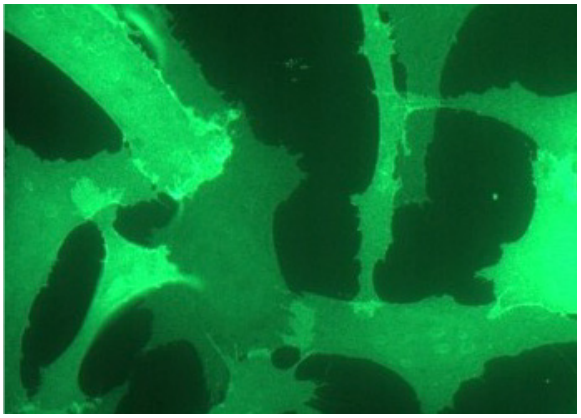
Typical Results

Total EFC Detection Data



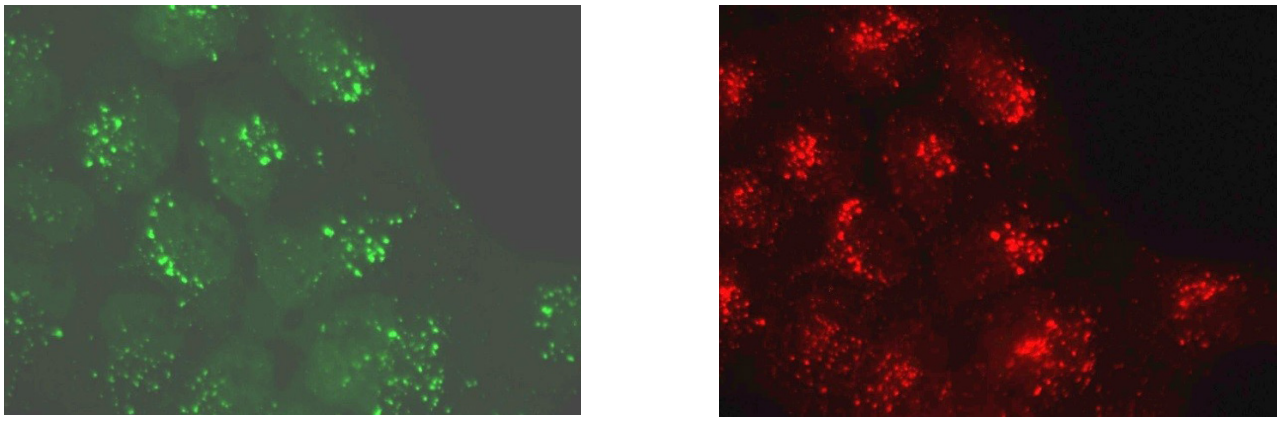
PathHunter CHO-K1 β-Arrestin2-EA Parental Cells or native cells were seeded in a 384-well plate and incubated overnight at 37°C and 5% CO_2. Following cell lysis in the absence (left bar) and presence (right bar) of excess Enzyme Donor (ED), luminescence signal was detected using the PathHunter Detection Kit (Cat. No. 93-0001) according to the Total EFC Detection protocol. Data are plotted as RLU in millions (mean ± standard deviation) and show that luminescence is detected with the EA Parental cell in the presence of ED, but not for the native cells.

Localization Data for MEM-EA

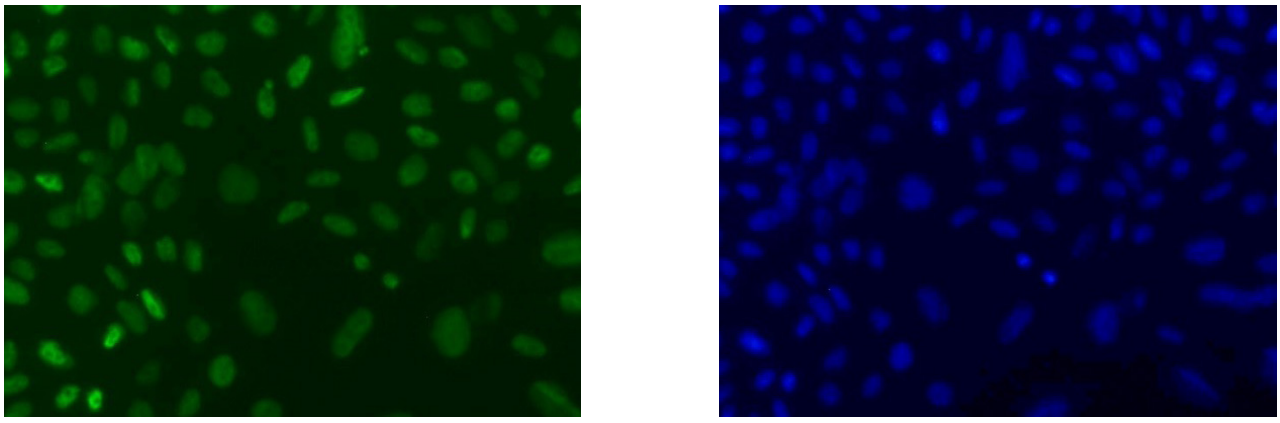


Plasma membrane localization of the MEM-EA reporter protein in U2OS cells revealed by immunofluorescence with anti-EA antibody.

Localization Data for ENDO-EA and NUC-EA

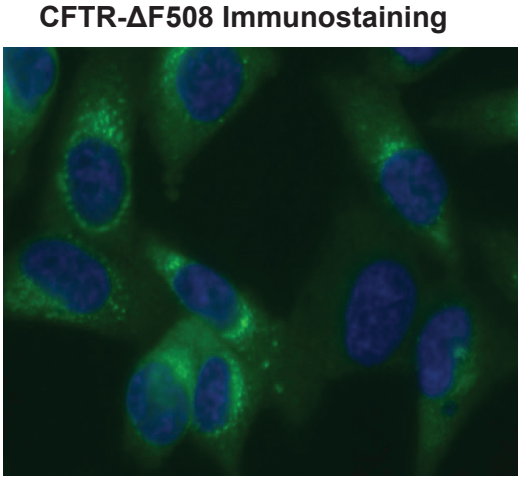


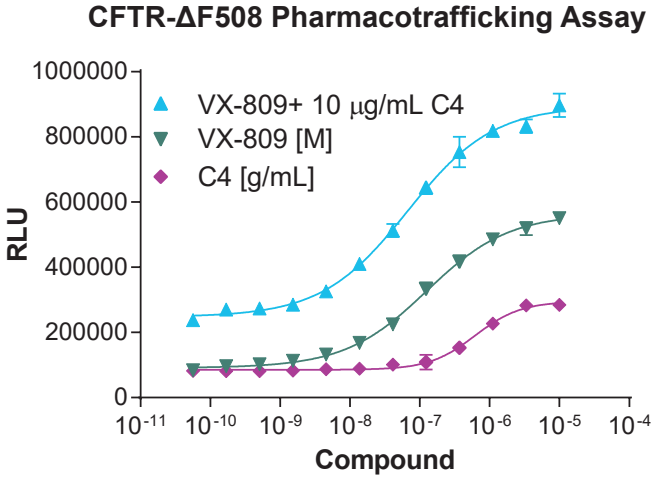
U2OS ENDO-EA Parental cells were stained with anti-EA (green; left panel) and anti-EEA1 (red; right panel). EEA1 is early endosome antigen 1 localized to endosomes (Note: the epitopes recognized by anti-EEA1 antibody used (c45b10; Cell Signaling Technologies) are not present in the ENDO-EA protein sequence). The co-localization of green and red staining in the cells reveals that the ENDO-EA protein is expressed in early endosomes.



Expression of the NUC-EA reporter protein in nucleus as evidenced by co-localization of anti-EA staining (green; left) and the commonly used nucleic acid stain, DAPI (blue; right) in CHO-K1 NUC-EA cells.

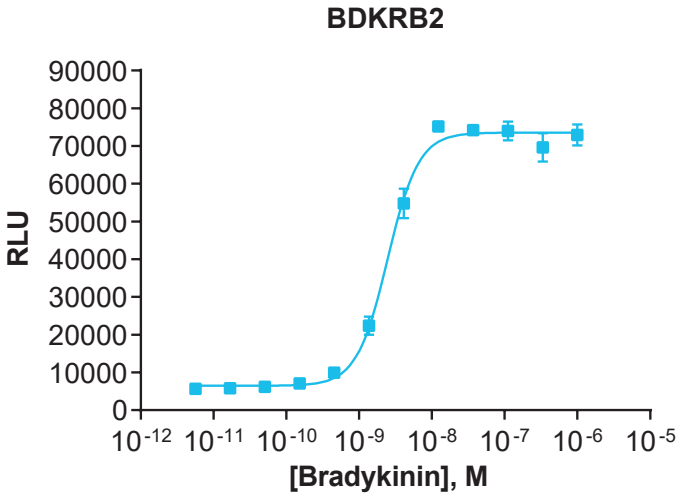
Localization and Assay Data for MEM-EA Using a PathHunter Pharmacotrafficking Assay





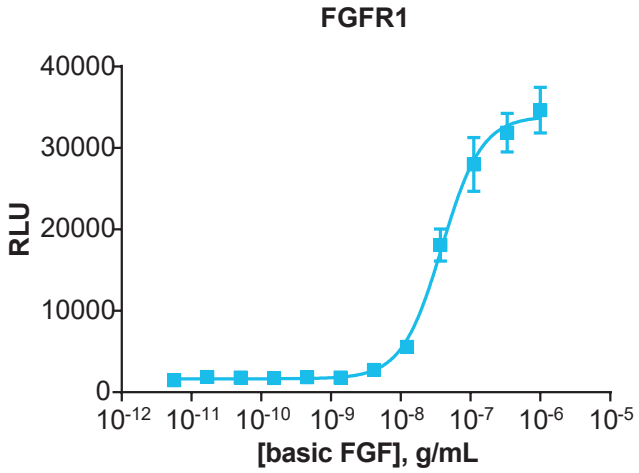
Analysis of a mutant form of the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) containing a single point deletion CFTR-ΔF508 was conducted. This common deletion in cystic fibrosis patients causes the protein to misfold, thus preventing efficient trafficking and leading to ER retention (immunofluorescence image, left). Using the PathHunter CFTR-ΔF508 Pharmacotrafficking assay (created using the U2OS MEM-EA parental cell line and CFTR-ΔF508-PK1 plasmid vector) with a combination of 2 corrector (or pharmacochaperone) compounds, C4 and VX-809, stabilizes the mutant receptor, allowing for proper trafficking. Dual treatment results in elevated signal (right, red curve) indicating an additive effect, which is the expected behavior of the combination of the two compounds.

β-Arrestin Recruitment Assay Data



A stable pool of PathHunter HEK 293 β-Arrestin2-EA parental cells expressing a PK-tagged receptor (BDKRB2) were plated in a multi-well plate and stimulated with a control agonist (bradykinin), using the following assay conditions: AssayComplete™ Cell Plating 0 Reagent with a cell incubation time of 24 hours and agonist incubation time of 90 minutes at 37°C. Results gave an EC_{50} of 2.5 nM and large S:B of 11.

SH2 Recruitment Kinase Receptor Assay Data



FGFR1-PK was stably co-expressed in the PathHunter PLCG1(SH2)-EA parental cells allowing creation of a functional assay for basic FGF. Basic FGF (5.6 x 10^{-12} to 1 x 10^{-6} g/mL) was added to 10K FGFR1-PK PLCG1-EA cells per well in a 384-well plate for 16 hours at 37°C, followed by addition of PathHunter Detection Reagent for 1 hour, and reading luminescence on a standard laboratory plate reader. The signal to background and EC_{50} for basic FGF in FGFR1-PK PLCG1-EA cells were 23.4 and 8.6 nM, respectively.

Supplemental Information

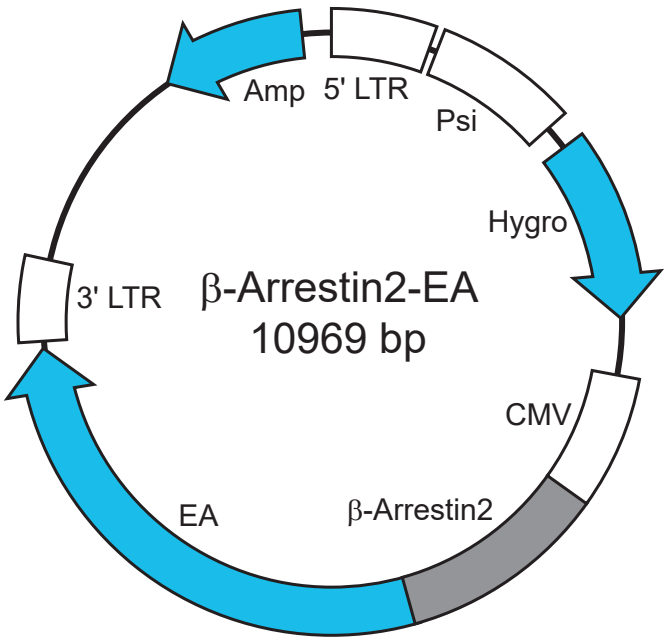
GPCR ProLink™ Vector Guide Use the following tables to determine the recommended PK vector for your GPCR of interest. For orphan GPCRs, we recommend using PK1 vector. Note ARMS2-PK2 vectors can also be used for the GPCR targets that recommend the ARMS1-PK1 vector.

Human GPCR ProLink Vector Guide

GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag
ADCYAP1R1	PK1	CHRM5	ARMS2-PK2	GLP2R	PK1	NTSR1	PK1
ADORA1	PK1	CMKLR1	PK1	GPR1	PK1	OPRD1	PK1
ADORA2B	PK1	CMKOR1	PK1	GPR109A	PK1	OPRK1	PK1
ADORA3	PK1	CNR1	PK1	GPR119	ARMS2-PK2	OPRL1	PK1
ADORA3	ARMS2-PK2	CNR2	PK1	GPR120	PK1	OPRM1	PK1
ADRA1B	PK1	CRHR1	PK1	GPR35	ARMS2-PK2	OXTR	PK1
ADRA2A	ARMS2-PK2	CRHR2	PK1	GPR55	PK1	P2RY11	PK1
ADRA2B	ARMS2-PK2	CRTH2	PK1	GPR81	PK1	P2RY12	PK1
ADRA2C	ARMS2-PK2	CX3CR1	PK1	GPR84	PK1	P2RY2	PK1
ADRB1	PK1	CXCR1	PK1	GPR91	PK1	P2RY4	PK1
ADRB2	PK1	CXCR2	PK1	GPR92	PK1	P2RY6	ARMS2-PK2
AGTR1	PK1	CXCR3	PK1	GRPR	PK1	PPYR1 (NPY4)	PK1
AGTRL1	PK1	CXCR4	PK1	HCRTR1	PK1	PRLHR	PK1
AVPR1A	PK1	CXCR5	ARMS1-PK2	HCRTR2	PK1	PROKR1	PK1
AVPR1b	PK1	CXCR6	PK1	HRH1	ARMS2-PK2	PROKR2	PK1
AVPR2	PK1	DRD1	PK1	HRH2	PK1	PTAFR	PK1
BDKRB1	ARMS2-PK2	DRD2L	ARMS2-PK2	HRH3	ARMS2-PK2	PTGER2	PK1
BDKRB2	PK1	DRD2S	ARMS2-PK2	HRH4	PK1	PTGER3	ARMS2-PK2
BRS3	ARMS2-PK2	DRD3	ARMS2-PK2	HTR1A	ARMS2-PK2	PTGER3	ARMS2-PK2
C5AR1	PK1	DRD4	ARMS2-PK2	HTR1B	ARMS1-PK1	PTGER4	PK1
C5L2	PK1	DRD5	PK1	HTR1E	ARMS2-PK2	PTGIR	PK1
CALCR	PK1	EDG1	PK1	HTR1F	ARMS2-PK2	PTHR1	PK1
CALCR + RAMP2	PK1	EDG2	PK1	HTR2C	PK1	PTHR2	PK1
CALCR + RAMP3	PK1	EDG3	PK1	HTR5A	ARMS2-PK2	SALPR	ARMS1-PK2
CALCRL + RAMP1	PK1	EDG4	PK1	HTR6	PK1	SCTR	PK1
CALCRL + RAMP2	PK1	EDG5	PK1	KISS1R	PK1	SSTR1	PK1
CALCRL	PK1	EDG6	PK1	LHCGR	PK2	SSTR2	PK1
CCKAR	PK1	EDG7	PK1	LTB4R	PK1	SSTR3	PK1
CCKBR	PK1	EDG8	PK1	MC1R	ARMS1-PK1	SSTR4	ARMS2-PK2
CCR1	PK1	EDNRA	PK1	MC3R	PK2	SSTR5	PK1
CCR10	PK1	EDNRB	PK1	MC4R	PK1	TACR1	PK1
CCR2	PK1	F2R	PK1	MC5R	PK1	TACR2	PK1
CCR3	PK1	F2RL1	PK1	MCHR1	PK1	TACR3	PK1
CCR4	PK1	F2RL3	PK1	MCHR2	ARMS2-PK2	TBXA2R	PK1
CCR5	PK1	FPR1	PK1	MLNR	PK1	TRHR	PK1
CCR6	PK1	FPRL1	PK1	MRGPRX2	PK1	TSHR	PK1
CCR7	PK1	FSHR	ARMS1-PK2	MTNR1A	ARMS1-PK2	UTR2	PK1
CCR8	PK1	GALR1	PK1	MTNR1B	ARMS2-PK2	VIPR1	PK1
CCR9	PK1	GALR2	PK1	NMU1R	PK1	VIPR2	PK1
CHRM1	ARMS1-PK1	GCGR	PK1	NPBWR1	ARMS2-PK2	XCR1	PK2
CHRM2	PK1	GHSR1a	PK1	NPBWR2	ARMS2-PK2		
CHRM3	ARMS1-PK1	GIPR	PK1	NPY1R	PK1		
CHRM4	PK1	GLP1R	PK1	NPY2R	PK1		

Ortholog GPCR ProLink Vector Guide							
GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag
cEDG5	PK1	mCCR8	PK1	mEDNRA	PK1	mOXTR1	PK1
mADCYAP1R1	PK1	mCCR9	PK1	mEDNRB	PK2	mP2RY6	ARMS2-PK2
mADCYAP1R1	PK1	mCMKLR1	PK1	mFPR1	PK1	mP2RY12	PK1
mADORA2B	PK1	mCNR1	PK1	mGALR2	PK2	mPPYR1 (mNPY4R)	ARMS2-PK2
mADORA3	PK1	mCNR2	PK1	mGHSR1a	PK1	mPTAFR	PK1
mAGTRL1	PK1	mCRHR1	PK1	mGHSR1a	PK1	mPTAFR	PK1
mBRS3	PK1	mCRTH2	PK1	mGLP1R	PK1	mSSTR2	PK1
mC5AR1	PK1	mCXCR2 (IL8RB)	PK1	mGPR1	PK1	mSSTR5	PK1
mCCKAR	PK1	mCXCR3	PK1	mHTR2A	PK1	mUTR2	PK1
mCCR1	PK1	mCXCR4	PK1	mKISS1R	PK1	mVIPR1	PK1
mCCR2	PK1	mCXCR5	PK1	mLTB4R1	PK1	rCRTH2	PK1
mCCR3	PK1	mCXCR6	PK1	mMCHR1	PK1	rEDG5	PK1
mCCR4	PK1	mCXCR7	PK1	mNPY1R	PK1	rOPRM1	PK1
mCCR5	PK1	mDRD5	PK1	mNPY2R	PK1	rPROKR1	PK1
mCCR6	PK1	mEDG1	PK1	mOPRD1	PK1	rPROKR2	PK1
mCCR6	PK1	mEDG5	PK1	mOPRK1	PK1	sEDG5	PK1
mCCR7	PK1	mEDG6	PK1	mOPRM1	PK1		

β-Arrestin2-EA Vector Map



SH2 Recruitment Parental Cell Line Guide

Use the following table to determine the recommended SH2 recruitment parental cell line (e.g. SHC1(SH2)-EA or PLCG1(SH2)-EA) for your receptor/kinase of interest. If your receptor is not shown in the table, we suggest trying both SH2 recruitment parental cell lines to see which one will provide a meaningful functional assay for the receptor/kinase that you are studying. Please keep in mind that you will need to introduce the receptor of interest with a PK1 tag, and possibly also introduce an unlabelled co-receptor.

RTK and CTK SH2 Recruitment Parental Cell Line Guide					
Target	Receptor Kinase	Parental Cell Line	Target	Receptor Kinase	Parental Cell Line
DDR1	RTK	SHC1	INSRb	RTK	PLCG1
EphA5	RTK	PLCG1	cINSRa	RTK	PLCG1
EphA7	RTK	PLCG1	cINSRb	RTK	PLCG1
EphB1	RTK	SHC1	mINSRa	RTK	PLCG1
EphB2	RTK	SHC1	mINSRb	RTK	PLCG1
EphB3	RTK	SHC1	PDGFRa	RTK	PLCG1
EphB4	RTK	SHC1	PDGFRb	RTK	PLCG1
ErbB1	RTK	SHC1	PRLR-JAK2	CTK	SHC1
ErbB4	RTK	SHC1	rhTrkA-rhP75	RTK	SHC1
FGFR1	RTK	PLCG1	rTrkA-rP75	RTK	SHC1
FGFR2	RTK	PLCG1	Tie2	RTK	SHC1
FGFR4	RTK	PLCG1	TrkA	RTK	SHC1
FGFR4-β-Klotho	RTK	PLCG1	TrkA-P75	RTK	SHC1
c-Ret-GFRα1	RTK	SHC1	TrkB	RTK	SHC1
c-Ret-GFRα2	RTK	SHC1	TrkB-P75	RTK	SHC1
GHR-JAK1	CTK	PLCG1	TrkC	RTK	SHC1
IGF1R	RTK	SHC1	TrkC-P75	RTK	SHC1
INSRa	RTK	PLCG1			

Generation of a Stable Clone from a Stable Cell Pool (optional)

While working with a double stable pool expressing both β-Arrestin2-EA and GPCR-PK (or other PL/PK-tagged protein) fusion proteins is an option, generating a stable clonal cell line may be desirable for 3 reasons: 1) obtaining preferred response (e.g. a large assay window S:B), 2) more homogeneous expression levels of both β-Arrestin2-EA and GPCR-PK fusion proteins (and possibly more homogeneous responses) and 3) whose responses may be more stable over time.

If the desired response is achieved in the stable pool (e.g. S:B >2 and EC_{50} in expected range), the cell pool is a good candidate for clonal dilution. We recommend freezing down several vials of the original cell pool (at least 1 x 10^6 cells per vial) as a backup and 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 limiting dilution of your stably transfected cells.

1. Starting from a cell density of 200,000/mL, prepare serial 1:10 dilutions of cells in growth medium without antibiotics until a final concentration of 20 cells/mL is achieved.
2. Seed 50 µL of cells/well into 384-well tissue culture-treated plates.
3. Wrap plates in parafilm to slow evaporation, and place in 37°C and 5% CO_2 humidified incubator.
4. Allow cells to grow for ~6 to 8 days (for HEK 293, U2OS, and A549) and visually inspect wells to search for single colonies.
5. Mark the colonies and incubate another 6 to 9 days (e.g. 12 to 17 days total depending on doubling time of cells).
6. Once the cells reach confluency in the 384-well plate, lift cells and transfer into a 96-well plate.
7. Allow cells to reach confluency, then transfer to a 48-well plate, then a 24-well plate, followed by a 6-well plate.

During this process, each well contains a clonal population of stably transfected cells that can be maintained in normal growth medium. It is recommended to add selective antibiotics (e.g. Hygromycin B and G418) to maintain selective pressure on both constructs only after cells are transferred from 24-well to a 6-well dish.

8. Once cells reach 80% to 90% cell confluency in a 24-well plate, there should be sufficient cells available to evaluate functional response of clones to desired ligand.
9. Seed plates and perform initial screening experiments with ligand as indicated in Section IV (β-Arrestin Recruitment Assay and Detection).
    a. Do a single test with a small number of replicates with and without a high concentration of agonist.
    b. Select a subset of functionally positive "wells" (cells) and move to 6-well plate.
    c. Once these grow up under selection in 6-well plate, test cells for full dose response to agonist.
    d. Select final clone candidates based on dose response (e.g. correct potency and largest signal to background ratio).
10. Once clones with appropriate functional response are identified, it is recommended to freeze down sufficient vials (at least 4, at 1 x 10^6 cells per vial) to serve as an early passage from which a master working cell bank can be generated. We also highly recommend evaluating the stability of each clone over at least 10 passages. This can be done by evaluating expression of each construct by Western blot, and/or evaluating the functional response to ligand at multiple passages (e.g. at passage 2, 6, and 10).

Confirmation of Fusion Proteins by Total EFC Detection Assay (Optional)

Total EFC detection assays involve adding an excess of one EFC component (EA or ED) to lysed cells to fully complement the reciprocal EFC partner protein, and thereby allowing the determination of relative levels of expression. Total EFC detection assays may be useful for intermediary characterization, troubleshooting, or characterization in the absence of a well characterized functional ligand.

The following two optional protocols can be used to test for expression of Target-PL/PK and of Reporter-EA, respectively, in your cells. Note for full functional characterization of your assay cell line, we recommend running a functional assay to demonstrate the expression levels, inducible interaction, and EFC of the Target-PK and Reporter-EA fusion proteins.

Performing total EFC tests for Target-PL/PK and Reporter-EA require additional detection kits PathHunter PL/PK Detection Kit (Cat. No. 93-0812 Series) and PathHunter EA Cell Line Confirmation kit (Cat. No. 93-1096S), respectively.

A. Confirm Expression of the Target-PK Fusion Protein

Using the detection reagents in optional product PathHunter PL/PK Detection Kit (Cat. No. 93-0812 Series), perform the following assay to qualitatively assess Target-PL/PK expression.

1. Assay Plate Preparation
    a. Prepare a white wall, clear bottom 384-well tissue culture treated assay plate (Cat. No. 92-0013) as follows:
        i. Leave 8 wells empty (no cells) for testing the +ED assay positive control.
        ii. Add 2,500 Target-PL/PK expressing EA parental cells per well, in 20 µL cell culture medium to 8 replicate wells.
        iii. As another control (optional), add 2,500 EA parental cells (not expressing Target-PL/PK) per well in 20 µL cell culture medium to 8 replicate wells.
    b. Place the seeded plate in a 37°C and 5% CO_2 humidified incubator overnight, prior to detection.
2. Prepare Working Solutions and Controls
    a. Create two separate working solutions (one +EA and one –EA) with per well volumes as indicated in the table below.
    b. For the +ED positive control wells (see A.1.a.i), prepare 20 µL per well of a 1:4 diluted solution of ED (Positive Control Peptide) in PBS [5 µL/well ED and 15 µL/well PBS].

Create positive control and working solutions just prior to performing the detection assay.

Create enough of both working solution for all wells (volumes in table are per well).

To Detect	Working Solutions	EA Reagent (µL)	PBS (µL)	Cell Lysis Buffer (µL)	Substrate Reagent (µL)	Total per Well (µL)
Signal: Target-PK	+EA working solution	5	0	5	20	30
Background	-EA working solution	0	5	5	20	30

3. Reagent Additions and Detection

    a. For the +ED positive control wells (see A.1.a.i), add 20 µl of 1:4 diluted solution of ED (Positive Control Peptide) per well.

    b. Add 30 µL +EA working solution or -EA working solution, each to half of the wells for the following assay conditions to be tested:

        i. Presumed Target-PL/PK expressing EA Parental cells

        ii. EA Parental cells not expressing Target-PL/PK (optional)

        iii. +ED assay positive controls

    c. Incubate assay plate for 30 to 60 minutes at ambient temperature, in the dark, followed by reading the plate on a luminometer.

B. Confirm Expression of the Reporter-EA Fusion Protein

The expression of Reporter-EA in the supplied EA parental cells has already been determined at DiscoverX (see total EFC data on the datasheet for your parental cell line) and has been demonstrated to be stable for at least 10 passages. Performing this test for Reporter-EA expression is probably not essential, but may be used for confirmation and troubleshooting. To determine relative levels of Reporter-EA expression, use detection reagents in optional product PathHunter EA Cell Line Confirmation kit (Cat. No. 93-1096S), according to the following assay procedure.

1. Assay Plate Preparation

    a. Prepare a white wall/clear bottom 384-well tissue culture treated assay plate (Cat. No. 92-0013) as follows:

        i. Leave 8 wells empty (no cells) for testing the +EA assay positive control.

        ii. Add 2,500 Reporter-EA expressing cells per well, in 20 µL cell culture medium to 8 replicate wells.

        iii. As another control (optional), add 2,500 native cells (not expressing Reporter-EA) per well in 20 µL cell culture medium to 8 replicate wells. For example, if using U2OS β-Arrestin2-EA parental cells, native cells would be U2OS cells.

    b. Place the seeded plate in a 37°C and 5% CO_2 humidified incubator overnight, prior to detection.

2. Prepare Working Solutions and Controls

    a. Create two separate working solutions (one +ED and one –ED) with per well volumes as indicated in the table below.

    b. For the +EA positive control wells (wells without cells), add 20 µL of a 1:4 diluted solution of EA Positive Control (5 µL/well EA Positive Control and 15 µL/per well PBS).

Create positive control and working solutions just prior to performing the detection assay.

Create enough of both working solution for all wells (volumes in table are per well).

To Detect	Working Solutions	ED Reagent (µL)	PBS (µL)	Cell Lysis Buffer (µL)	Substrate Reagent (µL)	Total per Well (µL)
Background	-ED working solution	0	5	5	20	30
Signal: Reporter-EA	+ED working solution	5	0	5	20	30

3. Reagent Additions and Detection

   a. Add 30 µL +ED working solution or -ED working solution, each to half of the wells for the following assay conditions to be tested.

      i. Reporter-EA Parental expressing cells

      ii. Native cells not expressing Reporter-EA (optional)

      iii. +EA assay positive controls

   b. Incubate assay plate for 30 to 60 minutes at ambient temperature, in the dark, followed by reading the plate on a luminometer.

Frequently Asked Questions

What method is best to introduce my Target-PL/PK plasmid into the EA parental cells?

- Any standard method of gene delivery (such as electroporation, lipid transfection, or viral transduction) appropriate for the cell background (U2OS, CHO-K1, or HEK 293) is appropriate. Methods with higher efficiency are likely to allow more rapid generation of stable transfectants.

How much of my Target-PL/PK plasmid should I use to transfect the EA parental cells?

- _ Follow a standard method or the method recommended by the manufacturer of the chosen gene delivery method for the U2OS, CHO-K1, or HEK-293 cells.

After transfection and G418 selection, I am unable to detect any expression of my Target-PL/PK fusion protein (by functional response, by total EFC detection assay, or by immunoblotting).

- Ensure that the target protein insert contains a start codon and Kozak sequence, does not contain a stop codon, and that it was cloned in-frame with the 3' PL/PK-tag sequence.

After selection of a double transfected cell line, why is my assay signal:background (S:B) low?

- Isolation of clones from the cell pool often results in identification of a clone with better S:B and gives insight into the responses of clones making up the cell pool. Also, assay optimization of ligand incubation time, incubation temperature, and cell plating reagent (e.g. serum types and concentrations) can improve assay window (S:B).
- Assay window for some assays (e.g. those where expression of EA and/or PL/PK-tagged proteins are low or interaction of the two EFC components is low) may increase by using the more sensitive PathHunter Flash Detection Kit (Cat. No. 93-0247 Series) in place of the standard PathHunter Detection Kit (Cat. No. 93-0001 Series).

- If S:B is suboptimal, in addition to cloning from the existing pool, repeating pool generation with less or more of the PK-protein expressing plasmids being transfected may yield a pool (or a subsequent clone) with a preferable S:B. In the specific case where the background is high (thereby attenuating S:B), obtaining a pool or clone with lower expression of PK-tagged protein or, where relevant, the untagged co-receptor or CTK, may decrease the high background and improve the S:B. Typically, this is achieved empirically by screening and selecting for a pool with more optimal S:B (and appropriate pharmacology).

Can ENDO-EA and ENDO-MEM be used to study peripheral membrane proteins as well as membrane spanning proteins such as GPCRs?

- While we have not validated ENDO-EA and ENDO-MEM for use with cytoplasmic PK-tagged peripheral membrane proteins, in principle this should be feasible.

Can ENDO-EA and ENDO-MEM be used to study type II (N_{in}-C_{out}) membrane proteins?

- We have not validated ENDO-EA and ENDO-MEM for use with PK-tagged type II proteins. ENDO-EA and MEM-EA are both facing the cytosol and so must the PK-tag. Thus, any use of ENDO-EA and MEM-EA parentals for type II membrane proteins would require the PK-tag on the 5' end of the target protein. All of our ProLink vector have a 3' tag and thus are incompatible with type II membrane proteins.

Which Enzyme Donor (ED) tag should I use for my application?

- The ability of ED tags to bind to and complement the Enzyme Acceptor (EA) increases from PK1 to PK2 and then to ePL. Weak protein:protein interactions and some nuclear translocation assays may use PK2. Assays with strong, direct protein:protein interactions will usually use PK1. In addition to using PK1 and PK2, assays of GPCRs (especially those that don't naturally or robustly recruit β-arrestin2 may use either of the two ARMS containing PK2 vectors (consult the tables in the Supplemental Information section of this user manual). Assays for nuclear translocation will usually use ePL since the interaction between the ePL-tagged protein under study and the NUC-EA may not interact directly. Assays for protein stability and degradation use ePL tags and involve addition of the EA protein at the time of detection. Finally, especially if your initial assay is unsatisfactory, obtaining an optimized assay may benefit from empirical determination of which tag is best for your protein of interest and assay.

Which SH2 EA Parental cell line should I use for my receptor tyrosine kinase or cytokine receptor?

- Refer to the SH2 Recruitment Parental Cell Line Guide in the Supplemental Information section of this user manual.

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

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