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User Manual

PathHunter® Signaling Pathway Reporter Assay

For Detection of Ligand-Mediated Activation of Signaling Pathways

Document Number: 70-406 Revision 0

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Table of Contents

Please read this entire user manual and cell line-specific datasheet before proceeding with the assay.

For additional information or Technical Support, see contact information below.

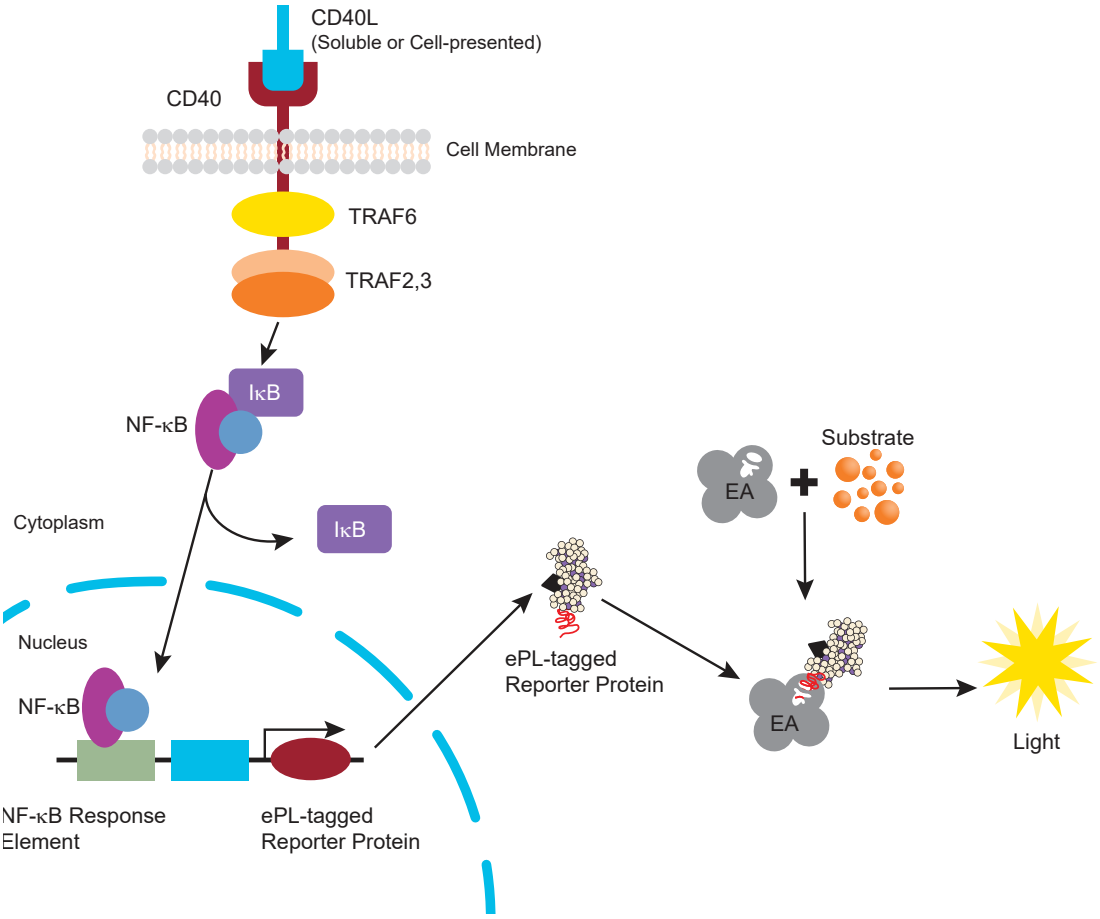
Overview

The PathHunter Signaling Pathway Reporter Assays are stable clonal cell lines used for monitoring the activation of various signaling pathways. Ligands (for example, CD40, IL-6 etc.) bind to distinct receptors and trigger specific signaling pathways. Transcription factors are then activated, followed by their nuclear translocation. Binding of these activated transcription factors to specific promoter elements regulates downstream gene transcription, leading to protein expression.

Assay Principle

The PathHunter assay relies on the established Enzyme Fragment Complementation (EFC) technology. EFC uses a split β-galactosidase (β-gal) enzyme, which consists of the Enzyme Donor (ePL) and the Enzyme Acceptor (EA) fragments. Independently, these fragments have no β-gal enzymatic activity. However, when forced to complement, they form an active β-gal enzyme that hydrolyzes a substrate to produce a chemiluminescent signal.

Each stable cell line is engineered to express a synthetic DNA reporter construct, which contains a pathway-specific transcription factor response element. The response element, in turn, drives the expression of a proprietary reporter protein tagged with ePL. An activated signaling pathway induces the expression of the ePL-tagged reporter protein. The level of the expressed ePL-tagged reporter protein can then be measured by addition of the detection reagent containing EA, which forces complementation of the two enzyme fragments. The resulting active enzyme hydrolyzes the substrate present in the PathHunter detection reagents to generate a chemiluminescent signal.



Principle of Signaling Pathway Reporter Assays: This figure represents the NF-κB Signaling Reporter construct, which regulates PathHunter EFC Reporter expression via upstream NF-κB response elements controlled by NF-κB transcription factor binding. Upstream signaling events result in NF-κB nuclear translocation and subsequent response-element binding. Signaling activity is measured by monitoring PathHunter EFC Reporter abundance after complementary enzyme acceptor (EA) addition, resulting in enzyme fragment complementation to create an active β-gal enzyme, capable of substrate hydrolysis and production of a chemiluminescent signal.

Materials Provided

Components	
2 vials of cells	Refer to cell line-specific datasheet for shipped cell density

It is not recommended to substitute the Cell Plating (CP) Reagent that has been specified in the datasheet for the cell line.

Storage Conditions

Cells must arrive in a frozen state on dry ice, and should be transferred to the vapor phase of 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 long-term storage, store vials in the vapor phase of liquid nitrogen.

Additional Materials Required

Refer to the cell line-specific datasheet to determine the appropriate media and reagents required for the specific cell line used in the assay.

Material	Ordering Information
Control antibody/ligand	Refer to the cell line-specific datasheet
AssayComplete™ Cell Culture Kit	Refer to the cell line-specific datasheet
AssayComplete Cell Plating Reagent	Refer to the cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009 (for adherent cells)
AssayComplete Protein Dilution Buffer	92-0023 Series*
AssayComplete Thawing Reagent	Refer to the cell line-specific datasheet
AssayComplete Freezing Reagent	Refer to the cell line-specific datasheet
PathHunter® PL/PK Detection Kit	93-0812
96-Well White, Flat-Bottom TC-Treated, Sterile Plates with Lid, 10 plates/pack	92-0027
384-Well White, Flat-Bottom, TC-Treated, Sterile Plates with Lid, 10 plates/pack (if applicable)	92-0015
96-Well Green, V-bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Dulbecco's Phosphate-Buffered Saline (PBS)	Corning, Cat. No. 21-031-CM or similar
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Multimode or luminescence reader	Refer to discoverx.com/instrument-compatibility
Single and multichannel micropipettes and pipette tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microtubes	
Tissue culture disposable pipettes (1 mL-25 mL) and tissue culture flasks (T25 and T75 flasks, etc.)	
Cryovials for freezing cells	
Hemocytometer	
Humidified tissue culture incubator (37°C and 5% CO_2)	

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

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 vials). The following procedures are for safe storage, handling and removal of cryovials from the vapor phase of liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.
2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, cryovials must be stored in the vapor phase of liquid nitrogen.

Contact technical support immediately if cells received were already thawed.

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

3. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container.

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

4. Wait for at least one minute for any liquid nitrogen that may be present inside the vial to evaporate.

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

Cell Culture Protocol – Adherent cells

The following procedures are for thawing adherent cells from cryovials, seeding and expanding the cells, and freezing the cells once they have been propagated.

Cell Thawing

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

1. Pre-warm the AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes, as indicated on the cell line-specific datasheet.
2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask inside a sterile tissue culture hood. Set aside for Step 6. DO NOT add selection antibiotics to the thawing reagent.
3. Remove the cryovials from -80°C or liquid nitrogen vapor storage and immediately place them in 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 vapor storage, use tongs to place them immediately in 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 cryovials containing the cells briefly (30 seconds to 1 minute) in a 37°C water bath, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.

Do not leave the frozen cell vials in the water bath for longer than 1 minute. Prolonged thawing at 37°C may result in cell death.

5. Decontaminate the external surface of the vials by spraying and wiping them with 70% ethanol. Transfer them to a tissue culture hood.
6. Using a sterile pipette, gently transfer the thawed cells to the pre-filled T75 flask. Incubate the flask at 37°C and 5% CO_2.
7. Maintain the cells in culture until they are 70-80% confluent. Then, proceed to Cell Recovery instructions. Do not split if cells are below this confluency, or growth issues may occur.

Cell Recovery

The following is a protocol for ensuring maximal cell recovery once they become 70-80% confluent in a T75 flask.

1. Pre-warm the 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 it in a sterile tissue culture hood.

3. Gently aspirate the media from the T75 flask.
4. Add 5 mL of PBS into the T75 flask, and gently tip the flask side to side to ensure that the cells are rinsed.
5. Gently aspirate PBS from the flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the flask. Gently rock the flask back and forth to ensure that the inner surface of the flask is uniformly covered with trypsin.
7. Incubate the flask 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 observe the cells under a microscope to confirm cell detachment. Gently tap the edge of the flask to detach cells from the surface, if necessary. If the cells do not detach easily, then return the flask to the incubator for additional 1 to 2 minutes and repeat this step until all cells are in suspension.
9. Add 4 mL of the AssayComplete™ Thawing Reagent to the T75 flask. Using a pipette, gently rinse the cells with the reagent.
10. Gently pipet the cell suspension up and down several times to generate a single cell suspension without any clumps.
11. Split the cells conservatively for the first passage after thawing, using the AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
U2OS	1:2
HEK 293	1:3

For example, for U2OS cells, add 4 mL of the AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 2.5 mL (1/2 of the total cell suspension in the flask) into each new tissue culture flask.

12. Add 5 mL of AssayComplete Thawing Reagent to a new T75 or T225 flask, followed by addition of the cell suspension (volume determined in Step 11). Add an additional volume of the thawing reagent to reach a final volume of 15 mL for a T75 flask, or 45 mL for a T225 flask.
13. Transfer the flask to a tissue culture incubator and incubate the cells at 37°C and 5% CO_2.

Cell Propagation

The cells can be propagated after a successful recovery, which can be determined by monitoring them under a microscope. Healthy cells should adhere uniformly to the surface of the flask, with only a few cells remaining in suspension.

1. If the cells appear healthy, exchange the AssayComplete Thawing Reagent with 15 mL of AssayComplete Cell Culture Reagent for a T75 flask (45 mL for T225 flasks), supplemented with the recommended concentration of selection antibiotic. Refer to the cell line-specific datasheet to determine the correct Cell Culture Kit, recommended antibiotic, and antibiotic concentration for the cell line.

Cell culture media is prepared by mixing the components provided in the cell line-specific AssayComplete Cell Culture Kit, and appropriate selection antibiotics. Refer to the Cell Culture Kit's datasheet for instructions on using its components.

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

To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

3. Once the cells have reached 70-80% confluency, split the cells every 2 to 3 days, based on the doubling time of the cell line. Use the AssayComplete™ Cell Culture Reagent supplemented with the proper selection antibiotics (as indicated on the cell line-specific datasheet) to split the cells. Typical split ratios for common cell backgrounds are indicated in the table below.

Cell Background	Suggested Split Ratio
U2OS	1:3
HEK 293	1:5

Cell Freezing

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

1. Remove T75 (or T225) flasks from the incubator and place them in a sterile tissue culture hood.

Care should be taken while handling flasks to avoid contamination.

2. Slowly aspirate the media from the flasks.
3. Add 10 mL of PBS into each T75 flask (or 15 mL for a T225 flask), and swirl it 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 that the surface of the flask is uniformly 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, gently tap the edge of the flask to detach cells from the surface.
9. Add 5 mL of the cell culture media to each T75 flask (or 15 mL to each T225 flask).
10. Using a pipette, slowly rinse the cells from the surface of the flask with the added media. Slowly pipette up and down several times to achieve a single cell suspension with no cell clumps.
11. Transfer the cell suspension from the T75 flask into a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add an additional volume of the cell culture media to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks). Rinse the flask to collect the remaining cells, then transfer it into the conical tube. Slowly pipette up and down several times to ensure that a single cell suspension is formed.
12. To determine the concentration of cells in the suspension:
    - 12.1. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    - 12.2. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.
    - 12.3. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells 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, resuspend the cells to the desired concentration (e.g. 1-2 x 10^6 cells/mL) with ice-cold AssayComplete™ Freezing Reagent (as defined in the cell line-specific datasheet).

16. Make aliquots by transferring 1 mL of cell suspension into the labeled 2 mL cryogenic tubes. Seal the tubes tightly.

17. Freeze cells in a -80°C freezer at a controlled rate of -1°C/minute overnight in 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.

18. Transfer the vials into vapor phase of liquid nitrogen for long-term storage.

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

Cell Culture Protocol – Suspension Cells

The following procedures are for thawing suspension cells from cryovials, seeding and expanding the cells, and freezing them once they have been propagated. Refer to the cell line product datasheet for the specific AssayComplete products listed in the protocol below.

Cell Thawing

1. Add 9 mL of the AssayComplete Thawing Reagent in a T25 flask and place it in a humidified 37°C and 5% CO_2 incubator for 15 minutes to equilibrate pH and temperature of the reagent.

2. Remove the cell cryovials from -80°C or liquid nitrogen vapor phase storage and immediately place them in dry ice.

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

3. Place the cryovials containing the cells briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellets are almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.

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

4. Decontaminate the surface of the vials by spraying and wiping them with 70% ethanol. Transfer them to a tissue culture hood.

5. Gently transfer the thawed cells into the pre-filled T25 flask using a pipette, and incubate at 37°C and 5% CO_2 for 48 hours before proceeding with cell propagation

Cell Propagation

1. Passage the suspension cells in the T25 flask when they have reached a density of 1 x 10^6 cells/mL by taking an aliquot of cells to determine density. Do not let the cells exceed 3 x 10^6 cells/mL.

    1.1. Remove cells from the flask and transfer them to a conical tube (if necessary, add 5 mL of cell culture media to the flask, and rinse to collect the remaining cells. Transfer this additional volume to the conical tube).

    1.2. Centrifuge at 180 x g for 3 minutes at room temperature to pellet cells.

    1.3. Decant the supernatant or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.

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

   Note: If passaged every other day, we recommend seeding at a density of 2 x 10^5 cells/mL. If the interval between passages is longer than two days, we recommend seeding at a density of 1 x 10^5 cells/mL.

Flask Size	T25	T75	10 cm Dish
Final Culture Volume	10 mL	20 mL	10 mL

Cell Freezing

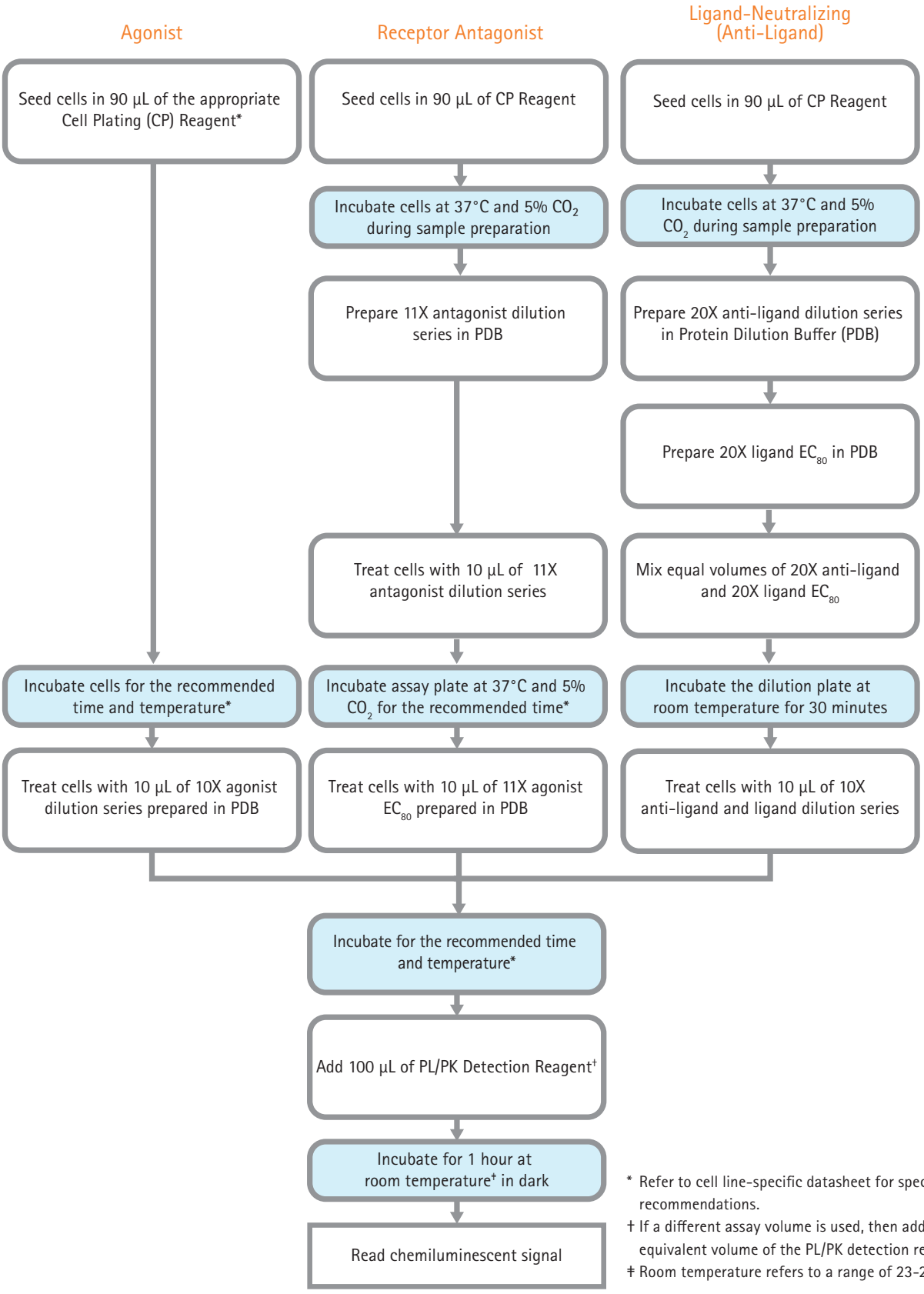
Note: The following procedure is for freezing cells from T75 flasks. If smaller flasks are used, adjust the volumes accordingly. This protocol assumes that cells have reached the desired cell density in a sufficient number of flasks to fill the desired number of cryovials. The cells will be cryopreserved in aliquots of 1 mL/vial, at the desired number of cells per vial (e.g. 2 x 10^6 per vial).

1. It is recommended to freeze the cells at a low passage number (2 to 3 passages). For optimal assay performance, ensure that the cells are in logarithmic growth phase at the time of freeze down.
2. Remove the cell suspension from the flask and transfer it to a conical tube (if necessary, add an additional 5 mL of cell culture media to the flask and rinse to collect the remaining cells. Transfer this additional volume to the conical tube).
3. Take an aliquot of the cells to determine the cell number.
4. Centrifuge the tube at 180 x g for 3 minutes at room temperature to pellet the cells.
5. Decant the supernatant, or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.
6. Immediately resuspend the cells in ice-cold AssayComplete™ Freezing Reagent to a concentration of 2 x 10^6 cells/mL.
7. Make aliquots by transferring 1 mL of cell suspension into the labeled 2 mL cryogenic tubes. Seal the tubes tightly.
8. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight in a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short-term storage, vials can be stored in the -80°C freezer for a maximum of two days.
9. Transfer the vials into the liquid nitrogen vapor phase for long-term storage.

Keep cells on ice during this time to maintain cell viability

Protocol Schematic

Quick-Start Procedure: Based on the type of sample being tested (agonist, antagonist or anti-ligand), select the appropriate protocol from the following scheme and perform the indicated steps in a 96-well plate.



* Refer to cell line-specific datasheet for specific recommendations.

† If a different assay volume is used, then add an equivalent volume of the PL/PK detection reagent.

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

Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies

The following protocol is designed for evaluating the ability of ligands or agonistic antibodies added as soluble reagents to activate the signaling pathway in the PathHunter Signaling Pathway Reporter Assay, in a 96-well format.

For assays to be run in a 384-well plate, refer to the table below for assay reagent volumes. For agonist experiments in a 384-well plate, the agonist dilution series should be prepared at 5X the final desired concentration.

Assay Reagents (Volume per Well)	96-Well Plate	384-Well Plate**
Cell Plating Volume (µL)*	90	20
Ligand (µL)	10	5
Working Detection Solution (µL)	100	30
Total Assay Volume (µL)	**200**	**55**

*Refer to the cell line-specific datasheet for the recommended cell density.

**For an assay in a 384-well plate, a recommended cell number per well would be half the recommended cell number per well for a 96-well plate. Additional optimization of the cell number may be required.

1. Preparation and Plating: Adherent Cells

The following protocol provides steps for harvesting and preparing adherent cells for plating in an assay plate. This protocol assumes that cells have reached a 70-80% confluency and have been cultured in the recommended cell culture media. For cell line-specific information on the appropriate AssayComplete™ Cell Plating Reagent, Cell Culture Kit, control ligand, incubation times and temperature, please refer to the cell line-specific datasheet.

1.1. Ensure that the cells are in the logarithmic growth phase prior to use in the assay.

1.2. Warm the AssayComplete Cell Plating Reagent and cell culture media in a clean 37°C water bath for 15 minutes. Refer to the cell line-specific datasheet for the recommended AssayComplete Cell Plating Reagent and cell culture media.

1.3. Dissociate the cells and resuspend them in fresh media.

1.3.1. Aspirate the media from the T75 flasks. Add 10 mL of room temperature PBS into each flask, and gently swirl it to rinse the cells. Aspirate PBS from the flask.

Use calcium- and magnesium-free PBS for rinsing cells prior to detachment.

1.3.2. Dissociate the cells by adding 1.5 mL of AssayComplete Cell Detachment Reagent. Gently rock the flask back and forth to ensure that the surface of the flask is uniformly covered.

Do not use trypsin for this step; especially with assays interrogating membrane-anchored receptors (e.g. GPCRs). Use of trypsin can negatively affect assay results.

1.3.3. Incubate the flask at 37°C and 5% CO_2 in a humidified incubator for 5 minutes, or until the cells have detached.

1.3.4. Remove the flask from the incubator and confirm that the cells have detached by viewing under a microscope. Gently tap the edge of the flask to detach cells from the inner surface, if necessary.

1.3.5. Add 8-10 mL of the cell culture media to each flask and pipette up and down a few times to dissociate cells.

1.4. Remove an aliquot of the cells from the flask and determine the cell density (e.g. using a hemocytometer or automated cell counter).

1.5. Transfer an appropriate volume of the cell suspension to a conical tube, depending on the number of cells required for the desired number of samples to be run in the assay. Centrifuge at 300 x g for 4 minutes at room temperature to pellet cells.

1.6. Decant the supernatant or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.

1.7. Resuspend the cell pellet in the recommended AssayComplete™ Cell Plating Reagent. Based on the number of cells in the pellet from Step 1.5, resuspend the pellet in an appropriate volume to achieve the desired concentration of cells (e.g. to achieve 5,000 cells/well in 90 µL, resuspend cells at 5.5 x 10^4 cells/mL). Refer to the cell line-specific datasheet for the recommended concentration of cells.

1.8. Pour the cell suspension into a sterile reagent reservoir. Transfer 90 µL of the cell suspension into each well of a 96-well assay plate using a multichannel pipette

1.9. Incubate the assay plate at 37°C and 5% CO_2 before proceeding with ligand/antibody preparation. Refer to the cell line-specific datasheet for recommended cell incubation time.

2. Preparation and Plating: Suspension-Type Signaling Pathway Reporter Cells

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

2.1. Warm the AssayComplete Cell Plating reagent in a clean 37°C water bath for 15 minutes.

2.2. Take an aliquot of cells (in suspension) from the flask, and determine the cell density by first counting cells, and then calculating the cell concentration per mL. Use the calculated concentration to determine the total number of cells in the tissue culture flask.

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

2.4. Centrifuge at 180 x g for 3 minutes at room temperature to pellet cells.

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

2.6. Resuspend the cell pellet to the desired concentration (e.g. to achieve 10,000 cells/well in 90 µL, resuspend cells at 1.1 x 10^5 cells/mL) in the pre-warmed AssayComplete Cell Plating Reagent.

2.7. Using a multichannel pipette and reagent reservoir, seed 90 µL of the cell suspension into one 96-well assay plate.

2.8. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line datasheet for the recommended cell incubation time. Proceed to the Preparation: Soluble Ligand or Agonist Antibody section.

3. Preparation: Soluble Ligand or Agonist Antibody

When optimizing assay conditions, it is recommended to include a standard curve of the control ligand or antibody to verify that the kit components are working optimally.

Prepare serial dilutions of the control ligand or agonist antibody in the master dilution plate by making a 3-fold, 11-point serial dilution in the AssayComplete Protein Dilution Buffer (PDB), or an appropriate ligand dilution buffer (as specified on the ligand datasheet).

The control ligand/agonist antibody should be prepared at 10X the final screening concentration.

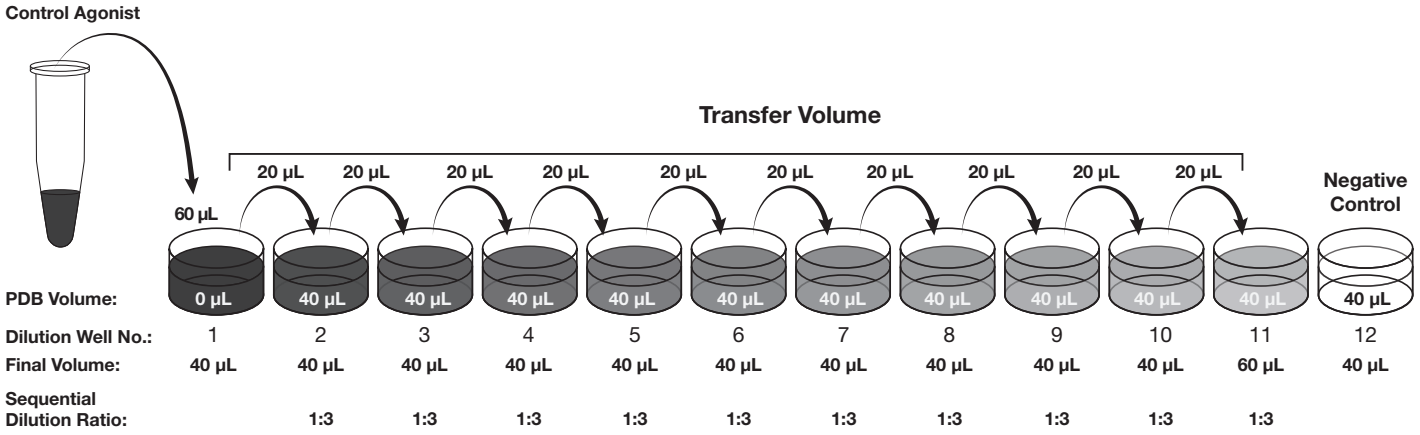
3.1. Add 40 µL of PDB to dilution Wells A2 to A12 of the master dilution plate. This is sufficient volume for 2 rows. The dilution volume may be adjusted according to the number of wells desired.

3.2. Prepare the control ligand or antibody in PDB (or the ligand-specific buffer) at 10X the top screening concentration. Refer to the cell line-specific datasheet for the recommended top screening concentration of the control ligand.

3.3. Add 60 µL of the highest concentration of the control ligand or antibody to Well A1 of the master dilution plate.

3.4. Using a clean pipette tip, transfer 20 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 20 µL from Well A2 into Well A3, and mix well. Repeat this process until Well A11, resulting in an eleven-point, 1:3 dilution series. No sample is transferred to Well A12 as this is the negative control well.

3.5. Set up serial dilutions for additional ligands or soluble agonist antibodies in a similar manner, using the ligand diluent recommended for the cell line on the cell line-specific datasheet.



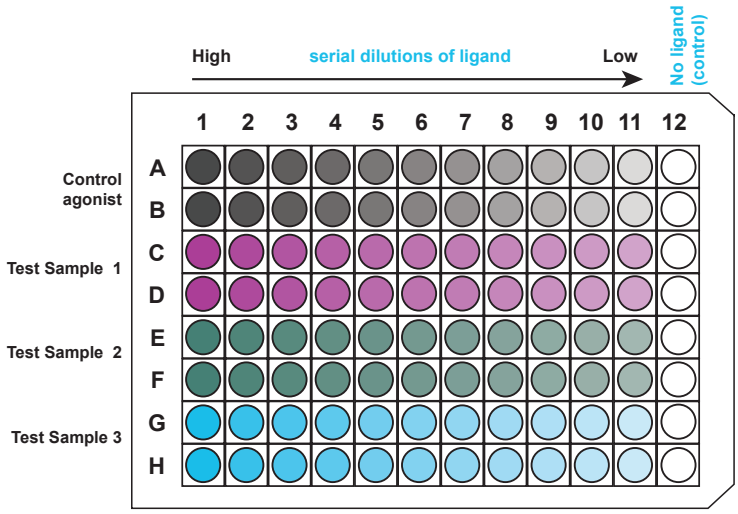
Control Antibody Serial Dilutions: Make 11 three-fold serial dilutions of the control ligand/antibody in a separate dilution plate.

4. Addition: Soluble Ligand or Agonist Antibody

4.1. Transfer 10 µL of each 10X ligand/antibody serial dilution (prepared in Step 3) into the appropriate wells of Rows A and B of the 96-well assay plate. The Representative Assay Plate Map shows the recommended format of sample addition in duplicates.

4.2. Cover the plate with a lid and incubate at the specific temperature and time indicated on the cell line-specific datasheet.

Representative Assay Plate Map



Assay Plate Map: This plate map shows an 11-point dose curve, with 2 data points at each concentration for one reference and three test samples per plate with a 1:3 serial dilution scheme.

5. Detection

5.1. Prepare a stock of the working detection solution (PL/PK) in a 15 mL polypropylene tube or a reagent reservoir. The detection kit includes EA Reagent, Lysis Buffer and Substrate Reagent. Mix the kit components in the ratio indicated in the table below:

Working Detection Solution (PL/PK) for a 96-well Format		
Components	**Volume Ratio**	**Volume per Plate (mL)**
EA Reagent	1	2
Lysis Buffer	1	2
Substrate Reagent	4	8
Total Volume		**12**

5.2. Transfer 100 µL (or a volume equivalent to the assay volume) of the working detection solution to each well of the assay plate.
Optional: Place the plate on an orbital shaker at 350 rpm for 1 minute to achieve uniform mixing.

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

6. Reading: Assay Plate

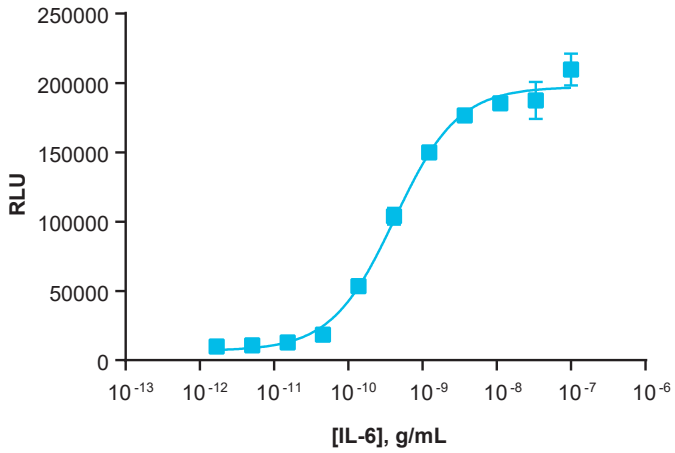
Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

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

Typical Results

The following graph is a typical example of a dose-response curve for the PathHunter HepG2 STAT3 Pathway Reporter Assay generated using the protocol outlined in this user manual. Soluble IL-6 has been used to activate an endogenous IL-6 receptor. The data shows a potent, dose-dependent activation of the STAT3 signaling pathway.

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



EC_{50} (ng/mL)	0.42
S/B	20.3

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Detailed Assay Protocol: Receptor Antagonists or Pathway Inhibitors

The following protocol is for evaluating the ability of antagonistic (anti-receptor) antibodies or pathway inhibitors to inhibit ligand-induced activation of the signaling pathway in a 96-well format cell-based assay. For assays to be run in a 384-well plate, refer to the table below for assay reagent volumes. Antagonist experiments run in a 384-well plate use a 6X antagonist dilution series and an agonist EC_{80} dose at 6X the final screening concentration.

Assay Reagent	96-Well Plate	384-Well Plate**
Number of Cells	Refer to cell line-specific datasheet	Refer to cell line-specific datasheet*
Cell Plating Volume* (µL)	90	20
Antagonist (µL)	10	5
Agonist EC_{80} (µL)	10	5
Working Detection Solution (µL)	110	30
Total Assay Volume (µL)	**220**	**60**

* Refer to the cell line-specific datasheet for the recommended cell density.

**For an assay in a 384-well plate, a recommended cell number per well would be 1/2 the recommended cell number per well for a 96-well plate. Additional optimization of the cell number may be required.

1. Preparation and Plating: Adherent Cells

1.1. Prepare and plate the cells in a final volume of 90 µL per well for running the assay, as described in Step 1 of the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies.

2. Preparation and Plating: Suspension-Type Signaling Pathway Reporter Cells

2.1. Prepare and plate the cells in a final volume of 90 µL per well for running the assay, as described in Step 2 of the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies.

3. Preparation: Receptor Antagonist or Pathway Inhibitor

The anti-receptor antibodies or inhibitors should be prepared at 11X the final screening concentration.

3.1. Prepare serial dilutions of the antagonist receptor antibodies or inhibitors to be tested, as described for soluble ligand or agonist antibodies in Step 3 of the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies.

4. Addition: Receptor Antagonist or Pathway Inhibitor

4.1. Transfer 10 µL of the 11X antagonist antibody/inhibitor serial dilutions to the appropriate wells of Rows A and B of the 96-well assay plate, as indicated on the Representative Assay Plate Map (e.g. control antagonist/inhibitor in rows A and B; test antagonists in rows D and E).

4.2. Cover the plate with a lid and incubate for 1 hour at 37°C and 5% CO_2.

5. Preparation: Agonist (Stimulation)

5.1. Prepare the agonist at a fixed concentration (e.g. at the EC_{80}) in PDB as an 11X working stock solution.

5.2. Add 40 µL in each well of a new row on the master dilution plate for easy dispensing into the assay plate.

6. Addition: Agonist (Stimulation)

6.1. Transfer 10 µL of the prepared 11X agonist stock solution for assay stimulation into the appropriate wells of plated cells containing the receptor antibody/inhibitor. The Representative Assay Plate Map shows the recommended format of sample addition and testing in duplicates.

6.2. Cover the plate with a lid and incubate at 37°C and 5% CO_2 in a humidified incubator. Refer to the cell line-specific datasheet for the recommended assay incubation time.

7. Detection

7.1. Prepare the working detection solution and add it to each well of the assay plate, as described in Step 5 in Detailed Assay Protocol: Soluble Ligand or Antibodies.

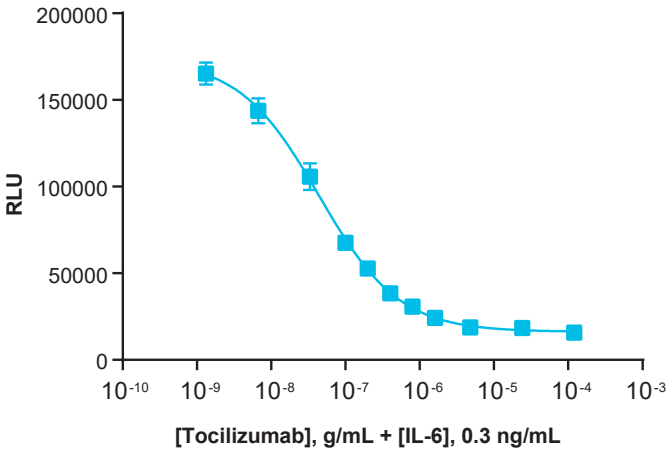
8. Reading: Assay Plate

8.1. Read assay plates as described in Step 6 in Detailed Assay Protocol: Soluble Ligand or Antibodies.

Typical Results

The following graph is an example of a dose-response curve for the PathHunter HepG2 STAT3 Pathway Reporter Assay generated using the protocol outlined in this user manual. The data shows dose-dependent inhibition of IL-6 activation by Tocilizumab, an anti-IL-6RA antibody.

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



IC_{50} (ng/mL)	44.1
S/B	10.5

Detailed Assay Protocol: Ligand Neutralizing or Anti-Ligand Antagonists

The following suggested protocol is for evaluating the ability of neutralizing (anti-ligand) antagonists (antibodies or low molecular weight inhibitors) to inhibit the ligand-induced activation of the signaling pathway in a 96-well format cell-based assay. For running the assay in a 384-well plate, refer to the table below for specific reagent volumes. Anti-ligand experiments run in a 384-well plate use an antibody and agonist mix that is 5X the final screening concentrations.

Assay Reagent (Volume per Well)	96-Well Plate	384-Well Plate**
Cell Plating Volume* (µL)	90	20
Antibody and Agonist mix (µL)	10	5
Working Detection Solution (µL)	100	30
Total Assay Volume	**200**	**55**

* Refer to the cell line-specific datasheet for the recommended cell density.

**For an assay in a 384-well plate, a recommended cell number per well would be half the recommended cell number per well for a 96-well plate. Additional optimization of cell number may be required.

1. Preparation: Anti-Ligand Sample

1.1. Prepare anti-ligand sample dilutions as described in Step 3 in the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies, at 20X the desired final concentration.

2. Preparation and Pre-incubation: Ligand (EC_{80}) and Anti-Ligand Sample Dilution Series

2.1. Prepare a 20X working stock solution of a fixed concentration of the ligand (e.g. the EC_{80}) in PDB.

2.2. In a V-bottom dilution plate, mix an equal volume of the ligand working stock solution and the anti-ligand sample dilution series (e.g. mix 50 µL of the ligand stock solution and 50 µL of the anti-ligand dilution series). Mix well by slowly pipetting up and down several times.

2.3. Incubate this 10X dilution series mixture of the ligand and anti-ligand antibody at room temperature for 30 minutes. Optimal incubation time should be determined empirically.

3. Preparation and Plating: Adherent Cells

3.1. Prepare and plate the cells in a final volume of 90 µL per well for running the assay, as described in Step 1 in the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies.

4. Preparation and Plating: Suspension-Type Signaling Pathway Reporter Cells

4.1. Prepare and plate the cells in a final volume of 90 µL per well for running the assay, as described in Step 2 of the Detailed Assay Protocol: Soluble Ligand or Agonist Antibodies.

5. Addition: Pre-Incubated Anti-Ligand and Ligand Mixture

5.1. Transfer 10 µL of the 10X mixture prepared in Step 2 containing the ligand and anti-ligand antibody dilution series, into the appropriate wells of plated cells. The Representative Assay Plate Map shows the recommended format of sample addition in duplicates.

5.2. Cover the plate with a lid and incubate at 37˚C and 5% CO_2 in a humidified incubator. Refer to the cell line-specific datasheet for recommended incubation time.

6. Detection

6.1. Prepare the working detection solution and add it to each well of the assay plate, as described in Step 5 in Detailed Assay Protocol: Soluble Ligand or Antibodies.

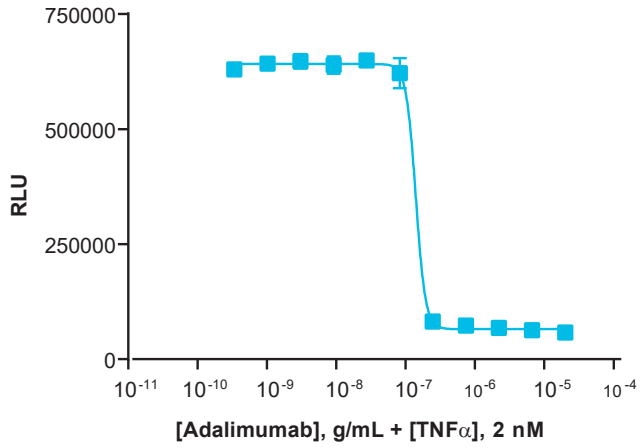
7. Reading: Assay Plate

7.1. Read assay plates as described in Step 6 in Detailed Assay Protocol: Soluble Ligand or Antibodies.

Typical Results

The following graph is an anti-ligand dose-response curve for the PathHunter U2OS NF-κB Pathway Reporter Assay, generated using the protocol outlined in this user manual. The data shows dose-dependent inhibition of TNFα-mediated NF-kB signaling by the anti-TNFα antibody, Adalimumab.

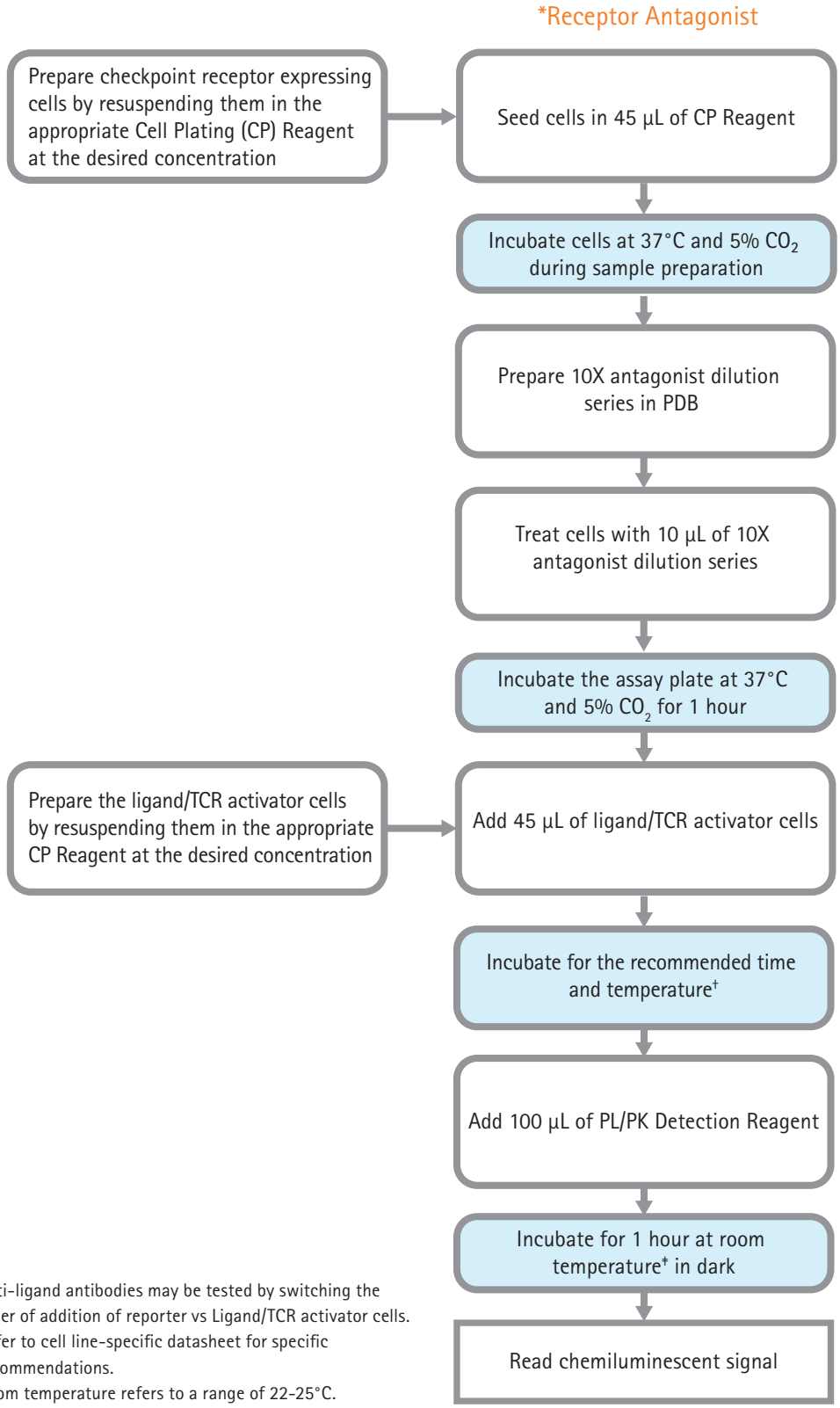
The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.



EC_{50} (ng/mL)	140
S/B	10

Protocol Schematic : Co-culture Reporter Assay for Inhibitory Checkpoint Receptors

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



* Anti-ligand antibodies may be tested by switching the order of addition of reporter vs Ligand/TCR activator cells.

† Refer to cell line-specific datasheet for specific recommendations.

‡ Room temperature refers to a range of 22-25°C.

Detailed Assay Protocol: Co-culture Reporter Assay for Inhibitory Checkpoint Receptors

The following detailed protocol is specific for evaluating the ability of antibodies and small molecule inhibitors to block checkpoint receptor-induced signaling in the PathHunter Functional Pathway Reporter Assays, when stimulated with TCR Activator cells co-expressing the relevant ligand, in a 96-well plate format.

For assays that will be run in a 384-well plate, refer to the table below for recommendation for assay reagent volumes. For agonist experiments in a 384-well plate, the agonist dilution series should be prepared at 5X the final desired concentration.

Assay Reagent (Volume per Well)	96-Well Plate	384-Well Plate
Signaling Cell Suspension Volume* (µL)	45	10
Antibody (µL)	10	5
Ligand Cell Suspension Volume* (µL)	45	10
Working Detection Solution (µL)	100	30
Total Assay Volume	**200**	**55**

*Refer to the cell line-specific datasheet for the recommended cell density.

1. Preparation and Plating: Functional Pathway Reporter Cells

1.1 Warm the AssayComplete™ Cell Plating Reagent in a clean 37°C water bath for 15 minutes.

1.2 Take an aliquot of the checkpoint receptor signaling cells (in suspension) from the flask to count cell density. Refer to the Cell Culture Protocol-Suspension Cells for instructions on cell thawing, propagation and freezing.

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

1.4 Centrifuge at 180 x g for 3 minutes at room temperature to pellet cells.

1.5 Decant the supernatant or carefully remove the media with a 10 mL pipette, without disturbing cell pellet.

1.6 Resuspend the cell pellet at the desired concentration (e.g. to achieve 20,000 cells/well in 45 µL resuspend cells at 4.4 x 10^5 cells/mL) in pre-warmed AssayComplete Cell Plating Reagent.

1.7 Using a multichannel pipette and reagent reservoir, seed 45 µL of the cell suspension into a 96-well assay plate. Optionally, test samples can be run on multiple plates, in triplicates or other variations.

1.8 Incubate the assay plate in a 37°C and 5% CO_2 humidified incubator until ready for antibody addition.

2. Preparation: Antibody

When optimizing assay conditions, it is recommended to include a positive control dilution curve of the control antibody, to verify that the assay components are working optimally. Refer to the cell line-specific datasheet to identify the appropriate control antibody and reagents.

Prepare serial dilutions of the control antibody in a 96-well dilution plate by making 11-point, 3-fold serial dilutions in AssayComplete Protein Dilution Buffer (PDB) or any other appropriate dilution buffer. The antibody dilutions should be prepared at 10X the final screening concentration. The control antibody dilution series will be added to Rows A and B of the 96-well assay plate. The test antibody dilution series will be added to Rows C and D of the plate.

2.1 For 11-point dilutions of control or test antibodies, use a V-bottom dilution plate (or non-binding tubes).

2.2 Add 40 µL of PDB to Wells 2 to 12 of the dilution plate (one row per antibody).

2.3 For test or control antibodies, add an appropriate volume of PDB to Well A1, such that the total volume of the diluted antibody is 60 µL.

2.4 Add an appropriate volume of control or test antibody to Well A1. Mix by slowly pipetting up and down several times.

2.5 Using a clean pipette tip, transfer 20 µL of antibody from Well A1 to Well A2. Mix thoroughly by slowly pipetting up and down several times. Replace the pipet tip and transfer 20 µL from Well A2 to Well A3, and mix well. Repeat this process until Well A11, resulting in an 11-point, 1:3 dilution series. No sample is transferred to Well A12, as this is the negative control.

2.6 Set up serial dilutions for additional antibodies in a similar manner.

3. Addition: Antibody

3.1. Add 10 µL of each 10X antibody serial dilution from Step 2 in duplicate, to the cells that had been plated in a 96-well plate (refer to Step 1).

3.2. Replace the lid back on to the plate, and incubate in a 37˚C and 5% CO_2 incubator for 1 hour.

4. Addition: Ligand/TCR Activator Cells

Ensure that the ligand/TCR activator cells (e.g. U2OS PD-L1/TCR Activator for the PD-1 Pathway Reporter Assay) in culture are in the logarithmic growth phase at the time of using these for the assay.

4.1. Warm AssayComplete™ Cell Plating Reagent and cell culture media recommended in the cell line datasheet for the ligand-presenting cell line, in a clean 37°C water bath for 15 minutes.

4.2. Dissociate the ligand/TCR activator cells and resuspend them in fresh media:

4.2.1 Aspirate the media from the T75 flasks

4.2.2 Add 10 mL of PBS (room temperature) into each flask, and swirl each flask to rinse the cells. Aspirate PBS from the flask.

Use calcium- and magnesium-free PBS for rinsing cells prior to detachment.

4.2.3 Dissociate the cells by adding 1.5 mL of AssayComplete Cell Detachment Reagent.

4.2.4 Rock the flask back and forth gently to ensure that the surface of the flask is covered.

4.2.5 Incubate the flask at 37˚C and 5% CO_2 in a humidified incubator for 5 minutes, or until cells have detached.

4.2.6 Remove the flask from the incubator and confirm that the cells have detached from the flask's surface by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface.

4.2.7 Add 8-10 mL of the cell culture media to each flask and pipette up and down 2-3 times to dissociate cells.

4.3 Remove an aliquot of the ligand/TCR activator cells from the flask and determine cell density (e.g. with a hemocytometer or Coulter Counter).

4.4 Transfer an appropriate volume of the cell suspension to a conical tube, depending on the number of cells required for the desired number of samples to be run in the assay. Centrifuge at 300 x g for 4 minutes at room temperature to pellet cells.

4.5 Decant the supernatant or carefully remove media with a 10 mL pipette, without disturbing the cell pellet.

4.6 Resuspend the cell pellet in AssayComplete™ Cell Plating Reagent. Based on the number of cells in the pellet, in Step 4.5 above, resuspend the pellet in an appropriate volume to achieve the desired concentration of cells (e.g. to achieve 30,000 cells/well in 45 µL, resuspend cells at 6.7 x 10^5 cells/mL). For the recommended concentration of ligand TCR activator cells, see the cell line-specific datasheet.

4.7 Using a multichannel pipette and reagent reservoir, add 45 µL of the ligand/TCR activator cell suspension into all wells containing cells in the 96-well assay plate.

4.8 Incubate the assay plate at the recommended temperature and time, as indicated on the product datasheet.

5. Detection

5.1 Add 100 µL (or a volume equivalent to the assay volume) of PathHunter PK/PL Detection mix to each well of the assay plate.

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

6. Reading: Assay Plate

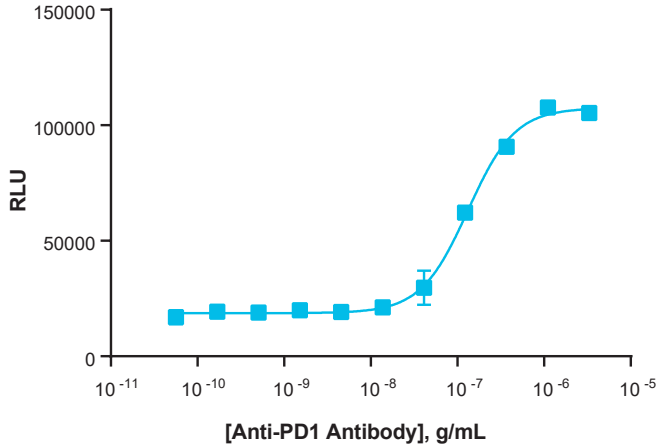
6.1 Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for an imager.

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

Typical Results

The following graph is a typical example of a dose-response curve for the PathHunter PD-1 NFAT Reporter Assay in co-culture with U2OS PD-L1 TCR Activator cells, generated using the protocol outlined in this user manual. The data shows a potent, dose-dependent inhibition of PD-1 activation in Jurkat NFAT cells with an anti-PD-1 antibody.

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



EC_{50} (ng/mL)	132.1
S/B	4.6

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Sub-optimal cell growth conditions	Refer to the datasheet for cell culture conditions.
	High DMSO/solvent concentration	Maintain DMSO/solvent at low concentrations (<1%) in serial dilutions of samples.
	Incorrect agonist used or incorrect agonist incubation time	Refer to the datasheet for recommended agonist and assay conditions.
	Incorrect preparation of agonist	Refer to the specific datasheet to ensure proper handling, dilution, and storage of agonist.
	Sub-optimal time course for induction	Optimize time course of induction with control agonist.
Decreased response	Higher passages give reduced performance	PathHunter cells are stable for at least 10 passages. Use low passage cells whenever possible.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should be prepared just prior to use.
	Problem with cell growth, cell viability, or cell density	Refer to the Cell Culture Protocol sections of this user manual for cell culture conditions.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 1 second/well.
Experimental S/B does not match datasheet value	Incorrect incubation temperature	Check and repeat the assay at the correct incubation temperature, as indicated on the cell line-specific datasheet
	Sub-optimal incubation time	Refer to the cell line-specific datasheet and optimize incubation time with control agonist.
Slow cell growth	U2OS grows slower than Jurkat	Doubling time is cell type-specific, therefore cell health should be monitored using a microscope.
	Slow-growing cells	Use functionally-validated and optimized AssayComplete™ media and reagents to improve assay performance.
EC_{50} is right-shifted	Ligand not stored properly	Follow directions for ligand storage as indicated in the ligand datasheet. Store in small aliquots at -20°C. Do not freeze/thaw more than twice.
	Incorrect top dose for the dose-response curve	Consult the dose-response curve on the cell line-specific datasheet for appropriate top concentration of ligand.
	Quality of ligand used in the assay	Using ligands from different vendors can affect assay performance. Use the ligand supplied by Eurofins DiscoverX as a positive control to ensure that the assay works well, before testing ligands or molecules from other sources.
	Problems with plate type and sample stability	Hydrophobic samples should be tested for solubility and may be diluted in buffer containing 0.1% BSA.
		Non-binding surface plates may be necessary for hydrophobic samples.
	Trypsin used for cell harvesting/plating	Use only AssayComplete Cell Detachment reagent to harvest cells for the assay.

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

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