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User Manual

PathHunter® Checkpoint Signaling Assay – Immunoglobulin Superfamily (IgSF)

For Chemiluminescent Detection of IgSF Checkpoint Receptor Signaling

Document Number 70-394 Rev 3

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Contents

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

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

Overview

The PathHunter Checkpoint Receptor Signaling Assay is a simple, highly specific assay to enable discovery and development of small molecule and biologic drugs targeting immune checkpoint receptors, belonging to the Immunoglobulin Superfamily (IgSF), such as PD-1, CD28 and ICOS.

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 ED 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.

The PathHunter Checkpoint Signaling Assay consists of human cells engineered to stably express an ED-tagged immune checkpoint receptor, while EA is fused to the phosphotyrosine-binding SH2 domain of an intracellular signaling protein such as Grb2 or SHP1. Engagement of the receptor by its ligand or agonist antibodies results in phosphorylation of the receptor's cytosolic tail. The SH2-domain fused to EA binds the phosphorylated receptor, forcing complementation of ED and EA, resulting in formation of an active β-gal enzyme, which hydrolyzes the substrate to generate a chemiluminescent signal.

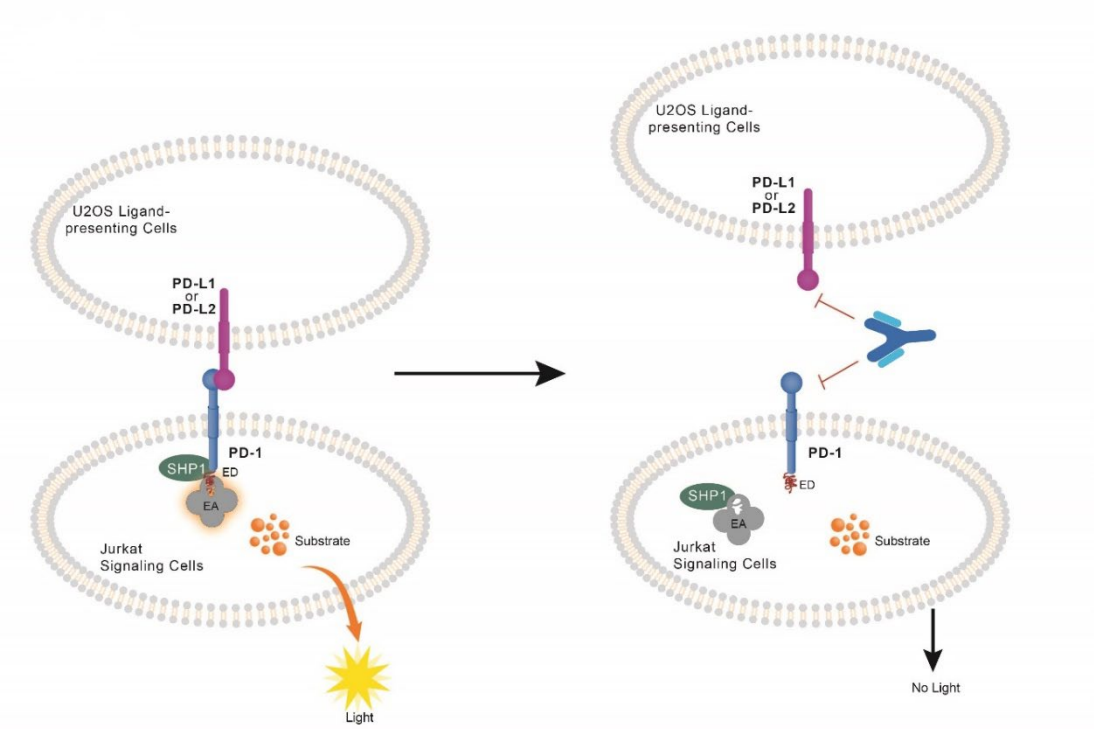


Figure 1. Signaling Assay for Inhibitory Checkpoint Receptors: Full-length PD-1 receptor was engineered with a small β-gal fragment (ED in red) fused to its C-terminus, and the SH2-domain of SHP-1 was engineered with the complementing β-gal fragment (EA). These constructs were stably expressed in Jurkat cells, while untagged full-length PD-L1 or PD-L2 was stably expressed in U2OS cells (ligand-presenting cells). Ligand engagement, through co-culture with ligand-presenting cells, results in phosphorylation of PD-1, leading to the recruitment of SHP-1-EA. This forces complementation of the EFC components to create an active □-gal enzyme. This active enzyme hydrolyzes substrate to generate chemiluminescence signal, as a measure of receptor activity. Addition of an antagonist that blocks PD1 signaling (e.g. antibodies to PD-1, PD-L1 or PD-L2), will prevent enzyme complementation resulting in a loss of signal.

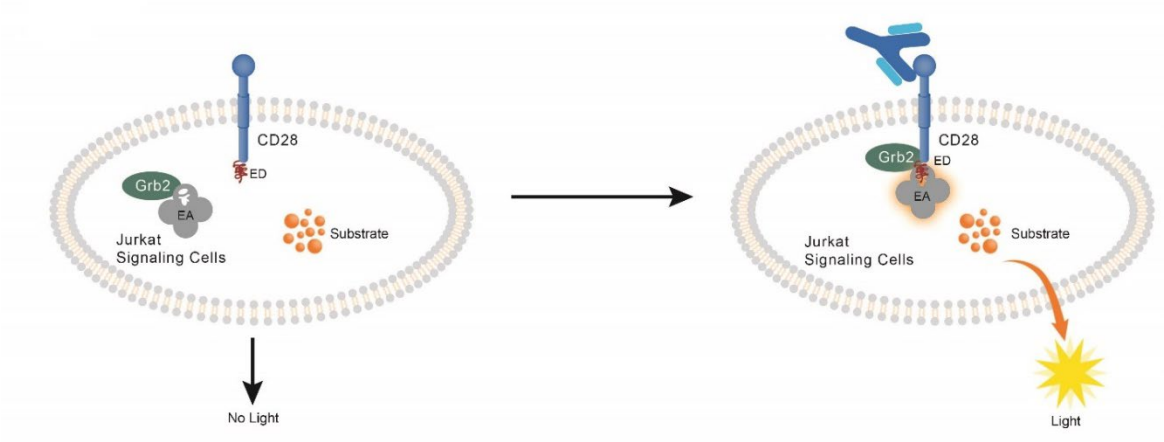


Figure 2. Signaling Assay for Activating Checkpoint Receptors: Full-length CD28 receptor was engineered with a small β-gal fragment (ED in red) fused to its C-terminus, and the SH2-domain of Grb2 was engineered with the complementing β-gal fragment (EA). These constructs were stably expressed in Jurkat cells. Receptor engagement with an agonist or an activating antibody results in phosphorylation of CD28, leading to the recruitment of Grb2-EA. This forces complementation of the EFC components to create an active β-gal enzyme. This active enzyme hydrolyzes substrate to generate chemiluminescence signal, as a measure of receptor activity.

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	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
Detection Kit	Refer to the cell line-specific datasheet
Ligand cell line (for co-culture assays)	Refer to the cell line-specific datasheet
96-Well White, Flat-Bottom TC-Treated, Sterile Plates with Lid, 10 plates/pack	92-0027
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.

    Contact technical support immediately if cells received were already thawed.

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.

    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 37ºC 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
CHO-K1	1:5
U2OS	1:2
HEK 293	1:3

For example, for CHO-K1 cells, add 4 mL of the AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5 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 15mL 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.

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

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.


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

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.

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
CHO-K1	1:10
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.

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 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.

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.

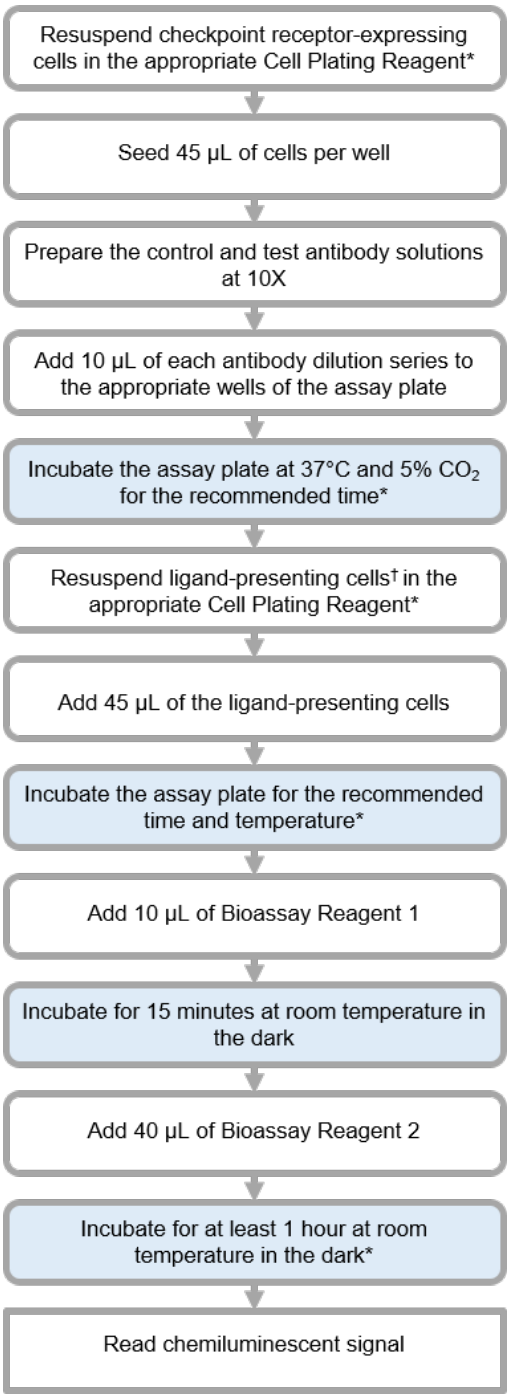
8. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight in a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short-term storage, vials can be stored in the -80°C freezer for a maximum of two days.

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: Signaling Assay for Inhibitory Checkpoint Receptors

Quick-Start Procedure: In a 96-well tissue culture treated plate perform the following steps:



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

† For testing an anti-ligand antibody, seed ligand-presenting cells first and pre-incubate with the antibody, followed by addition of signaling cells for the indicated time and temperature.

Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptor

The following detailed protocol is specific for evaluating the ability of anti-receptor antibodies and small molecule inhibitors to block checkpoint receptor-induced signaling in the PathHunter Checkpoint Receptor Signaling Assays in a 96-well plate format, using an anti-receptor antibody for illustration. For product specific information on AssayComplete™ Cell Culture Medium, Cell Plating Reagent, Control Antibody, incubation times and temperature, refer to the cell line-specific data sheet.

Note: If anti-ligand antibodies are to be tested, the cell plating order should be reversed, i.e. ligand cells should be added to the assay plate first, followed by addition of antibody, followed by the addition of receptor cells.

1. Preparation and Plating: Checkpoint Receptor Cells

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

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

1.3. 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.

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 the cell pellet.

1.6. Resuspend the cell pellet in the recommended AssayComplete™ Cell Plating Reagent. Based on the number of cells in the pellet from Step 1.3, resuspend the pellet in an appropriate volume to achieve the desired concentration of cells (e.g. to achieve 20,000 cells/well in 45 µL, resuspend cells at 4.4 x 10^5 cells/mL). Refer to the cell line-specific datasheet for the recommended concentration of cells.

1.7. Pour the cell suspension into a sterile reagent reservoir. Using a multichannel pipette, transfer 45 µL of the cell suspension into each well of a 96-well assay plate.

1.8. Incubate the assay plate at 37°C and 5% CO_2 until the antibody dilution series is ready to be added. Refer to the cell line-specific datasheet for recommended cell incubation time.

2. Preparation Antibody

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

Prepare serial dilutions of the control antibody in a master dilution plate by making 11-point, 3-fold serial dilutions in AssayComplete Protein Dilution Buffer or another appropriate dilution buffer. The 96-well dilution plate should be prepared at 10X of the final screening concentration.

2.1. Add 40 µL of PDB to Wells A1 to A12 of the master dilution plate.

2.2. The total volume of test or control antibodies should be 60 µL. Therefore, add an appropriate volume of PDB to Well A1, such that the total volume of the diluted antibody is 60 µL.

2.3. Add the appropriate volume of control or test antibody to Well A1. Mix well by slowly pipetting up and down.

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

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

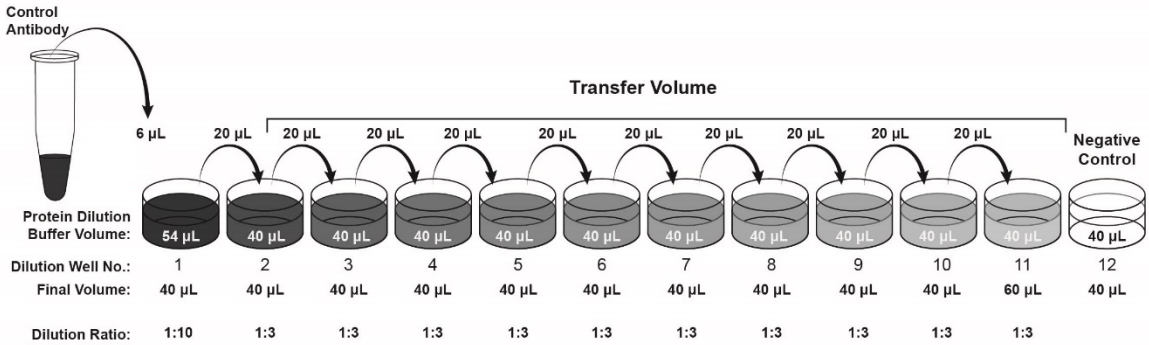


Figure 3. Antibody Dilution Series: Make eleven 3-fold serial dilutions of the control antibody in a master dilution plate.

3. Addition: Antibody

3.1. Transfer 10 µL of each 10X antibody serial dilution prepared in Step 2 into the appropriate wells of the 96-well assay plate. Figure 4. Representative Assay Plate Map shows the recommended format of sample addition to the plate in duplicates.

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

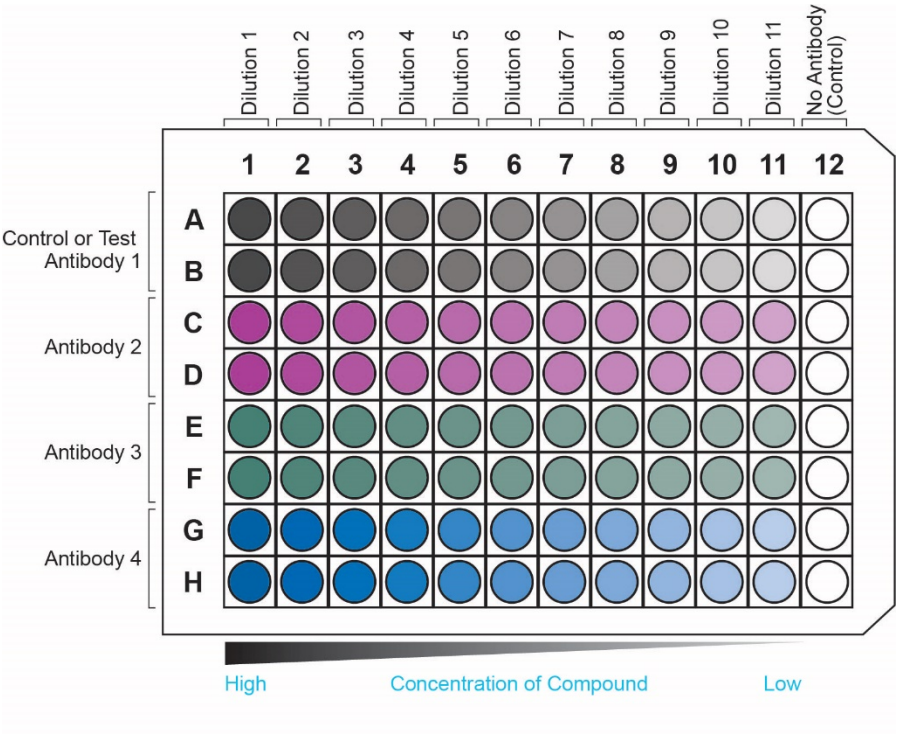


Figure 4. Representative Assay Plate Map: An example of 11-point serial dilution curves in duplicate with four different antibodies.

4. Addition: Ligand-Presenting Cells

Note: If anti-ligand antibodies are to be tested, the cell plating order should be reversed, i.e. ligand cells should be added to the assay plate first, followed by addition and incubation with the antibody, followed by the addition of receptor cells.

4.1. Ensure that the ligand-presenting cells (e.g. U2OS PD-L1 for the PD-1 Signaling Assay) in culture ae in log phase growth at the time of use in the assay.

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

4.3. Dissociate the ligand-presenting cells and resuspend them in fresh media.

4.3.1. Aspirate the media from T75 flasks.

4.3.2. Add 10 mL of room temperature PBS into each flask, and swirl it to rinse the cells.

4.3.3. Aspirate PBS from the flask.

4.3.4. Dissociate 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.

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

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

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

4.4. Remove an aliquot of the ligand-presenting cells from the flask and determine the cell density (e.g. using a hemocytometer or cell counter).

4.5. Transfer an appropriate volume of cell suspension into 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.

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

4.7. Resuspend the cell pellet in AssayComplete Cell Plating Reagent. Based on the number of cells in the pellet, as determined in Step 4.4, 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 cell/mL. For the recommended concentration of ligand-presenting cells, see the cell-line specific datasheet.

4.8. Using a multichannel pipette and reagent reservoir, add 45 µL of the ligand-presenting cell suspension into all wells of the 96-well assay plate.

4.9. Incubate the assay plate at the recommended time and temperature, as indicated on the cell line-specific datasheet.

5. Detection

5.1. Add 10 µL of PathHunter Bioassay Detection Reagent 1 to each well of the assay plate.

5.2. Incubate the assay plate for 15 minutes at room temperature in the dark. The PathHunter Detection Reagents are light sensitive, therefore incubation in the dark is necessary.

5.3. Add 40 µL of PathHunter Bioassay Detection Reagent 2 to each well of the assay plate.

5.4. Incubate the assay plate at room temperature in the dark, for the time indicated on the cell line-specific datasheet.

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.

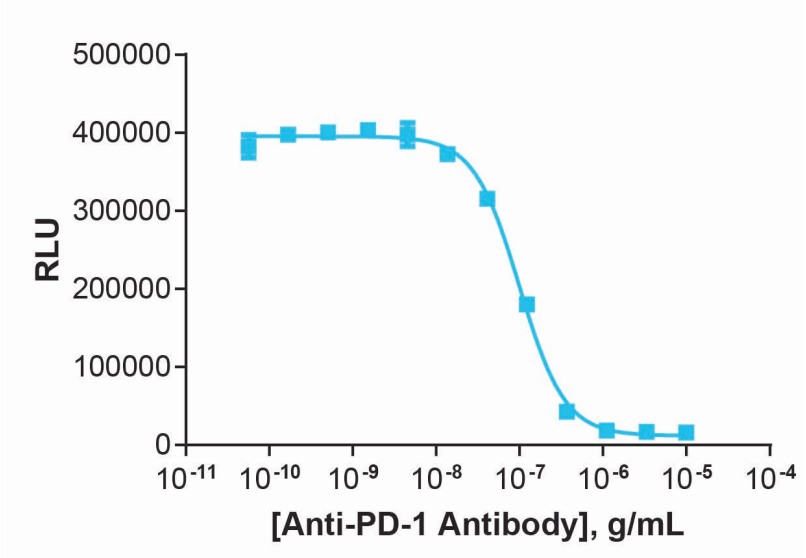
Data analysis can be performed using any statistical analysis software, such as GraphPad Prism, Softmax Pro, Gen5, Microsoft Excel, etc.

Typical Results

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

A.



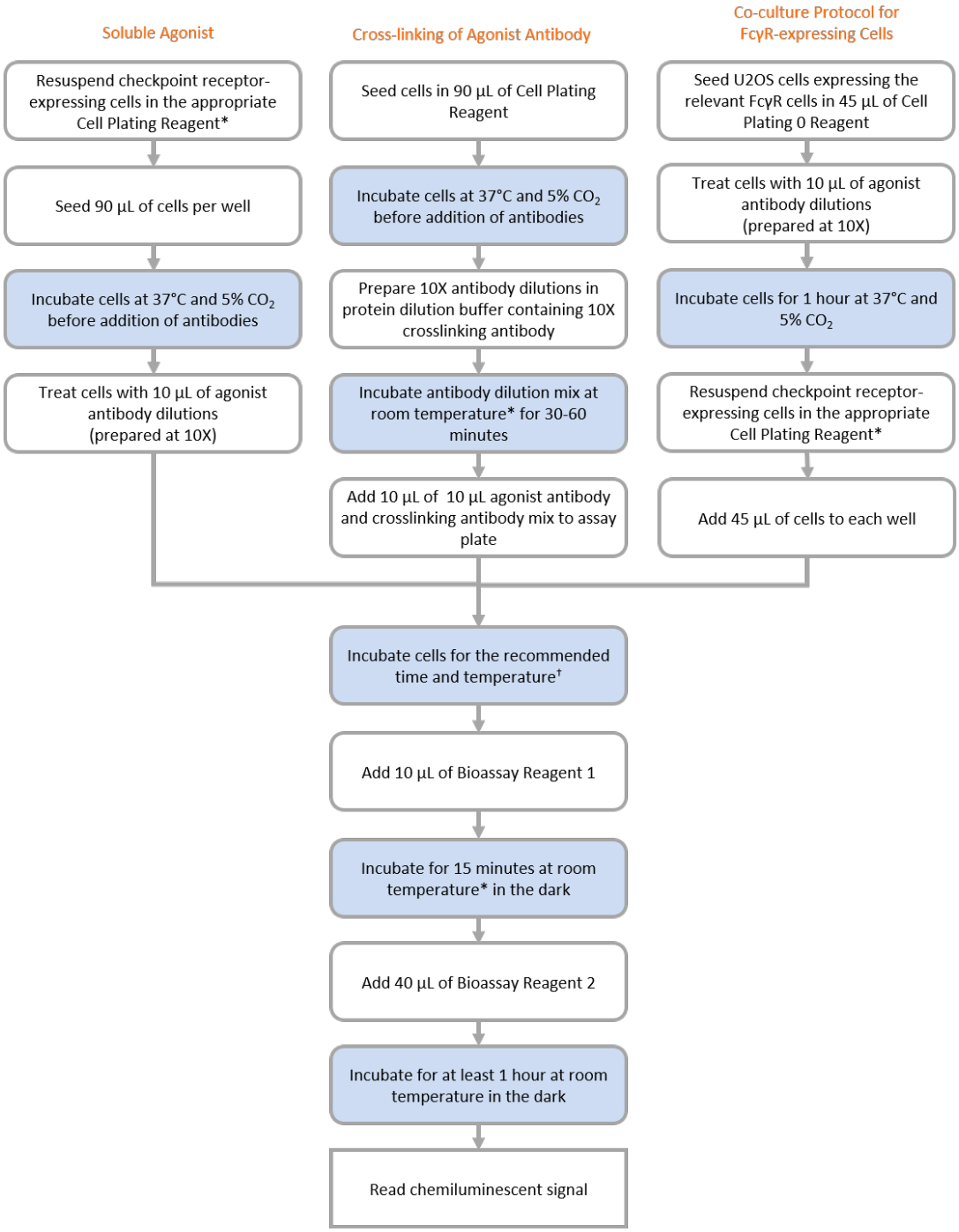
B.

EC_{50} (ng/mL)	99.6
S/B	25.2

Figure 5. Typical Results: Representative **A**, dose-response curve and **B**, EC_{50} and assay window for inhibition of PD-1 activation by an anti-PD-1 antibody, as measured in this assay.

Protocol Schematic: Signaling Assay for Activating Checkpoint Receptors

Quick-start procedure: In a 96-well tissue culture-treated plate, perform the following steps.



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

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

Detailed Assay Protocol: Signaling Assay for Activating Checkpoint Receptors

The following detailed protocol is specific for evaluating the ability of Fc-conjugated proteins or agonistic anti-receptor antibodies to activate checkpoint receptor signaling in the PathHunter Checkpoint Receptor Signaling Assays in a 96-well plate format.

For product specific information on AssayComplete™ Cell Culture Medium, AssayComplete Cell Plating Reagent, Control Antibody, incubation times and temperature, refer to the cell line-specific datasheet.

1. Preparation and Plating: Checkpoint Receptor Cells

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

1.2. Take an aliquot of the checkpoint receptor signaling cell line (in suspension) from the flask to count cell density.

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

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

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

1.6. Resuspend the cell pellet at the desired concentration (e.g. to achieve 50,000 cells/well in 90 µL resuspend cells at 0.55 x 10^6 cells/ mL) in pre-warmed AssayComplete Cell Plating Reagent. Note: Refer to the cell line-specific datasheet for recommended cell density in the assay.

1.7. Using a multichannel pipette and reagent reservoir, seed 90 µL of the cell suspension into one 96-well assay plate. Optionally, test samples can be run on multiple plates, in triplicates or other variations

1.8. Incubate assay plate in a 37°C and 5% CO_2 humidified tissue culture for the amount of time, as indicated in the cell line-specific datasheet, until ready to add the antibody dilution series.

2. Preparation: Antibody

2.1. Prepare serial dilutions of the antibody to be tested, as described in 2. Preparation: Antibody in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors.

3. Addition: Antibody

3.1. Add the antibody serial dilutions to the assay plate, as described in 3. Addition: Antibody in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors. Refer to Figure 4. Representative Assay Plate Map for the recommended format of antibody addition to the assay plate.

4. Detection

4.1. Add 10 µL of PathHunter Bioassay Detection Reagent 1 to each well of the assay plate.

4.2. Incubate the assay plate for 15 minutes at room temperature in the dark. The PathHunter Bioassay Detection Reagents are light sensitive, therefore incubation in the dark is necessary.

4.3. Add 40 µL of PathHunter Bioassay Detection Reagent 2 to each well of the assay plate.

4.4. Incubate the assay plate for at least 1 hour at room temperature in the dark. Refer to the cell line-specific datasheet for the recommended incubation time.

5. Reading: Assay Plate

5.1. Read assay plates as described in 6: Reading: Assay Plate in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors.

Detailed Assay Protocol: Cross-Linking Antibodies and Fc-Fusion Proteins

The following is a recommended protocol for testing the ability of cross-linked Fc-fusion proteins or agonistic anti-receptor antibodies to activate checkpoint receptor signaling in the PathHunter Checkpoint Receptor Signaling Assays in a 96-well plate format. Refer to cell line-specific data sheet for information regarding AssayComplete™ Cell Culture Media, Cell Plating Reagent, control ligand, incubation times and temperature required for each cell line.

1. Preparation and Plating: Activating Checkpoint Receptor Cells

1.1. Prepare and plate Activating Checkpoint Receptor Cells as described in Step 1 of the Detailed Assay Protocol: Signaling Assay for Activating Checkpoint Receptors.

2. Preparation: Cross-Linking Antibody

2.1. Prepare a stock solution of cross-linking antibody (e.g. anti-human Fc IgG antibody or other relevant antibody) at a concentration of 50-100 µg/mL in AssayComplete Protein Dilution Buffer. This will serve as the antibody dilution buffer for test and control antibodies.

2.2. The high dose of the dilution series should be prepared by mixing appropriate volumes of the primary test or control antibody with the cross-linking antibody in AssayComplete Protein Dilution Buffer such that the primary antibody is 10X the final high concentration and the cross-linking antibody is at 50-100 µg/mL. The subsequent serial dilutions of the primary test or control antibody in the 96-well dilution plate should use the cross-linking antibody solution generated in the step above to produce an 11-point, 3-fold dilution series prepared at 10X the final screening concentration. The control antibody dilution series will be in Rows A and B of the 96-well assay plate. The test antibody dilution series will be in Rows C and D of this 96-well assay plate. The final concentration of cross-linking antibody in the assay wells will be 5-10 µg/mL.

2.3. Incubate the dilution plate at room temperature for 30-60 minutes.

3. Addition: Cross-Linking Antibody

3.1. Add 10 µL of each 10X antibody serial dilution mixture to the appropriate number of replicate wells of plated cells.

3.2. Incubate the assay plate at 37˚C and 5% CO_2 incubator for the time indicated on the cell line-specific datasheet.

Add detection reagents and read assay plates as described in Steps 5 and 6 in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors.

Detailed Assay Protocol: Cross-linking Agonist Antibodies by Co-culture with FcγR-Expressing Cells

The following detailed protocol incorporates cross-linking of agonist antibodies in a physiologically relevant manner by co-culture with FcγR-expressing cells to enhance the response of activating checkpoint receptors in a 96-well format cell-based assay

1. Preparation and Plating: FcγR-Expressing Cells

1.1 Prepare and plate a fixed concentration (e.g. 20,000/45 µL) of appropriate FcγR-expressing cells in AssayComplete™ Cell Plating 0 Reagent as a 2.2X stock to achieve the recommended final density, as indicated in the cell line-specific datasheet (e.g. 10,000/well of FcγR expressing cells).

2. Preparation and Addition: Agonist Antibody

2.1 Prepare agonist antibody dilutions as described in 2. Preparation: Antibody in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors.

2.2 Add 10 µL of agonist antibody dilutions to plated FcγR cells.

2.3 Incubate cells for 1 hour at 37°C and 5% CO_2.

3. Preparation: Activating Checkpoint Receptor Cells

3.1. Prepare assay cells as described in 1. Preparation and Plating: Checkpoint Receptor Cells in the Detailed Assay Protocol: Signaling Assay for Activating Checkpoint Receptors. Cells should be plated in a final volume of 45 µL per well, at the density recommended in the cell line datasheet.

4. Addition: Activating Checkpoint Receptor Cell Addition

4.1. Add 45 µL of the prepared activating checkpoint receptor cells to the appropriate wells of plated FcγR cells containing agonist antibody as indicated on the previously described assay plate map.

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

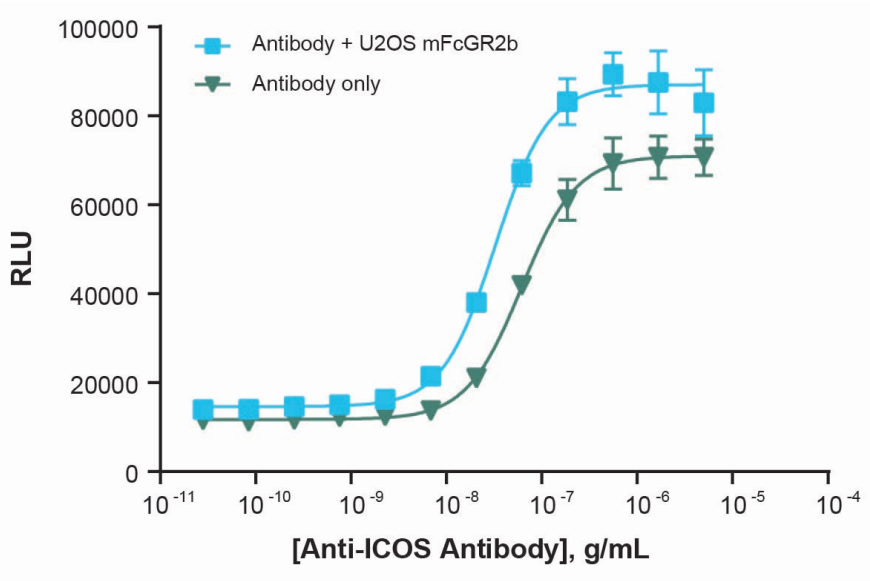
Add detection reagents and read assay plates as described in Steps 5 and 6 in the Detailed Assay Protocol: Signaling Assay for Inhibitory Checkpoint Receptors.

Typical Results

The following graph is a typical example of a dose-response curve for the PathHunter ICOS Signaling Assay used with or without FcγR cells. Using the protocol outlined in this user manual, anti-ICOS activating antibody presented with a U2OS mFcγR2b-expressing cell line shows a potent, dose-dependent activation of ICOS-expressing Jurkat Cells than with anti-ICOS antibody alone.

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

A



B

	Antibody Only	Antibody + U2OS mFcγR2b
EC_{50} (ng/mL)	61.4	26
S/B	6.3	6.4

Figure 6. Typical Results: Representative **A**, dose-response curve and **B**, EC_{50} and assay window for activation of ICOS-expressing Jurkat cells with anti-ICOS antibody, as well as with antibody presented with U2OS mFCγR-expressing cell line, as measured in this assay.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Sub-optimal cell growth conditions	Refer to the cell line-specific datasheet for cell culture conditions
	Incorrect control ligand/antobody used or incorrect incubation time	Refer to the datasheet for recommended agonist/antibody and assay conditions.
	Incorrect preparation of the control ligand/antibody	Refer to the cell line-specific datasheet to ensure proper handling, dilution, and storage of the ligand/antibody.
	Sub-optimal time course for induction	Optimize time course of induction with control ligand/antibody.
Decreased or no response	High passages give reduced performance	PathHunter cells are stable for at least 10 passages. Use low passage cells wherever possible.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should ideally 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 optimal cell culture conditions.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 second/well.
Experimental S/B does not match the value noted in the datasheet	Incorrect incubation temperature	Confirm assay conditions.
		Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Sub-optimal incubation time	Refer to the cell line-specific datasheet and optimize incubation time with the control ligand/antibody
	Incorrect ratio of ligand: receptor-expressing cells	Ensure that cell counts for both cell types are accurate. Ensure that botch cell types are growing in log phase before using in the assay.
EC_{50} is right-shifted	Improper storage of ligand/antibody	Follow directions for ligand/antibody storage as indicated in the ligand datasheet. Store in small aliquots at -20°C where appropriate. Do no freeze/thaw more than twice.
	Incorrect top dose for the dose-response curve	Refer to the dose-response curve on the cell line-specific datasheet for appropriate top concentration of the ligand/antibody
	Quality of ligand/antibody used in the assay	Antibodies from different vendors will perform differently in the assay. Use Eurofins DiscoverX-supplied ligand/antibody as a positive control to ensure that the assay works well before testing molecules from other sources.
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may diluted in buffer containing 1% BSA.
		Non-binding surface plates may be necessary for hydrophobic compounds.
High well-to-well variability in Z' study	Problems with plate type and compound stability	Z' studies should be performed with automation.
		It may be necessary to test plate types and compound stability.

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

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