User Manual

PathHunter® Jurkat SIRPα Signaling Bioassay Kit

For Detection of CD47-Induced SIRPα Signaling

For Bioassay Kits:

93-1135Y19-00129: 2-Plate Kit

93-1135Y19-00130: 10-Plate Kit

Document Number 70-411 Revision 2

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

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Overview

The PathHunter SIRPα Signaling Bioassay Kit provides a simple, highly specific assay to enable discovery and development of small molecule and biologic drugs targeting the CD47-SIRPα signaling axis. The bioassay kit contains all the materials needed for a complete assay, including cells, detection reagents, cell plating reagent, positive control antibody, and assay plates. The cryopreserved cells have been manufactured to ensure long-term assay reproducibility, and faster implementation from characterization to QC lot release. This bioassay has been optimized for a 96-well format. The protocol can be optimized further for running the assay in a high-throughput 384-well format.

Assay Principle

This bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, an Enzyme Donor (ED) and an Enzyme Acceptor (EA). Independently these fragments have no β-gal enzymatic activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

The PathHunter SIRPα Signaling Bioassay consists of human cells engineered to stably express an ED-tagged SIRPα receptor, while EA is fused to the phosphotyrosine-binding SH2 domain of the intracellular signaling protein, SHP1. Ligand or antibody-induced activation of the receptor 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 the formation of an active β-gal enzyme, which hydrolyzes the substrate to produce a chemiluminescent signal.

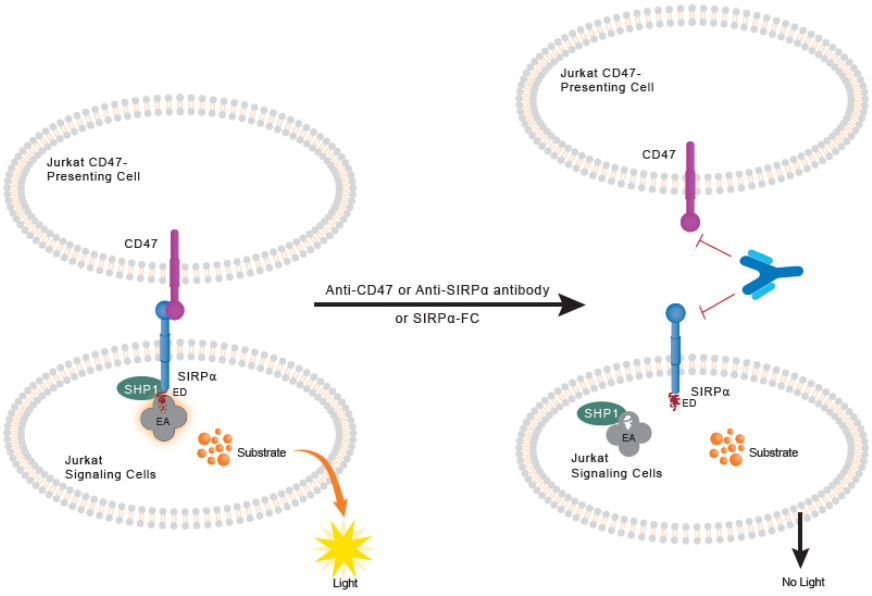


Figure 1: Assay Principle: A full-length SIRPα receptor is engineered with the ED fragment fused to its C-terminus, and an SH2-domain of SHP-1 is engineered with the complementary EA fragment. These constructs are stably co-expressed in Jurkat cells, while the untagged full-length ligand, CD47, is stably expressed in ligand-presenting cells. Ligand engagement, through co-culture with ligand-presenting cells, results in phosphorylation of the checkpoint receptor-ED fusion protein, leading to the recruitment of SHP-1-EA, forcing complementation of the EFC components to create an active β-gal enzyme. This active enzyme hydrolyzes substrate to create a chemiluminescence signal as a measure of receptor activity. In the presence of an anti-receptor or anti-ligand antibody, or a checkpoint receptor-FC fusion protein, the ligand-receptor engagement and EFC complementation are both disrupted and no chemiluminescence is observed.

Materials Provided

List of Components	93-1135Y19-00129	93-1135Y19-00130
PathHunter Jurkat SIRPα Signaling Bioassay Cells (3×10^6 cells in 0.2 mL per vial)	2	10
PathHunter Jurkat CD47-Presenting Ligand Bioassay Cells (5×10^6 cells in 0.2 mL per vial)	2	10
AssayComplete™ Cell Plating 0 Reagent (100 mL per bottle)	1	3
Anti-CD47 Antibody (20 µg per vial)	1	1
PathHunter Bioassay Detection Kit Detection Reagent 1 (bottle) Detection Reagent 2 (bottle)	 1 x 3 mL 1 x 12 mL	 1 x 15 mL 1 x 60 mL
96-Well White, Clear Flat-Bottom TC Treated, Sterile Plates with Lid	2	10

Storage Conditions

PathHunter Jurkat SIRPα Signaling Bioassay Cells and PathHunter Jurkat CD47-Presenting Ligand Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of bioassay cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately if the cells received were already thawed.

- Short-term (less than 24 hours): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (longer than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

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

AssayComplete™ Cell Plating 0 Reagent

Upon receipt, store at -20ºC. Once thawed, the Cell Plating Reagent can be stored at 4ºC for up to 4 weeks. For longer storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20ºC until needed. Avoid multiple freeze-thaw cycles.

To make aliquots suitable for testing one assay plate each, 25 mL of reagent per aliquot can be dispensed and frozen down.

Anti-CD47 Antibody

Upon receipt, store at -20ºC until ready to use (up to the expiration date listed in the kit's Certificate of Analysis). Centrifuge the vial prior to opening, to maximize recovery.

PathHunter Bioassay Detection Kit

Upon receipt, store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For long-term storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

For the ten-plate kit: If all the plates will not be used at the same time, we recommend making five aliquots for each of the two detection reagents. Each aliquot will be adequate for two assay plates. Make five aliquots of 3 mL each for Detection Reagent 1, and five aliquots of 12 mL each for Detection Reagent 2. Sufficient reagent volumes are provided in the kit to make these aliquots.

If the reagents will be used for a single plate, then the remaining detection reagents can be frozen. The detection reagents can be thawed and frozen for a total of three times without loss in performance.

96-Well White, Clear Flat-Bottom Tissue Culture-Treated Plates

Store at room temperature.

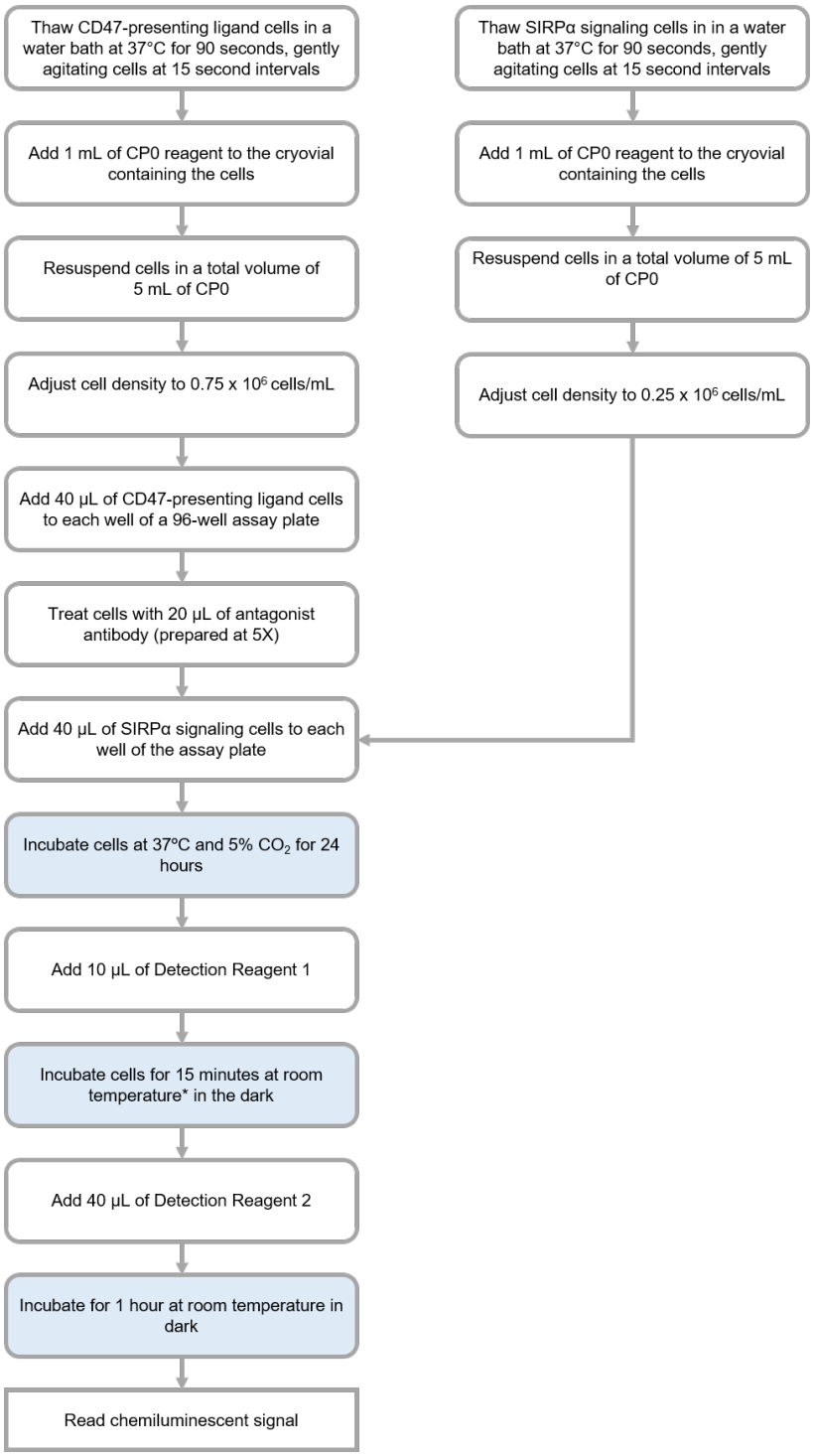
Additional Materials Required

The following equipment and additional materials are required to perform this assay:

Materials	**Ordering Information**
A 96-well green, V-bottom, untreated, non-sterile dilution plate	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micropipettors and pipette tips (20 µL – 1,000 µL)	
50 ml and 15 mL Polypropylene tubes	
1.5 mL Microcentrifuge tubes	
Tissue culture disposable pipettes (1 mL - 25 mL) and tissue culture flasks (T25 and T75 flasks, etc.)	
Water bath (37°C)	
Hemocytometer	
Humidified tissue culture incubator (37°C and 5% CO_2)	

Protocol Schematic

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



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

Detailed Protocol

The PathHunter SIRPα Signaling Bioassay is a co-culture model that utilizes cryopreserved SIRPα Signaling Bioassay Cells and CD47-Presenting Ligand Bioassay Cells to test therapeutic molecules that target the CD47-SIRPα signaling axis.

Day 1: Bioassay Cell Preparation

The following protocol is described for one assay plate that requires thawing and plating one vial each of the cryopreserved PathHunter Jurkat CD47-Presenting Ligand Bioassay Cells and Jurkat SIRPα Signaling Bioassay Cells.

1. Prior to thawing the cells, ensure that all the required materials are set up in the tissue culture hood. These include:
    a. Two sterile 25 mL reagent reservoirs, one labeled as ‘CD47’ and the other as ‘SIRPα’
    b. Two sterile 15 mL conical tubes, one labeled as ‘CD47’ and the other as ‘SIRPα’
    c. A pipette set to dispense 1 mL
    d. A multichannel pipette set to dispense 40 µL, and another one set to dispense 20 µL
    e. A bottle of AssayComplete™ Cell Plating 0 Reagent (CP0), pre-warmed in a 37°C water bath for 15 minutes
    f. A 96-Well White, Clear Flat-Bottom Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
    g. A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, to be used as the master dilution plate
2. Dispense 5 mL of pre-warmed CP0 into each of the labeled 15 mL conical tubes.
3. Remove the cryovials from the liquid nitrogen vapor and immediately place them in dry ice.
4. When ready to thaw the cells, place the vials in a floating rack. Ensure that there are no dry ice chips sticking to the vial surface, especially at the base of the vial.
5. Place the rack with the vials in a 37°C water bath for a total time of at least 90 to 100 seconds.
    5.1. After 30 seconds of placing the vials in the water bath, gently agitate floating rack for a few seconds, while keeping it in the water bath.
    5.2. Gently agitate the floating rack again for a few seconds, while keeping it in the water bath at 15 second intervals.
    5.3. At the end of the 90 second period, the cell pellet should be completely thawed.
6. Wipe the surface of the vial with 70% ethanol to disinfect and bring them into the tissue culture hood.

7. Add 1 mL of pre-warmed CP0 from each of the conical tubes to the corresponding cryovials. Add the reagent slowly along the walls of the cryovial tubes over a period of 5-10 seconds. Gently pipet up and down several times to uniformly resuspend the cells.

8. Transfer the cell suspension from each cryovial to the corresponding conical tubes, each containing the remaining 4 mL of CP0. Remove any remaining liquid from the cryovials to ensure maximum recovery of cells.

9. Count the number of viable cells for both CD47 and SIRPα cell suspensions, using a hemocytometer or an automated cell counter and determine the cell concentration.

    9.1. Dilute the Jurkat CD47-Presenting Ligand Bioassay cell suspension with CP0 to a final density of 0.75 x 10^6 cells/mL. Mix gently by slowly pipetting up and down. Place the tube in the tissue culture hood at room temperature for up to 30 minutes.

    9.2. Dilute the Jurkat SIRPα Signaling Bioassay cell suspension with CP0 to 0.25 x 10^6 cell/mL. Mix gently by slowly pipetting up and down. Place the tube in the tissue culture hood at room temperature up to 30 minutes.

    Note: If sample preparation will take longer than 30 minutes, the vial of the Jurkat CD47-presenting cells can be processed first and added to the assay plate, followed by sample preparation, and then the processing of the SIRPα signaling cell vial.

Day 1: Antagonist Antibody Preparation

The following protocol is designed for testing purified biologics. The PathHunter bioassay can also be run in the presence of high levels of serum or plasma without adversely impacting assay performance. In some assays, standard curves of the control can be prepared in neat serum or plasma and added directly to cells without further dilution. For best results, the optimized minimum required dilution of crude samples should be determined empirically.

A 1:2 dilution series of the control antibody (anti-CD47 antibody, included in the kit) has been used in this protocol. The volumes listed below are designed for running samples from one dose-response curve in duplicate on the assay plate (Refer to Figure 3: Representative Assay Plate Map). When evaluating the assay with your antibody, it is recommended to include a standard curve of the control antibody to verify that the assay is working optimally.

1. Add 100 µL of Cell Plating 0 Reagent (CP0) to Wells A2 to A12 of the master dilution plate.

2. Prepare the control antibody dose-response curve:
   The antibody dilutions are prepared at 5X the desired final concentration in the assay, with a top dose of 1 µg/mL, as it will be diluted 5-fold by adding 20 µL of antibody to 80 µL of the medium (40 µL from the ligand-presenting cells and 40 µL from the signaling cells) in the assay plate.

    2.1. Prepare serial dilutions of the control antibody in a 96-well master dilution plate by making 11-point, 2-fold serial dilutions in CP0:

        2.1.1. Prepare a 1:40 dilution of the control antibody in Well A1 of the master dilution plate: Dilute 5 µL of the supplied 200 µg/mL anti-CD47 antibody stock in 195 µL of CP0. This represents a 5X stock of the desired final highest concentration of 1 µg/mL.

2.1.2. Using a clean pipette tip, transfer 100 µL of the control antibody from Well A1 into Well A2 for a 1:2 dilution and mix thoroughly by pipetting up and down several times.

2.1.3. Replace the pipette tip and transfer 100 µL from Well A2 to A3, and mix well. Repeat this process until Well A11 is reached, resulting in an 11-point, 1:2 dilution series. No sample is transferred to Well A12, as this will serve as a negative control.

3. Set up serial dilutions for additional test antibodies in a similar manner, using new rows in the master dilution plate.

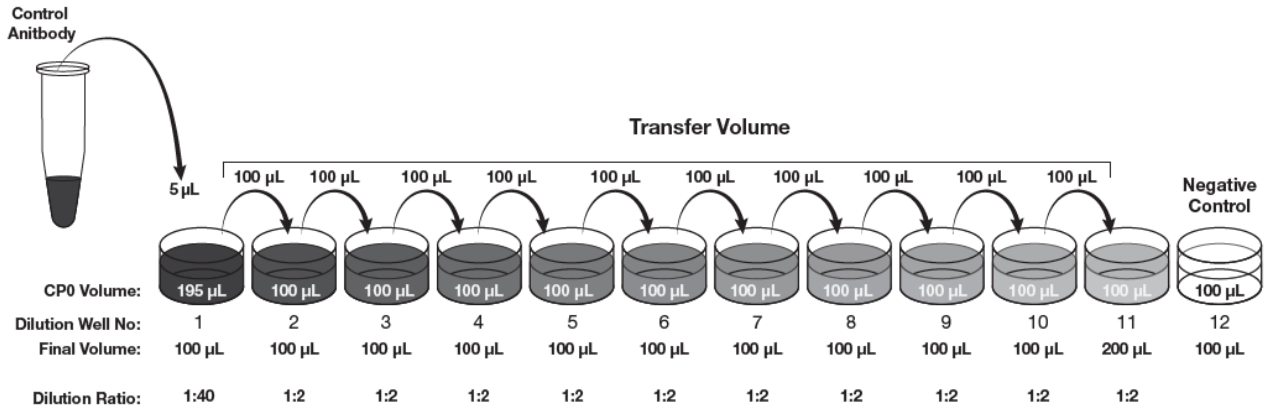


Figure 2. Agonist serial dilutions: Make serial dilutions of 1:2 for each antibody in a separate master dilution plate.

Day 1: Reagent Addition

1. Plating the CD47-Presenting Ligand Bioassay Cells:

1.1. Mix the cell suspension in the CD47-labeled conical tube by gently inverting the tube several times without introducing bubbles.

1.2. Transfer the contents of this tube into the sterile disposable reagent reservoir labeled as ‘CD47’.

1.3. Transfer 40 µL of the Jurkat CD47-Presenting Ligand Bioassay Cells to each well of the 96-well assay plate using a multichannel pipette.

2. Addition of Antibody Dilutions:

2.1. Transfer 20 µL of antibody dilutions prepared in Step 2 in Antagonist Antibody Preparation from the master dilution plate, to the appropriate wells of the 96-well assay plate, using a multichannel pipette. An example plate layout is shown below in Figure 3: Representative Assay Plate Map, where antibody from a single row of the master dilution plate would be aliquoted into 2 rows of the assay plate (e.g. Rows B and E).

3. **Plating the Jurkat SIRPα Signaling Bioassay Cells:**

   3.1. Mix the cell suspension in the SIRPα-labeled conical tube by gently inverting the tube several times without introducing bubbles.

   3.2. Transfer the contents of this tube into the sterile disposable reagent reservoir labeled as 'SIRPα'.

   3.3. Transfer 40 µL of the Jurkat SIRPα Signaling Bioassay Cells to each well of the 96-well assay plate using a multichannel pipette.

4. Place the lid on the assay plate. Gently mix the contents by slowly moving the plate back and forth 2-3 times on the surface of the tissue culture hood, in a criss-cross pattern.

5. Incubate the assay plate at 37ºC and 5% CO_2 in a humidified incubator for 24 hours.

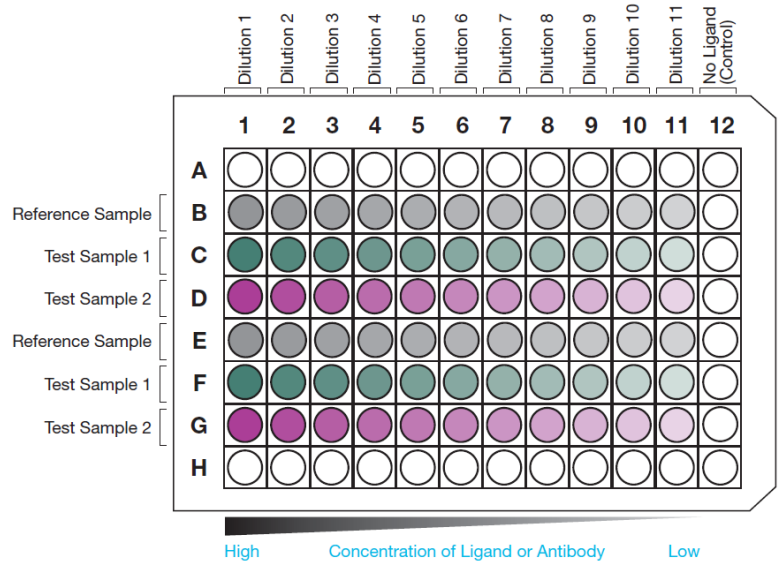


Figure 3. Representative Assay Plate Map: This plate map shows three interdigitated 11-point dose curves, with two replicates per dose point, for two test samples and one reference sample tested using the same dilution scheme.

Day 2: Detection

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

2. Incubate assay plate for 15 minutes at room temperature in the dark.

Detection Kit reagents are light sensitive, hence incubation in the dark is necessary.

3. Add 40 µL of Detection Reagent 2 to each well of the assay plate.

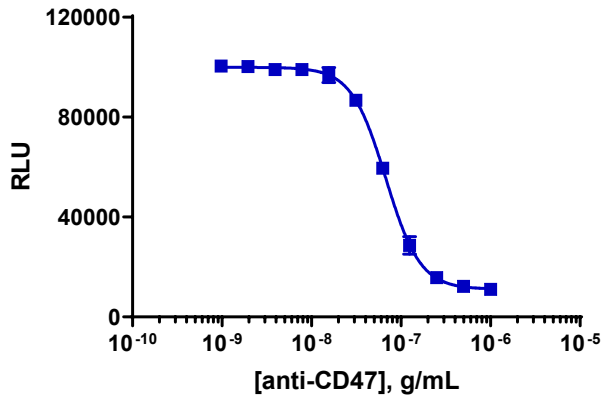
4. Incubate assay plate for 1 hour at room temperature in the dark.

5. 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 imager.

Typical Results

The following graph is an example of a typical dose-response curve for the SIRPα signaling bioassay generated using the protocol outlined in this manual. The data shows a potent, dose-dependent inhibition of the CD47-SIRPα signaling axis, when treated with the control anti-CD47 antibody.

A.



B.

IC_{50} (ng/mL)	67.6
S/B	9.1

Figure 4. Typical Results: Representative **A**, dose-response curve and **B**, the IC_{50} and assay window for the inhibition of the CD47-SIRPα signaling axis in the presence of an anti-CD47 antibody, as measured in this bioassay.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
Poor or no response	Incorrect thawing procedure	Refer to thawing instructions in the Bioassay Cell Preparation section of this user manual. The thawing process can have a significant impact on cell viability.
	Resuspended bioassay cells left at room temperature for too long	Resuspend bioassay cells can be incubated at room temperature (23-25°C) for 30 minutes (± 10 minutes) in a 15 mL conical tube. If preparation of antibody dilutions will take longer than 30 minutes (or if room temperature is >25°C), we recommend resuspending the ligand-presenting cells first, then thaw and process the signaling cell vial after preparation and addition of antibody dilutions to the assay plate.
	Incorrect ligand used or incorrect ligand incubation time	Refer to this user manual for recommended ligand/antibody and assay conditions.
	Incorrect preparation of control antibody	Refer to this user manual to ensure proper handling, dilution, and storage of control antibody.
	Sub-optimal time course for induction	Optimize time course of induction with ligand-presenting cells and reference antibody.
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 microplate reader	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 Certificate of Analysis	Incorrect incubation temperature	Confirm that correct assay conditions are used by consulting the protocol for this bioassay and Certificate of Analysis.
		Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
	Incorrect incubation time	Consult the Certificate of Analysis, and optimize incubation time with control ligand/antibody.
	Incorrect ratio of Ligand-presenting cells to Signaling cells	Ensure that the cell densities recommended for each of the bioassay cell types (ligand and signaling) are correct. Use of a hemocytometer to determine number of viable cells for both bioassay cell types is recommended.
IC_{50} is right- (or left-) shifted	Improper antibody handling or storage	Check antibody handling requirements.
	Incorrect ratio of Ligand-presenting to Signaling cells	Ensure that the cell densities recommended for each of the bioassay cell types (ligand-presenting and signaling) are correct. Use of a hemocytometer to determine number of viable cells for both bioassay cell types is recommended.
High variability between replicates	Contamination from tips	Changing tips during serial dilutions can help avoid carryover.
	Instrument calibration	The dispensing equipment should be properly calibrated, and the correct pipetting technique must be used.
		Non-binding surface plates may be necessary for hydrophobic compounds.

Poor detection of neutralizing antibodies	Incorrect ratio of ligand-presenting to signaling cells	To improve detection sensitivity of anti-drug neutralizing antibodies, a lower ratio of ligand will be desirable. Perform testing with altered ratios of Ligand-presenting: Signaling cells (e.g. 1:1 or 0.5:1).

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

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