User Manual

PathHunter® Sargramostim Bioassay Kit

For Chemiluminescent Detection of Sargramostim Activity

For Bioassay Kits with control

93-1078Y3-00111: 2-Plate Kit

93-1078Y3-00112: 10-Plate Kit

For Bioassay Kit without control

93-1078Y3-00190: 10-Plate Kit

Document Number 70-398 Rev1

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

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1. Overview

The PathHunter Sargramostim Bioassay Kit is a simple, robust, non-radioactive, dye free assay for quantitation of control-induced functional dimerization of two subunits of a receptor-dimer pair. This assay detects GM-CSF-induced heterodimerization of the CSF2RA and CSF2RB receptors comprising the GM-CSF receptor. The PathHunter Sargramostim Bioassay Kit with control contains all the materials needed to perform a complete assay, including cryopreserved, single-use cells, detection reagents, cell plating reagent, agonist for stimulating the cells, and assay plates. A 10-Plate PathHunter Sargramostim Bioassay Kit is also offered without control but contains all other components listed above to run the assay. This bioassay has been optimized for a 96-well plate format.

2. Assay Principle

This bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, the ProLink™ (PK), and the 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 Sargramostim Bioassay evaluates activity in the CSF2RA and CSF2RB Dimerization assay, an application of the Eurofins DiscoverX Dimerization Assay platform. The assay is designed to detect the control-GM-CSF- induced heterodimerization of the CSF2RA and CSF2RB subunits comprising the GM-CSF receptor. As shown in Figure 1, the bioassay cells have been engineered to co-express CSF2RB fused to PK, and CSF2RA fused to EA. Binding of GM-CSF to the cytokine-specific CSF2RA receptor leads to heterodimerization with CSF2RB, leading to activation of intracellular signaling pathways. Receptor dimerization forces the two β-gal enzyme fragments (PK and EA) into proximity, forcing complementation. The result is formation of a functional β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal. In this bioassay, Sargramostim binds to CSF2RA, stimulating receptor heterodimerization with the CSF2RB receptor, leading to an increase in assay signal.

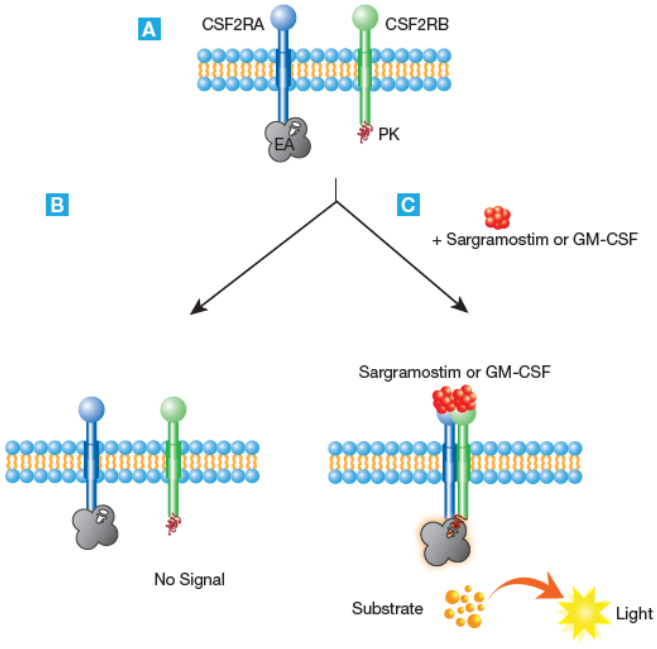


Figure 1. PathHunter Sargramostim Bioassay (Dimerization) Assay Principle.

3. Materials Provided in PathHunter Sargramostim Bioassay Kit

List of Components	93- 1078Y3-00111 (2-Plate Kit)	93- 1078Y3-00112 (10-Plate Kit)	93- 1078Y3-00190 (10-Plate Kit without control)
PathHunter U2OS CSF2RB/CSF2RA Bioassay Cells (0.6×10^6 cells in 0.1 mL per vial)	2	10	10
AssayComplete™ Cell Plating 22 Reagent (100 mL per bottle)	1 x 100 mL	2 x 100 mL	2 x 100 mL
AssayComplete Protein Dilution Buffer B5 (Bottle)	1 x 100 mL	2 x 100 mL	2 x 100 mL
Control Agonist (Recombinant Human GM-CSF) (5 µg per vial)	1	1	N/A*
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	 1 x 15 mL 1 x 60 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

***Note**: For 93-1078Y3-00190 control not provided in the kit, would need to be obtained separately if needed

4. Storage Conditions

PathHunter U2OS CSF2RB/CSF2RA 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 (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (greater 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 in dry ice in a covered container. Wait for 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 22 (CP22) Reagent

Upon receipt, store at -20°C. Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit's Certificate of Analysis), aliquot the reagent and store at -20°C until needed. Do not freeze-thaw more than three times. To make aliquots suitable for testing one assay plate each, 30 mL of reagent per aliquot can be dispensed and frozen down.

AssayComplete™ Protein Dilution Buffer 5 (PDB-B5)

Once thawed, Protein Dilution Buffer can be stored at 4°C 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 -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each, 10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on the stock sample

concentrations and should be adjusted accordingly.

Recombinant Human GM-CSF, Control agonist (If supplied in the kit)

Upon receipt, store at -20°C, until ready to use (up to the date listed in the kit's Certificate of Analysis). Centrifuge the vial before opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 50 µL of supplied reconstitution buffer to the 5 µg vial. Once prepared, the stock solution should be stored as suitable aliquots (e.g. 10 µL) at -20°C until needed. Do not freeze/thaw more than twice. Reconstituted control is stable for 12 months at -20°C to -80°C, or 1 week at 2-8°C.

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 Tissue Culture-Treated Plates

Store at room temperature.

5. Additional Materials and Equipment Recommended for Assay

The following equipment and additional materials are required to perform these assays:

Materials	Ordering Information
Recombinant Human GM-CSF	Eurofins DiscoverX (Part # 92-1317), or similar
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	Eurofins DiscoverX (Part # 92-0011), or similar
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Disposable reagent reservoir	Thermo Fisher Scientific, (Part # 8094) or similar
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettes and pipet tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	
Single and multichannel pipettors (e.g. P20, P100, P1000)	

6. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.1 mL of AssayComplete™ Freezing Reagent (refer to the CoA for cell number provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.


Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

PathHunter U2OS CSF2RB/CSF2RA Bioassay Cells must arrive in a frozen state on dry ice.


Contact technical support immediately, if cells received were already thawed.

1. 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, place vials in the vapor phase of liquid nitrogen storage.


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

2. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.

3. Proceed with the thawing protocol in the detailed assay protocol section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

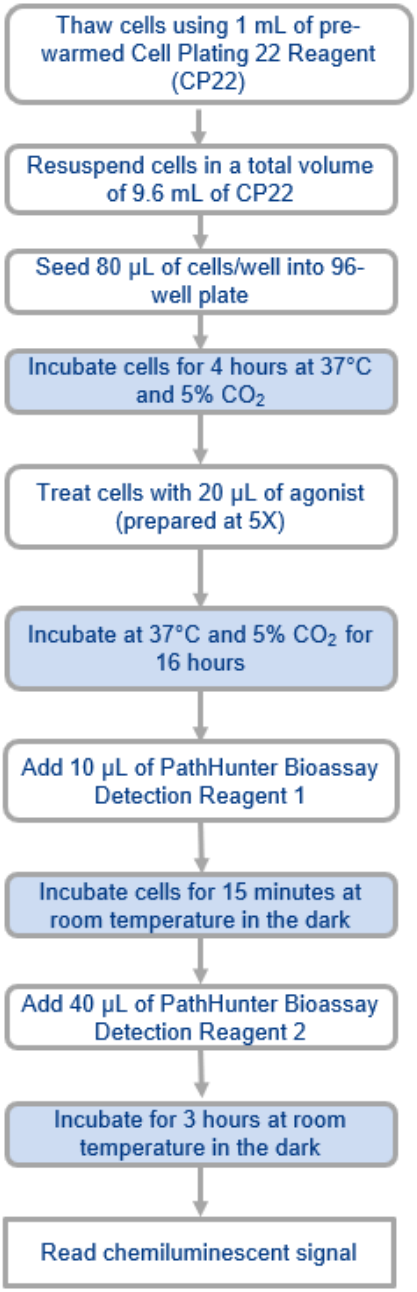
7. Protocol Schematic

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

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

Assay Name: _____Date: _____

Product Details: _____



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

8. Detailed Assay Protocol

This user manual provides a protocol for quantifying control mediated dimerization of the GM-CSF receptor, comprised of the CSF2RA and CSF2RB subunits. This assay is performed under aseptic conditions. Preparation of cells, Reference Standards/Test Samples are performed in a Biological Safety Cabinet following good aseptic technique.

All appropriate materials are either certified sterile or prepared aseptically.

If purchasing the bioassay kit without control, control can be sourced per the details in the Additional Materials and Equipment Recommended for Assay table.

8.1 Bioassay Cell Preparation (Day 1)

The following protocol is for thawing and plating frozen PathHunter U2OS CSF2RB/CSF2RA Bioassay Cells from cryovials.

1. Prior to thawing the cells, ensure that all the required materials are set up in the tissue culture hood. These include:

   a. One 25 mL reagent reservoir.

   b. One 15 mL conical tube.

   c. A micropipette (P1000) set to dispense 1 mL.

   d. A multichannel pipette and tips set to dispense 80 µL.

   e. A bottle of Cell Plating Reagent 22 (CP22, pre-warmed in a 37˚C water bath for 15 minutes).

   f. A white-walled, clear-bottom 96-well assay plate.

2. Dispense 9.6 mL of CP22 into the 15 mL conical tube.

3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.

4. Remove the cryovial from dry ice and ensure cap is tight. Holding vial by the cap, immediately thaw vial in 37°C water bath for 30 seconds (+/- 5 seconds), gently agitating the vial to thaw cells every 10 seconds.

   DO NOT LEAVE VIAL IN WATER BATH.

5. Visually inspect bottom of vial after 20 seconds. If pellet is thawed, remove vial from water bath, wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood. If ice is still visible, return vial to water bath for additional 10-15 seconds.

6. Add 1 mL of pre-warmed CP22 from the 15 mL conical tube, to the cryovial to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet up and down 3 times to uniformly suspend the cells.

7. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP22. Remove all the suspension from the cryovial tube to ensure maximum recovery of all the cells.

8. Replace the cap on the conical tube and mix by gentle inversion 2-3 times to ensure that the cells are

properly resuspended in the reagent, without creating any froth in the suspension. Pour it immediately into the sterile 25 mL reservoir.

9. Using a multichannel pipet, transfer 80 µL of the cell suspension to each well of the 96-well assay plate, one row at a time, using reverse pipetting. Mix cells in trough by pipetting up/down 2-3 times before aspirating and pipetting cells into each subsequent row in the assay plate.

10. Replace the lid on assay plate and leave the plate at room temperature in biosafety cabinet for 15 minutes (but no more than 30 minutes) to allow the cells to settle uniformly in the well, to minimize potential for edge effects.

11. Gently place the assay plate in a humidified tissue culture incubator set to 37˚C and 5% CO_2 for 4 hours before proceeding.

8.2 Sample Preparation (Day 1)

The following protocol is an example for preparing serial dilutions of Sargramostim, test sample and GM-CSF control agonist (optional). Sargramostim (Leukine) is supplied as a 250 µg/mL solution.

1. On day of assay, prepare working stock (5X) of Sargramostim in Protein Dilution Buffer B5 (PDB-B5) as detailed in Table 1 below. This will serve as the top concentration in the serial dilution curve prepared in Table 2.

Table 1. Example of Preparation of Sargramostim Reference Working Stock

Working Stock Solution	[Sargramostim], µg/mL	Volume 250 µg/mL Sargramostim, µL	Volume Protein Dilution Buffer-B5, µL
Sargramostim, Working Stock	15	15	235

***Note:** working stocks of test samples should be prepared similarly.

2. On the day of assay, prepare serial dilutions of Sargramostim reference in Row A of a fresh 96-well master dilution plate (MDP) at 5X the final concentration of each dose, in PDB-B5 as per Table 2 below. Sufficient volumes to run triplicate wells per dose in the assay plate are provided in the table. Volumes may be scaled up/down as appropriate.

   a. Add volume of sample diluent (PDB) to Row A of the MDP (as indicated in column 6) of Table 2.

   b. Transfer 200 µL of Sargramostim Working Stock prepared in Table 1 to well A1 of Row A in the MDP.

   c. Prepare serial dilutions by transferring the volume of Sargramostim (indicated in column 5 of Table 2) from the source well (indicated in column 4) to the destination well (indicated in column 1).

   d. Pipet up and down three times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 12, as this serves as the negative control.

Table 2. Example of Preparation of Sargramostim Reference Serial Dilutions

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well Location on Master Dilution Plate (MDP)	Concentration (5X) of sample on MDP Row A), ng/mL	Dilution Ratio	5X Sample Source Location	Volume 5X Sample, µL	Volume Ligand Diluent, µL	Final [Sample] in Assay (1X), ng/mL
Row A, Well 1	15,000	--	Working Stock from Table 1 (15 µg/mL)	200	-	3,000
Row A, Well 2	3,000	1:5	Row A, Well 1	50	200	600
Row A, Well 3	600	1:5	Row A, Well 2	50	200	120
Row A, Well 4	200	1:3	Row A, Well 2	100	200	40
Row A, Well 5	66.7	1:3	Row A, Well 4	100	200	13.3
Row A, Well 6	22.2	1:3	Row A, Well 5	100	200	4.4
Row A, Well 7	7.4	1:3	Row A, Well 6	100	200	1.5
Row A, Well 8	2.5	1:3	Row A, Well 7	100	200	0.5
Row A, Well 9	0.82	1:3	Row A, Well 8	100	200	0.16
Row A, Well 10	0.16	1:5	Row A, Well 9	50	200	0.03

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well Location on Master Dilution Plate (MDP)	Concentration (5X) of sample on MDP Row A), ng/mL	Dilution Ratio	5X Sample Source Location	Volume 5X Sample, µL	Volume Control Diluent, µL	Final [Sample] in Assay (1X), ng/mL
Row A, Well 11	0.03	5	Row A, Well 10	50	200	0.007
Row A, Well 12	0	--	--	--	200	0

3. In Row B of Master Dilution Plate, prepare test sample using same serial dilution scheme in Table 2 above.

4. Optional: Prepare the control agonist (Eurofins DiscoverX GM-CSF) dose response curve, which will serve as a positive control in this assay:

    a. Reconstitute GM-CSF (5 µg lyophilized powder) to 100 µg/mL stock solution by adding 50 µL of supplied reconstitution buffer.

5. Prepare intermediate and working stock of GM-CSF in Protein Dilution Buffer B5 (PDB-B5) as detailed in Table 3 below. The latter will serve as the top concentration in the serial dilution curve, prepared at 5X the final concentration in the assay, as shown in Table 4.

Table 3. Example of Preparation of GM-CSF Intermediate and Working Stocks

Working Stock Solution	[GM-CSF], µg/mL	Volume GM-CSF, µL	Volume PDB-B5, µL
GM-CSF, Intermediate Dilution 1	15	6.0 (of 100 µg/mL stock)	34.0
GM-CSF, Working Stock	1.5	12.5 (of 15 µg/mL stock)	112.5

6. On the day of assay, prepare serial dilutions of GM-CSF control agonist in Row C of the MDP, at 5X the final concentrations of each dose, in PDB-B5 as per Table 4 below. Sufficient volumes to run duplicate wells per dose in the assay plate are provided in the table. Volumes may be scaled up/down as appropriate.

    a. Add appropriate volume of sample diluent (PDB) to Row C of the MDP, as indicated in column 6 of Table 4.

    b. Transfer 100 µL of GM-CSF Working Stock prepared in Table 3 to well A1 of Row C of the MDP.

c. Prepare serial dilutions by transferring the volume of GM-CSF (indicated in column 5 of Table 4) from the source well (indicated in column 4) to the destination well (indicated in column 1).

d. Pipet up and down three times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 12, as this serves as the negative control.

Table 4. Example of Preparation of GM-CSF Control Serial Dilutions

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well location on Master Dilution Plate (MDP)	Concentration (5X) of sample on MDP, ng/mL	Dilution Ratio	Dilution (5X) Sample Source Well	Volume of Sample added, µL	Volume Control Diluent, µL	Final Concentration (1X) of Sample in Assay Plate, ng/mL
Row C, Well 1	1,500	--	Working Stock from Table 3 (1.5 µg/mL)	100	-	300
Row C, Well 2	500	1:3	Row C, Well 1	40	80	100
Row C, Well 3	166.7	1:3	Row C, Well 2	40	80	33.3
Row C, Well 4	55.6	1:3	Row C, Well 3	40	80	11.1
Row C, Well 5	18.5	1:3	Row C, Well 4	40	80	3.7
Row C, Well 6	6.2	1:3	Row C, Well 5	40	80	1.2
Row C, Well 7	2.1	1:3	Row C, Well 6	40	80	0.4
Row C, Well 8	0.69	1:3	Row C, Well 7	40	80	0.14
Row C, Well 9	0.22	1:3	Row C, Well 8	40	80	0.05
Row C, Well 10	0.076	1:3	Row C, Well 9	40	80	0.015
Row C, Well 11	0.025	1:3	Row C, Well 10	40	80	0.005

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well location on Master Dilution Plate (MDP)	Concentration (5X) of sample on MDP, ng/mL	Dilution Ratio	Dilution (5X) Sample Source Well	Volume of Sample added, µL	Volume Control Diluent, µL	Final Concentration (1X) of Sample in Assay Plate, ng/mL
Row C, Well 12	0	--	--	--	100	0

7. Add 20 µL from the Sargramostim reference curve from Row A on the MDP to the appropriate wells of the assay plate (e.g. Rows B, D and F) as shown in the Representative Assay Plate Map (Figure 2).

8. Add 20 µL from the test sample curve from Row B on the MDP to the appropriate wells of the assay plate (e.g. Rows C, E and G) as shown in the Representative Assay Plate Map (Figure 2).

9. Add 20 µL from the GM-CSF control curve from Row C on the MDP to appropriate wells of the assay plate (e.g. Rows A and H), as shown in the Representative Assay Plate Map (Figure 2).

10. Place the assay plate in the 37°C and 5% CO_2 incubator and incubate for 16 hours.

8.3 Addition of Detection Reagent

Day 2: Signal Detection

1. Thaw one aliquot of Bioassay Detection Reagent 1 from PathHunter Bioassay Detection kit, equilibrate to room temperature, and transfer 2.3 mL using a pipet into a sterile reservoir.

2. Remove assay plate from incubator and remove lid. Using a multichannel pipet, add 10 µL of the Bioassay Detection Reagent 1 from the reservoir into each row of the assay plate.

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

   Optional: place the plate onto an orbital shaker at 350 rpm for 1 minute to promote even mixing.

4. Incubate the assay plate for 15 minutes (+/- 5 minutes) at room temperature (22°C - 25°C) in the dark (e.g. in a drawer or covered location on the bench top).

5. Thaw one aliquot of Bioassay Detection Reagent 2 from PH Bioassay Detection kit, equilibrate to room temperature, and transfer 9.2 mL into a fresh sterile reservoir.


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

5. Retrieve assay plate and remove lid. Using a multichannel pipet, add 40 µL of the Bioassay Detection Reagent 2 from the reservoir into each well of the assay plate.

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

7. Incubate the assay plate for 3 hours (+/- 15 minutes) at room temperature (22°C - 25°C) in the dark (e.g. in a drawer or covered location on the bench top).

8. 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.
   - A luminescence reader usually collects signal from all wavelengths. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

9. Data analysis and plotting graph can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.)

Representative Assay Plate Map

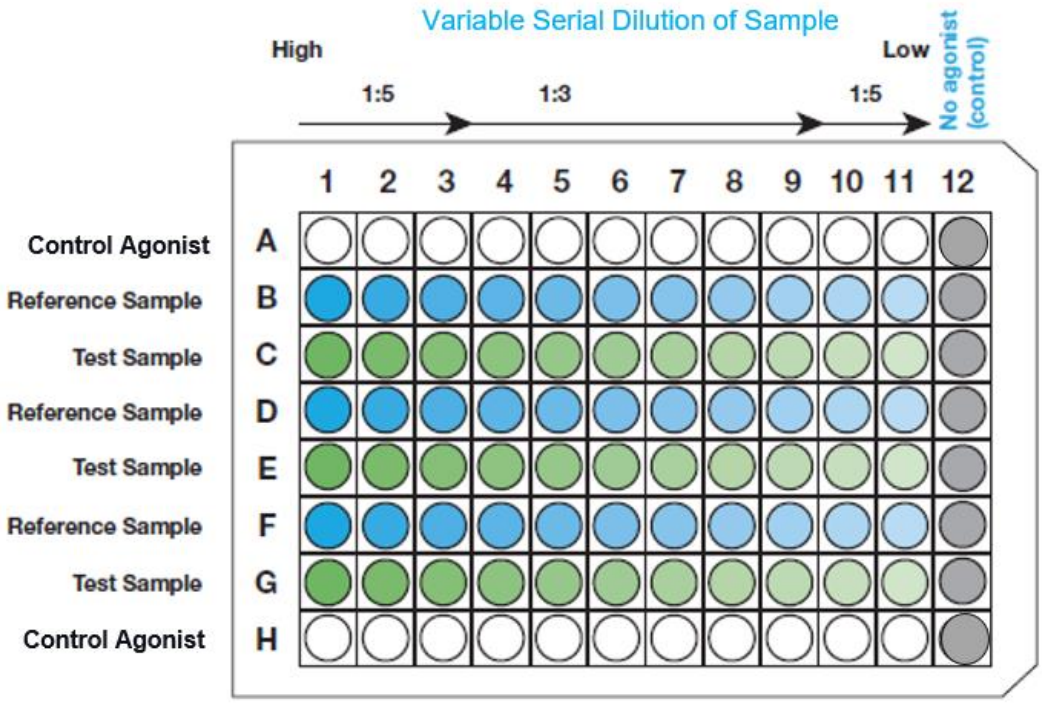


Figure 2. Representative Assay Plate Map

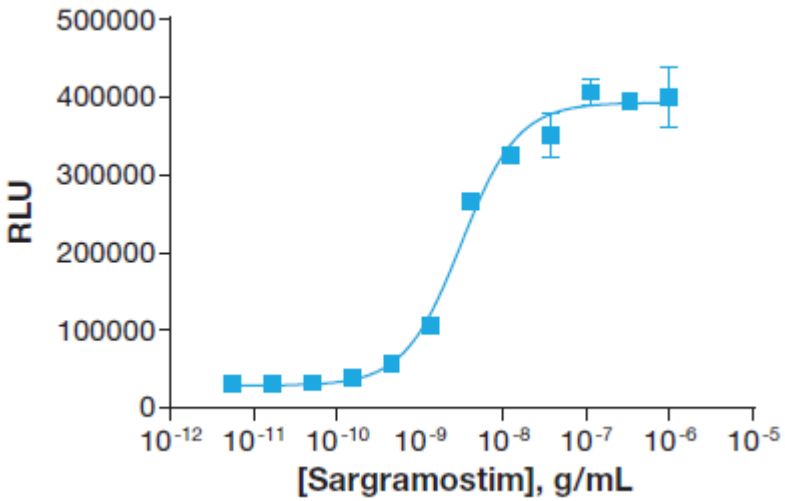
This plate map shows an 11-point dose response curve with 3 data points at each concentration for one reference and one test sample per plate, each prepared with a variable dilution scheme, as shown at top of figure. Note: the optional control agonist is run in rows A and H and is prepared using a 1:3 dilution series.

9. Typical Results

The following graph is an example of a typical dose-response curve for the PathHunter Sargramostim Bioassay Kit generated using the protocol outlined in this user manual. The data shows potent, dose-dependent dimerization of the GM-CSF receptor subunits (CSFR2A and CSF2RB), when treated with Sargramostim.

The plate was read on an EnVision® Multimode Plate Reader (with a 0.2 sec /well integration time) and data analysis was conducted using GraphPad Prism.

A



B

S/B	EC_{50} (ng/mL)
13.1	3.14

Figure 3. Typical Results:

Representative **A**, dose-response curve and **B**, EC_{50} and assay window for dose-dependent stimulation of dimerization of the components of the GM-CSF receptor (CSFR2A and CSF2RB) in U2OS cells when treated with a dose response of Sargramostim.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to the thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect control used or incorrect control incubation time	Refer to the datasheet for recommended control and assay conditions.
	Sub-optimal time course for induction	Optimize time course of induction with the agonist and antagonist.
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	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 Certificate of Analysis	Incorrect incubation temperature	Confirm assay conditions.
		Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Incorrect preparation of ligand (agonist or antagonist)	Some controls are difficult to handle. Confirm the final concentration of controls.
	Sub-optimal agonist challenge concentration	Perform the agonist curve to reassess EC_{80} with the control provided in the kit. Perform antibody titrations with EC_{80} and EC_{90} agonist challenge concentrations to re-optimize the assay window.
EC_{50} is right-shifted	Improper control handling or storage	Check the control handling requirements.
	Difference in agonist binding affinity	Refer to the Certificate of Analysis for the control provided in the kit to confirm that the control used is comparable.
	Problems with plate type	Hydrophobic compounds should be tested for solubility and may require evaluation in different dilution buffers.
	Problems with compound stability	Non-binding surface plates may be necessary for hydrophobic compounds.

For questions on using this product, please contact Technical Support at discoverx.com/support/

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