User Manual

PathHunter® Internalization Assays

For Detection of Single-Pass Membrane Receptor Internalization

Document Number 70-413 Revison 0

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

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Overview

PathHunter Internalization Assays are cell-based assays that measure internalization of single-pass membrane receptors, e.g. receptor tyrosine kinases (ErbB-2), immune checkpoint receptors (CD33), mediated by small molecule or biologic therapeutics. Measuring drug-mediated receptor internalization can provide insight into diseases, drug tolerance, unwanted side effects and helps in identifying safer drugs. These receptor internalization assays are valuable tools for therapeutics that require internalization, which is used as a key determinant for their efficacy, such as for antibody-drug conjugates (ADCs).

These assays utilize stable cell lines and a simple, homogenous protocol and can easily be implemented in a high-throughput format.

Assay Principle

The PathHunter Internalization Assay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, ProLink (PK) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

In this assay, the ProLink tag is fused to the C-terminus of the membrane-bound target protein, while the EA is localized to the early endosome. Antibody binding to the target induces internalization and transport of the receptor-antibody complex to the early endosome where the target protein-PK fusion protein co-localizes with EA in the endosome, resulting in complementation of PK and EA to form an active β-gal enzyme. Enzymatic activity is quantitatively measured using a chemiluminescent substrate in the PathHunter Detection Kit.

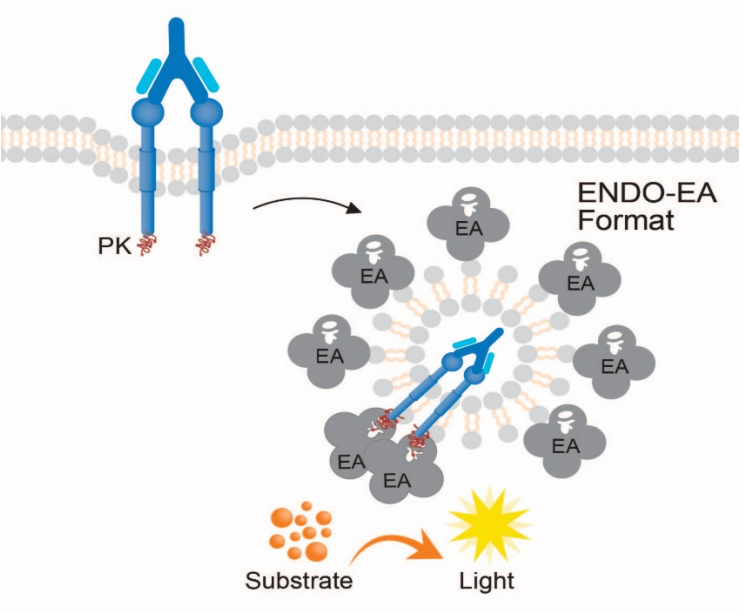


Figure 1. PathHunter Internalization Assay Principle: An example of measurement of receptor internalization using the internalization assay platform. Antibody binding to the PK- tagged target receptor induces internalization and transport via endosomes, which are tagged with the enzyme acceptor fragment, and the formation of a functional β-gal enzyme. The active enzyme then hydrolyzes the substrate and generate 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.

Contact technical support immediately if cells received were already thawed.

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
PathHunter Flash Detection Kit	93-0247
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. Hold the vials using the cap.. do not touch the sides of the via.....(please use standard language for this warning)

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

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 in a 37°C water bath briefly (30 seconds to 1 minute), 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 solution.

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	1:2

For example, for U2OS cells, add 1 mL of the AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 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.

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

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 recommended/appropriate Cell Culture Kit, recommended selection antibiotics, 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.

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 gently 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 solution.

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. Using a pipet, 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 flask (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 volume (0.5 mL or less) of the suspended cells in a separate tube.

    12.2. Transfer an appropriate portion of this cell suspension 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).

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

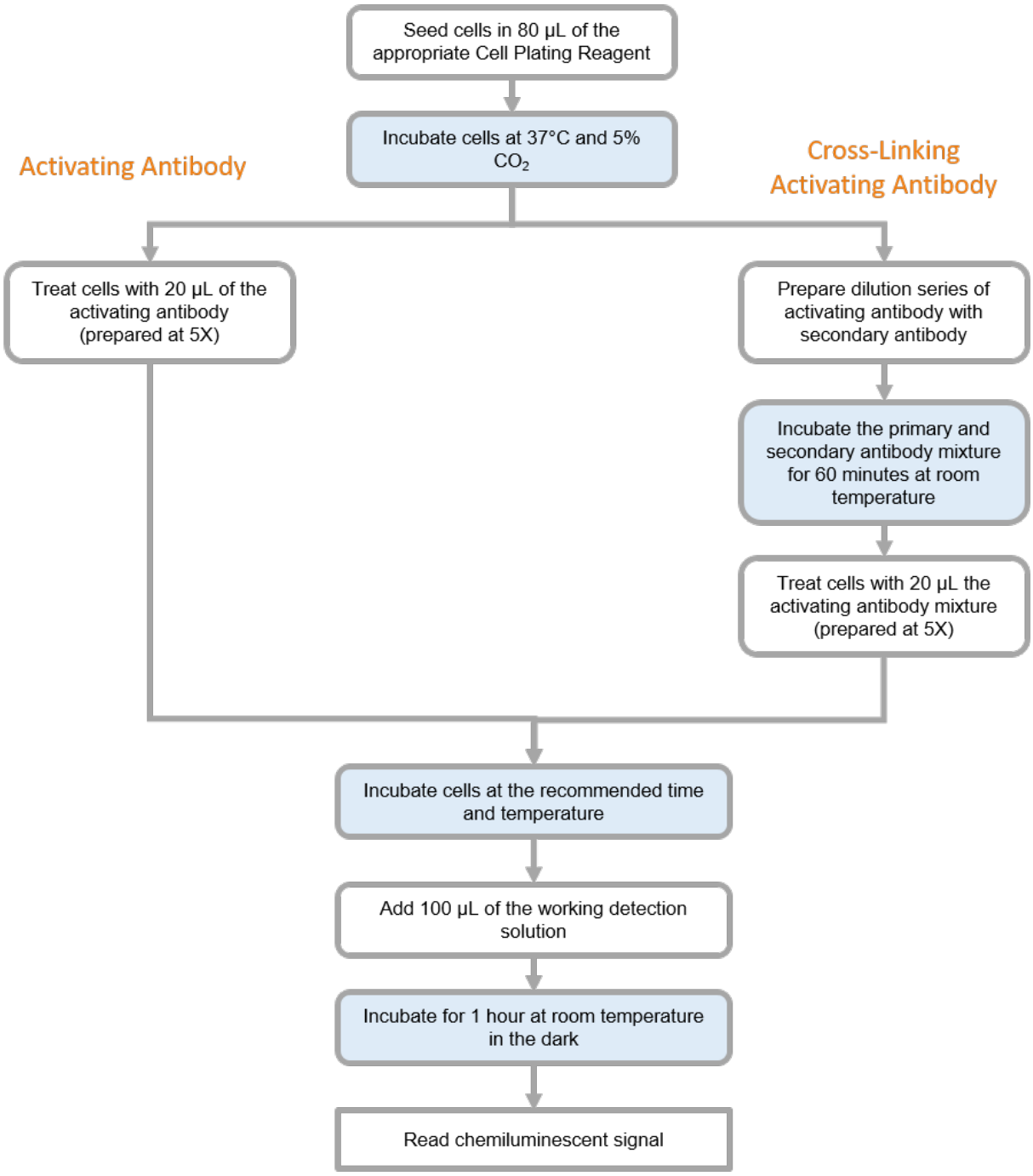
16. Make aliquots by transferring 1 mL of cell suspension into the labeled 2 mL cryogenic tubes. Seal the tubes tightly.

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.

Protocol Schematic

Quick-Start Procedure: Based on whether a cross-linking secondary antibody is required for the assay, 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 appropriate detection reagent.
‡Room temperature refers to a range of 23-25°C.

Detailed Assay Protocol: Activating Antibody

The following protocol is for evaluating the response with an activating antibody (an antibody that mediates receptor internalization) in a 96-well plate. Refer to the table below for assay reagent volumes.

Assay Reagents (Volume per Well)	96-Well Plate*	384-Well Plate**
Cell Plating Volume (µL)*	80	20
Activating Antibody (µL)	20	5
Working Detection Solution (µL)	100	25
Total Volume (µL)	**200**	**50**

* 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 of 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 mL of AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL for T225 flasks). 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 an 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. 62,500 cells/mL or 5,000 cells/80 µL). 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 80 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 the agonist antibody preparation.

2. Preparation: Activating Antibody

When optimizing assay conditions, it is recommended to include a standard curve of the control antibody to verify that the kit components are working optimally. The following protocol is for setting up dilution series for an activating antibody.

Prepare serial dilutions of the control antibody in the master dilution plate (96-well, V-Bottom, Untreated, Non-Sterile Dilution Plate) by making a 3-fold, 11-point serial dilution in the AssayComplete Protein Dilution Buffer (PDB), or an appropriate antibody dilution buffer (as specified on the antibody datasheet). The control activating antibody should be prepared at 5X the final screening concentration.

2.1. Add 60 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.

2.2. Prepare the control antibody in PDB (or an antibody-specific buffer). We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the antibody. Therefore, prepare a working concentration that is 1250X the expected EC_{50} per well to get a 5X working concentration. For example, for an expected EC_{50} of 10 ng/mL, prepare the highest working concentration at 12.5 µg/mL. This is 5X the final top concentration of 2500 ng/mL, and the expected EC_{50} will lie near the center of the dose-response curve.

2.3. Add 90 µL of the highest concentration of the control antibody to Well A1 of the master dilution plate.

2.4. Using a clean pipette tip, transfer 30 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 30 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.

2.5. 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.

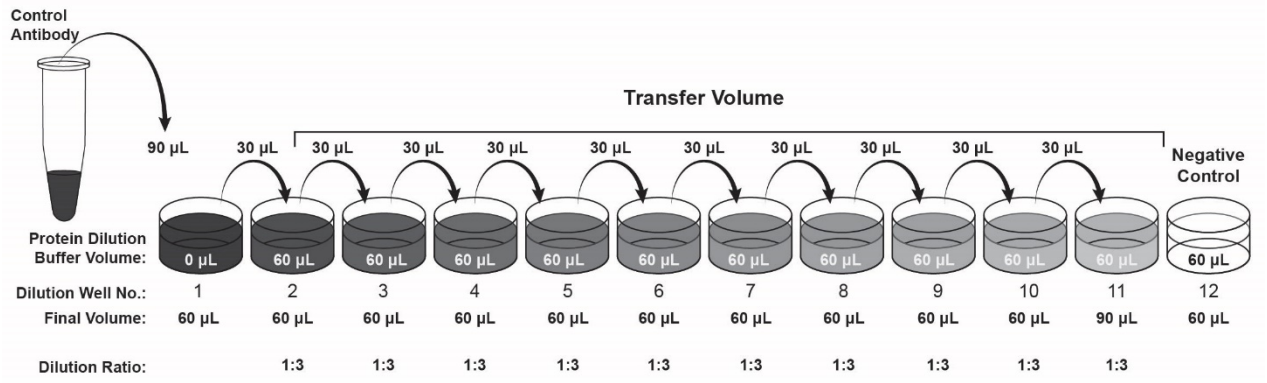


Figure 2. Antibody Serial Dilutions: Make 11 three-fold serial dilutions of the control antibody in a separate dilution plate.

3. Addition: Activating Antibody

3.1. Transfer 20 µL of the 5X activating antibody serial dilutions to the appropriate wells of the 96-well assay plate, as indicated on the Figure 3: Representative Assay Plate Map (e.g. control antibody in rows A and B; test antibody in rows C and D)

3.2. Cover the plate with a lid and incubate for the recommended time and temperature.

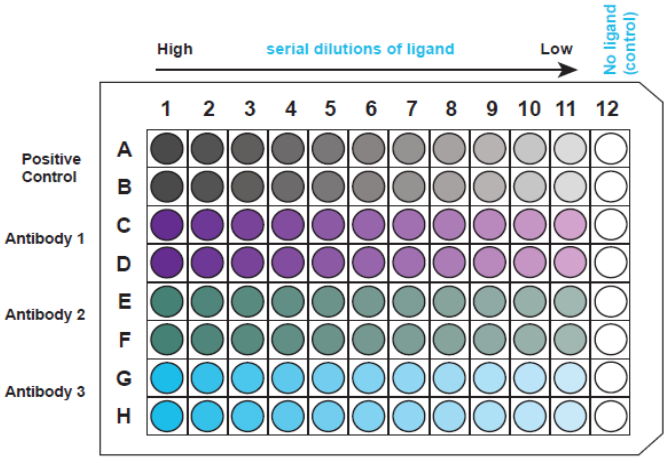


Figure 3. Representative Assay Plate Map: This plate map shows an 11-point dose curve, with 2 data points at each concentration for one reference and three test samples per plate with a 1:3 serial dilution scheme.

4. Detection

4.1. Prepare a stock of the working detection solution in a 15 mL polypropylene tube or a reagent reservoir. The PathHunter Flash detection kit includes Flash Cell Assay Buffer and Flash Substrate. Mix the kit components in the ratio indicated in the table below:

Working Flash Detection Solution for a 96-well Format		
Components	Volume Ratio	Volume per Plate (mL)
Flash Cell Assay Buffer	1	2.5
Flash Substrate	4	10
Total Volume		**12.5**

4.2. 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.

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

The Working Detection Solution is light sensitive, thus incubation in the dark is necessary.

5. 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.

The actual signal characteristics are affected over time by lab conditions such as temperature. The user should establish an optimal read time accordingly. Luminescence readers collect signal from all wavelengths. Some instrument manufacturers may include a cut-off filter at higher wavelengths, but usually wavelength setting is not required for luminescence readout.

To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

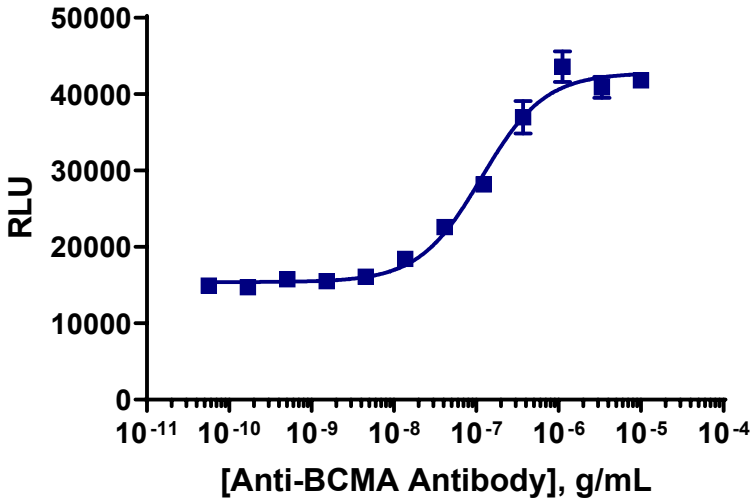
Data analysis can be performed using any statistical analysis software, such as GraphPad Prism, Softmax Pro, Gen5, Microsoft Excel, etc.

Typical Results

The following graph is an example of a dose-response curve for the PathHunter U2OS BCMA Internalization Assay generated using the protocol outlined in this user manual. The data shows dose-dependent BCMA internalization induced by a commercial anti-BCMA 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)	116
S/B	2.8

Figure 4. Typical Results: Representative **A**, dose-response curve and **B**, the EC_{50} and assay window observed for BCMA internalization when stimulated by treatment with an anti-BCMA antibody.

Detailed Assay Protocol: Activating Antibody with Cross-Linking

The following protocol is designed for evaluating activating antibody responses with the addition of cross-linking by using a secondary antibody to cluster the target antibody. Experiments with cross-linked activating antibodies are run in a 96-well tissue culture plate, and use a 5X stock of the activating antibody dilution series prepared with a fixed concentration of the cross-linking antibody. Refer to the table below for assay reagent volumes.

Assay Reagents (Volume per Well)	96-Well Plate*	384-Well Plate**
Cell Plating Volume (µL)*	80	20
Cross-linked Antibody Mix (µL)	20	5
Working Detection Solution (µL)	100	25
Total Volume (µL)	**200**	**50**

*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 of Cells

1.1. Prepare and plate the cells as described in section 1. Preparation and Plating: Adherent Cells in the Detailed Assay Protocol: Activating Antibody

2. Preparation and Addition: Cross-Linked Antibody

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

2.2. The highest 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 PDB, such that the primary antibody is 5X the desired final concentration and the cross-linking antibody is at 50-100 µg/mL.

2.3. 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 Step 2.1 above to produce an 11-point, 3-fold dilution series prepared at 5X 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 the assay plate (refer to Figure 3: Representative Assay Plate Map). The final concentration of the cross-linking antibody in the assay wells will be 10-20 µg/mL.

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

2.5. Add 20 µL of each 5X antibody serial dilution mixture to the appropriate number of replicate wells of plated cells.

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

3. Detection and Plate Reading

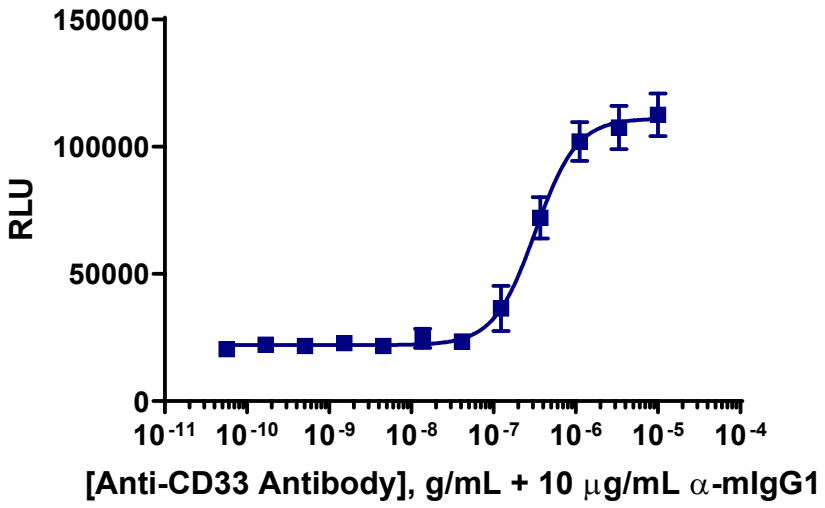
Add detection reagents and read assay plates, as described in Steps 4 and 5 in the Detailed Assay Protocol: Activating Antibodies.

Typical Results

The following graph is a typical example of a dose-response curve for the PathHunter U2OS CD33 Internalization Assay with cross-linking generated using the protocol outlined in this user manual. A commercial anti-CD33 antibody was used in combination with a secondary antibody for crosslinking to stimulate CD33 receptor internalization. The data shows potent, dose-dependent CD33 internalization when incubated with the cross-linked anti-CD33 antibody.

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

A.



B.

EC_{50} (ng/mL)	320.9
S/B	5.4

Figure 5. Typical Results: Representative **A**, dose-response curve and **B**, the EC_{50} and assay window for CD33 internalization with cross-linking.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Sub-optimal cell growth conditions	Refer to the datasheet for cell culture conditions.
	Incorrect activating antibody used or incorrect antibody incubation time	Refer to the datasheet for recommended activating antibody and assay conditions.
	Incorrect preparation of activating antibody	Refer to the specific datasheet to ensure proper handling, dilution, and storage of antibody.
	Sub-optimal time course for induction	Optimize time course of induction with control antibody.
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	Antibody not stored properly	Follow directions for antibody 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 antibody used in the assay	Differences in antibody vendors can also affect assay performance - be sure to use DiscoverX supplied antibody as a positive control to ensure that the assay works well in your hands before testing ligands or molecules from other sources.
	Problems with plate type.	Hydrophobic samples should be tested for solubility and may be diluted in buffer containing 0.1% BSA.
	Problems with sample stability.	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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