DiscoverX

User Manual

PathHunter®

Anakinra Bioassay Kit

For Chemiluminescent Detection of Anakinra Activity

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

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Overview

PathHunter Anakinra Bioassay kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study anakinra potency and neutralizing antibodies. The bioassay kits contain all the reagents needed for a complete assay including cells, detection reagents, cell plating reagent, positive control agonist, and assay plates. The pre-qualified, frozen cells have been manufactured for single-use and are provided in a ready-to-assay format that saves time and adds convenience.

Technology Principle: PathHunter Anakinra Bioassay

These assays utilize 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.

The PathHunter Anakinra Bioassay is an application of the DiscoverX Dimerization Assay platform, which can be used to detect ligand-induced functional dimerization of two subunits of a receptor-dimer pair. This assay detects IL-1α and IL-1β induced heterodimerization of the IL1R1 and IL1RAP receptors. The cells have been stably engineered to co-express IL1RAP fused to PK, and IL1R1 fused to EA. Activation of the IL1 receptor through IL-1α or IL-1β leads to receptor dimerization, which is an essential functional event in the receptor's signaling cascade. Receptor dimerization forces the two enzyme fragments to complement, resulting in the formation of a functional β-gal enzyme. The enzyme hydrolyzes a substrate to generate a chemiluminescent signal. Anakinra binds to IL1R1 and prevents its activation through IL-1α or IL-1β, thereby inhibiting receptor heterodimerization with the IL1RAP receptor. This leads to an inhibition of the signaling event and therefore a reduction in assay signal.

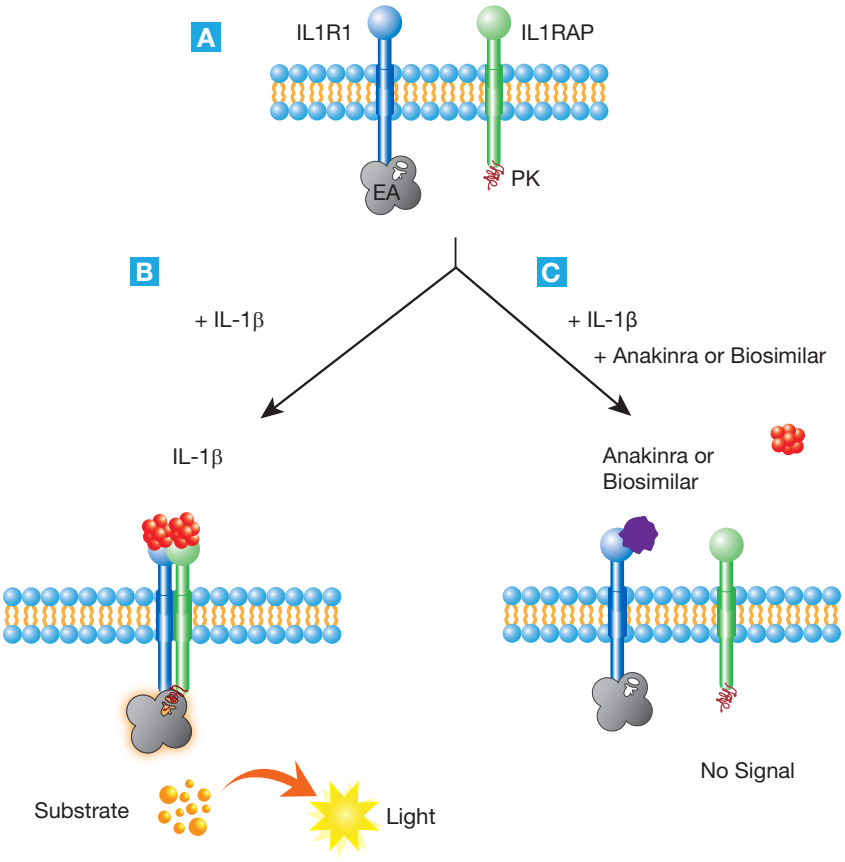


Figure 1. PathHunter Anakinra Bioassay Principle

Materials Provided

List of Components	93-1032Y3-00105	93-1032Y3-00106
PathHunter U2OS IL1R1/IL1RAP Bioassay Cells	2 vials	10 vials
PathHunter Bioassay Detection Kit Detection Reagent 1 (mL) Detection Reagent 2 (mL)	 2 8	 10 40
AssayComplete™ Cell Plating Reagent 5	1 X 100 mL	3 X 100 mL
Protein Dilution Buffer	1 X 50 mL	2 X 50 mL
Control Agonist (IL-1β)	1 vial	1 vial
96-Well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

PathHunter U2OS IL1R1/IL1RAP Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vials as soon as possible upon receipt.

If continued storage of the frozen vials is necessary, store as follows:

- Short term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for more 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 a lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid nitrogen. Upon thawing, if liquid nitrogen is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the liquid nitrogen tank.

PathHunter Bioassay Detection Kit

Store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For longer storage (up to the expiration date listed on the kit 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, 1 mL of Detection Reagent 1 per aliquot can be dispensed and frozen down. 4 mL of Detection Reagent 2 per aliquot can be dispensed and frozen down separately. Do not mix the two reagents during aliquoting.

AssayComplete Cell Plating Reagent 5 (CP5)

Once thawed, the CP5 can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit 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, 30 mL of reagent per aliquot can be dispensed and frozen down.

Protein Dilution Buffer (PDB)

Once thawed, the PDB can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit 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 your stock sample concentrations and should be adjusted accordingly.

Recombinant Human IL-1β Control Agonist

Store at -20°C until ready to use (up to the expiration date listed on the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 100 µL of Protein Dilution Buffer to the 10 µg vial. Reconstituted ligand is stable for 12 months at -20 to -80°C, or 1 week at 2-8°C.

96-Well Tissue Culture Treated Plates

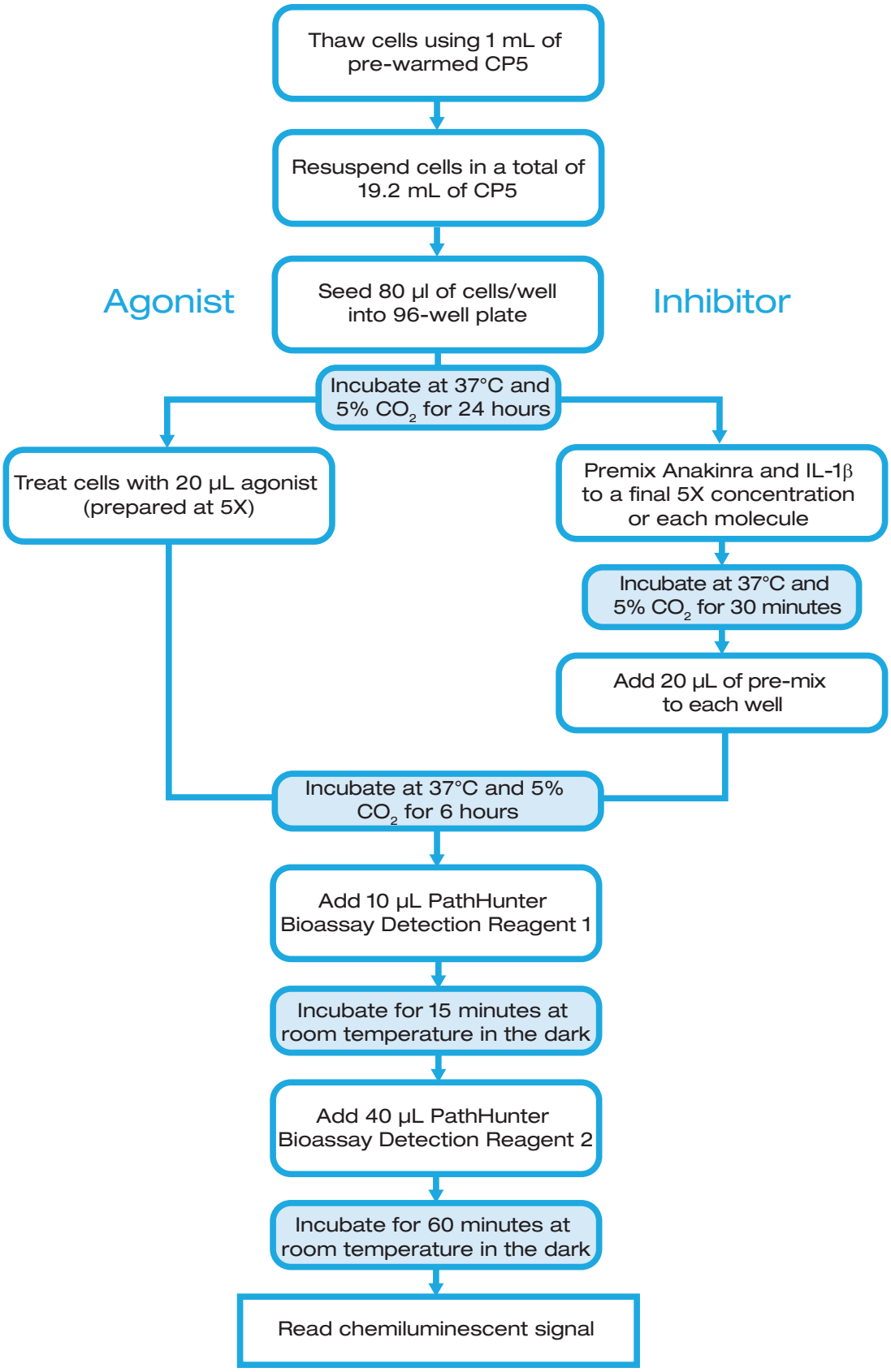
Store at room temperature.

Additional Materials Required

Material	Ordering Information
V-Bottom 96-well ligand dilution plates	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Single and multichannel micro-pipettors and pipette tips	
Disposable reagent reservoir	ThermoFisher Scientific, Cat. No. 8094 or similar

Protocol Schematic

In a 96-well tissue culture treated plate provided in the kit, perform the following steps:



Anakinra Bioassay Protocol

Day 1: PathHunter Bioassay Cell Preparation:

The following protocol is for thawing and plating frozen PathHunter U2OS IL1R1/IL1RAP Bioassay cells from cryovials.

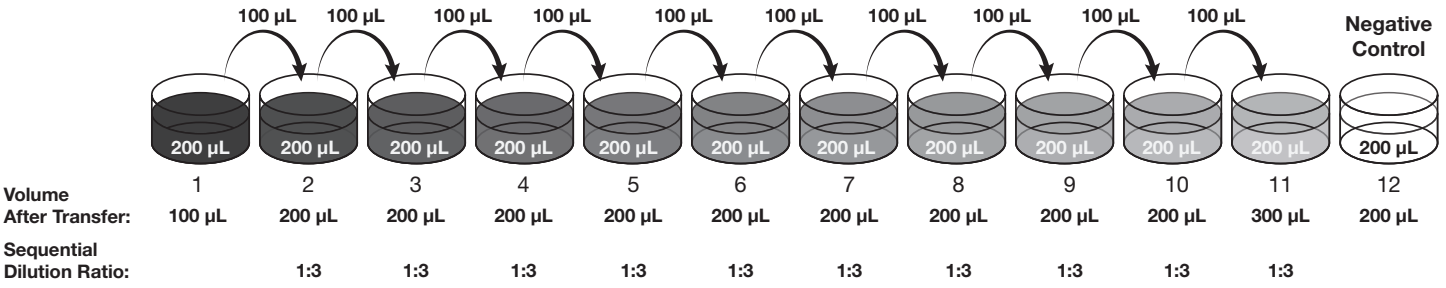
1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir.
    b. One 50 mL conical tube.
    c. A micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 80 µL.
    e. A bottle of CP5, pre-warmed in a 37˚C water bath for 15 minutes.
    f. A white-walled, clear-bottom 96-well assay plate.
2. Dispense 19.2 mL of CP5 into the 50 mL conical tube.
3. Remove the cryovial from liquid nitrogen and immediately place in dry ice.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% ethanol, and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

4. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP5 from the 50 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 18.2 mL of CP5. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Mix the tube by inversion to ensure the cells are properly mixed in the medium without creating any froth in the suspension and immediately pour the suspension into the 25 mL reservoir.
6. Add 80 µL of cells to each well of the 96-well assay plate using the multichannel pipette.
7. Let plate sit for 15 minutes at room temperature to allow cells to settle, avoiding the potential for edge effects.
8. Incubate for 24 hours at 37ºC and 5% CO_2 in a humidified tissue culture incubator.

Day 1: Sample Preparation

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



1. Prepare the reference agonist (IL-1β) dose response curve, which will serve as a positive control in this assay. The agonist is prepared at 5X the desired final concentration as it will be diluted by adding to the 80 µL of media present in the assay plate.

    a. Add 200 µL of PDB to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate).

    b. Add 100 µL of the reconstitution buffer to the IL-1β vial containing 10 µg of lyophilized powder to make a 100 µg/mL stock solution.

    c. Add 190 µL of PDB to well A1 of the master dilution plate. Add 10 µL of the 100 µg/mL IL-1β stock to this well. Mix thoroughly by pipetting up and down several times. This results in a 5 µg/mL solution (5X the final top concentration of 1 µg/mL).

    d. Using a clean tip, transfer 100 µL from well A1 into well A2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 into well A3. Mix by pipetting up and down several times. Repeat this process until well A11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to column 12 as this will serve as a negative control.

2. Prepare anakinra curve. Anakinra is prepared at 10X the desired final concentration. Top dose: 100 µg/mL.

    a. Add 200 µL of PDB to well B2 to B12 in a new row of the master dilution plate.

    b. Add 200 µL of anakinra prepared at 10X the desired final concentration (1000 µg/mL) to B1 of this row on the master dilution plate. The final concentration of the top dose of anakinra in the assay plate is 100 µg/ mL.

    c. Using a clean tip, transfer 100 µL from well B1 into well B2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well B2 into well B3. Mix by pipetting up and down several times. Repeat this process until well B11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to B12 as this will serve as a negative control.

    d. Transfer 40 µL from the anakinra curve to a separate pre-mixing row C on the master dilution plate.

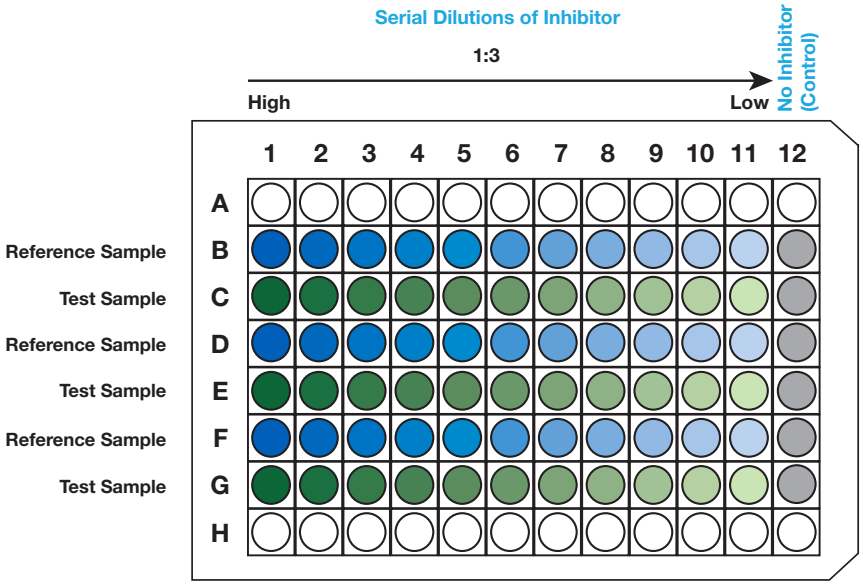
3. Prepare the agonist challenge for anakinra. The EC_{80} of the Recombinant Human IL-1β (DiscoverX Cat. No. 92-1296) was determined to be approximately 3 ng/mL. This EC_{80} should be empirically verified and optimized in each testing facility, particularly when IL-1β from a different vendor is being used.
   Prepare the agonist challenge at 10X the desired final concentration. For enough agonist for a single biosimilar curve run in triplicate, dilute 10 µL of the 100 µg/mL stock with 990 µL of PDB in an Eppendorf tube to make a working stock of 1 µg/mL. Then transfer 60 µL from this 1 µg/mL working stock in 1.9 mL of PDB to prepare 30 ng/mL solution (10X the final concentration of 3 ng/mL).
4. Transfer 40 µL from the IL-1β agonist challenge to wells 1-12 in row C (pre-mixing row) on the master dilution plate, which contains 40 µL of the anakinra dilution curve, for a 1:1 dilution. Mix the contents of the pre-mixing wells thoroughly by pipetting up and down several times.
5. Place the assay plate in the 37°C and 5% CO_2 incubator for 30 minutes.
6. Add 20 µL from the agonist reference curve on the master dilution plate to the appropriate wells of the assay plate.
7. Add 20 µL from each well of the anakinra and IL-1β pre-mix from the pre-mixing wells to the appropriate wells of the assay plate.
8. Place the assay plate in the 37°C and 5% CO_2 incubator for 6 hours.

Day 2: Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate.
2. Incubate the plate at room temperature for 15 minutes in the dark.
3. Using a multichannel pipette add 40 µL of Detection Reagent 2 to each well of the assay plate.
4. Incubate the plate at room temperature for 1 hour in the dark.
5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers of 5-10 seconds for imager.

PathHunter Bioassay Detection Reagents are light sensitive, thus incubation in the dark is necessary.

Representative Plate Map for Sample Curve



This plate map shows an 11-point dilution curve with three data points at each concentration for one reference and one test sample per plate, with a serial dilution scheme.

Troubleshooting Guide

Problem	Cause	Solution
No response	Improper thawing procedure	Refer to thawing instructions in this user manual. Thawing process can have a significant effect on cell viability.
	Improper ligand used or improper ligand incubation time	See certificate of analysis for recommended ligand and assay conditions.
	Improper preparation of ligand (agonist or antagonist)	Refer to specific datasheet to ensure proper handling, dilution and storage of ligand.
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope.
Low or no signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
Experimental S:B does not match datasheet value	Incorrect incubation temperature	Confirm assay conditions. Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
	Improper preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands.
EC_{50} is right-shifted	Improper ligand handling or storage	Make sure ligands are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the certificate of analysis.
	Contamination from tips	Changing tips during serial dilutions can help to avoid carryover.
High variability between replicates	Instrument calibration	Ensure dispensing equipment is properly calibrated, and proper pipetting technique is used.

For additional information or technical support, please contact Technical Support listed below.

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