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User Manual

PathHunter® U2OS IL-31RA/OSMRβ Dimerization Bioassay Kit

For Detection of Ligand-Induced Inhibition of IL-31RA and OSMRβ Dimerization

Document Number: 70-407 Revision 0

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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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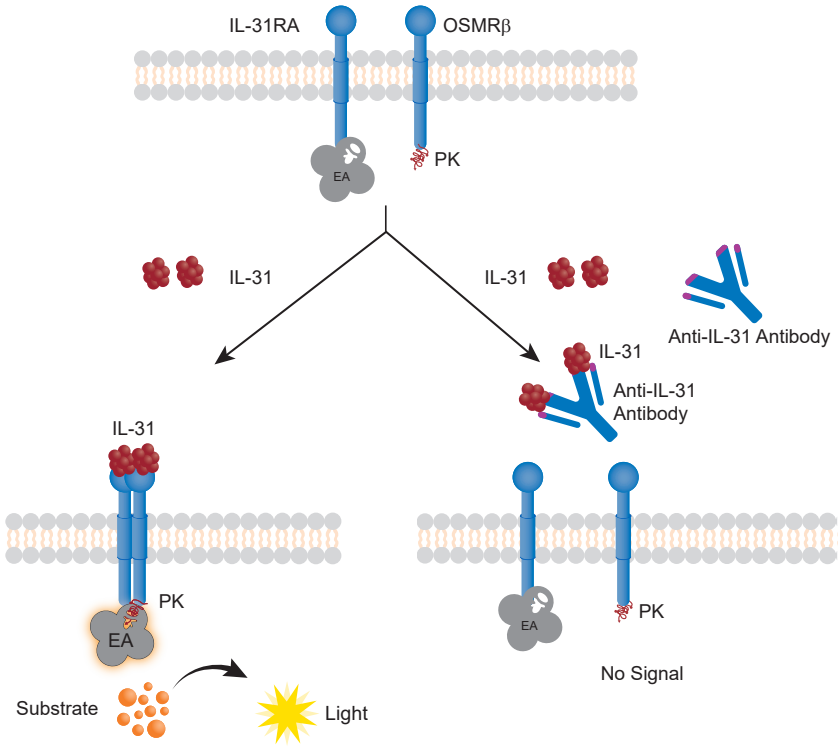
Overview

The PathHunter IL-31RA/OSMRβ Bioassay Kit provides a robust, highly sensitive and easy-to-use cell-based functional assay to study receptor-ligand interactions on the cell surface. This kit has been designed to rapidly measure potency of the biologic drug, or detect the presence of neutralizing antibodies against the biologic. The bioassay kit contains all materials needed to run the assay, including cryopreserved cells, detection reagents, cell plating reagent, control agonist, and assay plates. The ready-to-use cryopreserved cells have been manufactured for single-use to ensure assay reproducibility, and faster implementation from characterization to lot release. This assay has been optimized for a 96-well format.

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 IL-31RA/OSMRβ Bioassay is an application of the Eurofins DiscoverX's dimerization assay platform. The assay is designed to detect IL-31-induced functional dimerization of the Interleukin 31 receptor alpha chain (IL-31RA) and the Oncostatin-M receptor beta chain (OSMRβ). The cells have been engineered to co-express OSMRβ fused to PK, and IL-31RA fused to EA. Binding of an agonist, such as IL-31, to the IL-31RA, induces it to dimerize with OSMRβ, bringing the two receptor chains into close proximity and forcing complementation of the two enzyme fragments. This results in the formation of a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal. Blocking agonist binding using an anti-ligand or anti-receptor antibody can prevent this interaction, resulting in a dose-dependent reduction of the signal.



Materials Provided

List of Components	93-1002Y3-00083	93-1002Y3-00084
PathHunter U2OS IL-31RA/OSMRβ Bioassay Cells (1.2 x 10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 0 Reagent* (100 mL per bottle)	1	2
Recombinant Human IL-31 (10 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

*Cell Plating 0 Reagent is also used for diluting control agonist and antagonists in the bioassay.

Storage Conditions

PathHunter U2OS IL-31RA/OSMRβ 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.

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.

AssayComplete™ Cell Plating 0 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), 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.

Recombinant Human IL-31

Upon receipt, store at -20°C, until ready to use (up to the expiration date listed in the datasheet). Centrifuge the vial before opening to maximize recovery.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

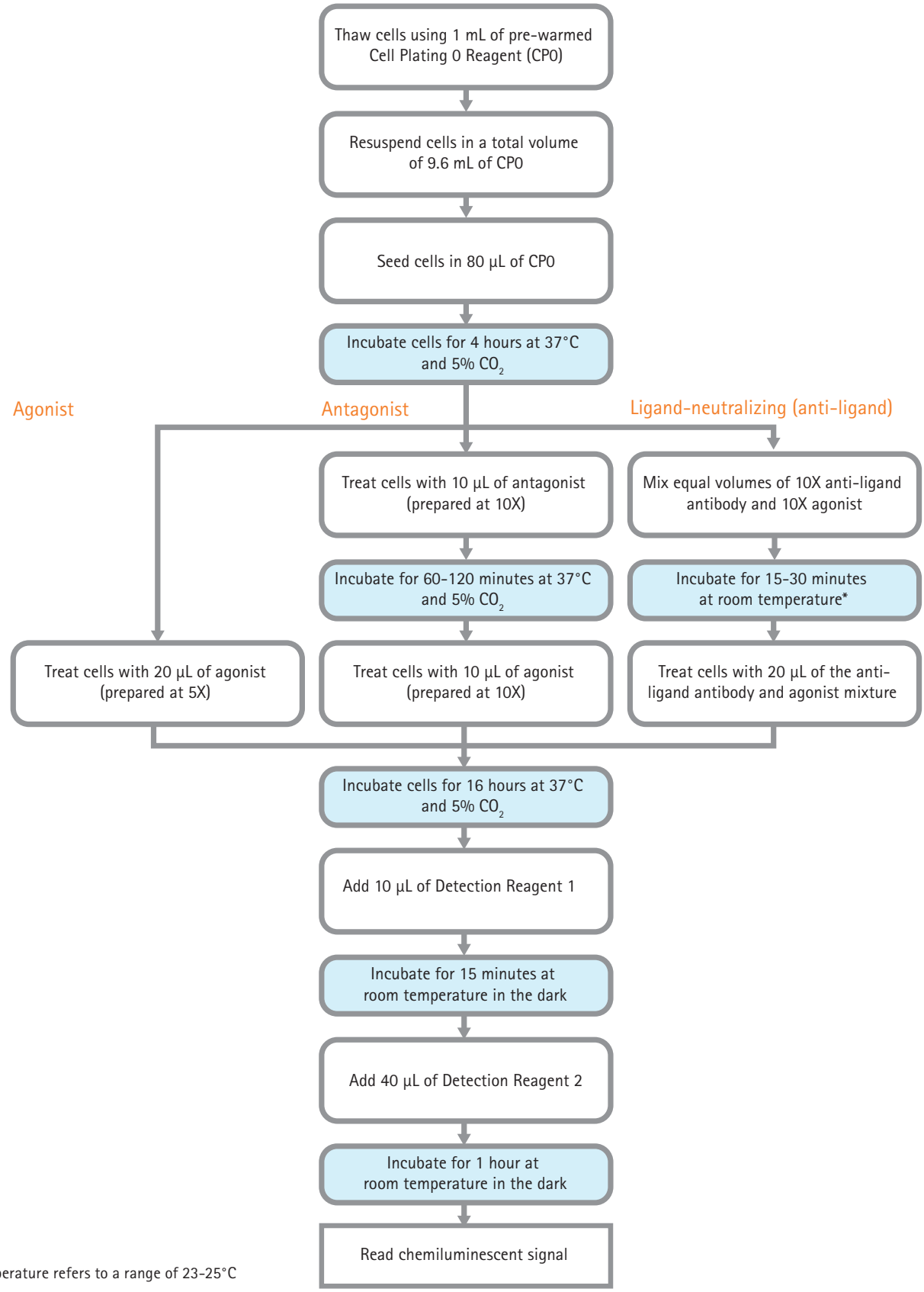
Additional Materials Required

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

Materials	Ordering Information
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	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
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettes and pipette tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	

Protocol Schematic

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



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

Detailed Protocol

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter U2OS IL-31RA/OSMRβ 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 sterile 25 mL reagent reservoir
    b. One sterile 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 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 9.6 mL of CP0 into the 15 mL conical tube.
3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.

DO NOT use a heated water bath to thaw the vial. Hold the cryovials by the cap; **DO NOT** touch the sides or bottom of the vial to avoid thawing of the cell pellet. Wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood.

4. Add 1 mL of pre-warmed CP0 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 the suspension up and down several times to uniformly resuspend the cells.
5. Transfer the cell suspension into the conical tube containing the remaining 8.6 mL of CP0. Aspirate all the suspension from the cryovial to maximize recovery of cells.
6. Gently invert the conical tube several times to ensure that the cells are uniformly suspended in the CP reagent, without creating any froth, and immediately transfer the suspension into the 25 mL reservoir.
7. Transfer 80 µL of the cell suspension to each well of the 96-well assay plate using a multichannel pipette. Replace the lid and leave the plate at room temperature for 15 minutes to allow the cells to settle uniformly in the well, to minimize potential edge effects.
8. Incubate the assay plate at 37°C and 5% CO_2 for 4 hours before proceeding with the assay.

Day 1: Sample Preparation

The following protocol is designed for testing purified biologics. The PathHunter assays can 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 1:3 serial dilution of the control agonist/ligand, IL-31, has been used in this protocol. The volumes listed below are designed for running samples from one dose-response curve in triplicate on the assay plate (Refer to the Representative Assay Plate Map). Different samples are prepared based on their mode of action, e.g., receptor antagonists that bind to the receptor and inhibit dimerization, or anti-ligand antibodies that bind to ligands and prevent them from binding to the receptor.

1. Add 200 µL of CP0 to Wells A2 to A12 of the master dilution plate.

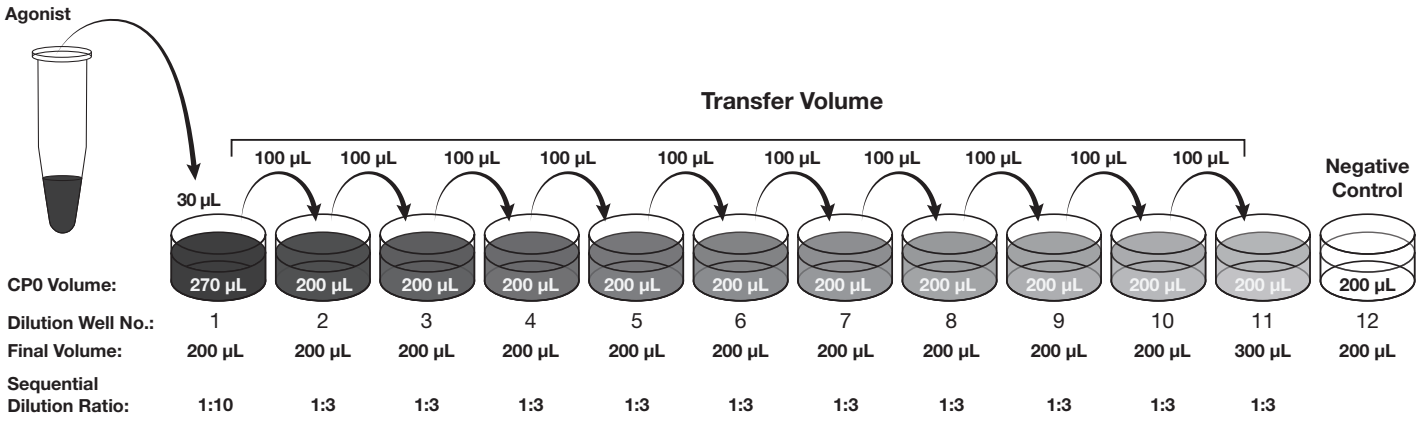
2. Prepare the IL-31 dose-response curve:
   The Recombinant Human IL-31 will serve as a positive control in this assay. The agonist is prepared at 5X the desired final concentration as it will be diluted by adding 20 µL to 80 µL of the medium present in the assay plate.

   2.1. Add 100 µL of the supplied Reconstitution Buffer to the vial containing 10 µg of the lyophilized IL-31 powder, to make a 100 µg/mL stock solution. Gently shake (do not vortex) for ten minutes to completely dissolve the powder.

   2.2. Make a 15 µg/mL intermediate solution of IL-31, by adding 30 µL of the 100 µg/mL solution prepared in Step 2.1, into 170 µL of CP0.

   2.3. Make a 1:10 dilution of the intermediate solution prepared in Step 2.2. Add 270 µL of CP0 to Well A1 of the master dilution plate. Add 30 µL of the 15 µg/mL intermediate solution to Well A1, and mix thoroughly by pipetting up and down several times. This results in a 1.5 µg/mL solution (5X the 300 ng/mL top dose).

   2.4. Using a clean pipette tip, transfer 100 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 100 µL from Well A2 into Well A3, and mix well. Repeat this process through Well A11, resulting in an eleven-point, 1:3 dilution series. No sample is transferred to Well A12, as this will serve as a negative control.



Agonist Serial Dilutions: Make 11 three-fold serial dilutions of the agonist in a master dilution plate.

3. Prepare any agonist test samples following the steps above.

4. Remove the assay plate from the incubator and place it in the tissue culture hood.

5. Transfer 20 µL from each well of the agonist dilution series from the master dilution plate to the appropriate wells of the assay plate.

6. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 16 hours.

7. Prepare the antagonist dilution series using the following protocol. If antagonist samples are not being tested, then skip Steps 7-11 and proceed to the Day 1: Ligand-Neutralizing Sample Preparation section or Day 2: Detection.

    7.1. The antagonist is prepared at 10X the desired final concentration, in 11-point dilution series, similar to that described for IL-31 in Steps 1 and 2. However, an appropriate top dose and dilution scheme (e.g. a 1:3 dilution scheme) must be determined empirically. No antagonist is added to any wells in column 12, as these are the negative control wells.

    7.2. Remove the assay plate from the incubator and place it in the tissue culture hood.

    7.3. Transfer 10 µL from each well of the antagonist dilution series from the master dilution plate to the appropriate wells of the assay plate, using a multichannel pipette.

    7.4. Incubate the assay plate at 37°C and 5% CO_2 for 60-120 minutes. The optimal incubation time for your antagonist must be determined empirically.

8. Prepare the agonist challenge for the antagonist dilution series: The EC_{80} of the supplied IL-31 was determined to be approximately 15 ng/mL. If IL-31 from a different vendor is used, the EC_{80} should be determined empirically, prior to testing samples. Prepare the agonist challenge at 10X the desired final concentration. The following steps provide an adequate amount of the agonist challenge for two antagonist curves run in triplicate:

    8.1. Transfer 20 µL of the 15 µg/mL intermediate solution prepared in Step 2.2, into 1980 µL of CP0. This results in a 150 ng/mL solution.

    8.2. Transfer 100 µL of the 150 ng/mL solution to 11 clean wells of one row (e.g. Wells A1-Wells A11) of a V-bottom 96-well plate. Add 100 µL of CP0 to Well 12.

9. Remove the assay plate from the incubator and place it in the tissue culture hood.

10. Transfer 10 µL of the 10X agonist challenge prepared in Step 8, to the appropriate wells (i.e. wells with the antagonist dilution series) of the assay plate, using a multichannel pipette.

11. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 16 hours.

Day 1: Ligand-Neutralizing Sample Preparation

The following protocol is used for the quantitation of anti-ligand samples, e.g. antibodies or Fc-fusion proteins, etc. The ligand is pre-incubated with the neutralizing sample for 15-30 minutes. However, the optimal incubation time for your sample should be determined empirically, before addition to the assay.

1. Prepare the anti-ligand antibody dilution series at 10X the final desired concentration in a new row (e.g. row B) of the master dilution plate.

2. Prepare the agonist challenge at 10X the final desired concentration. Transfer 5 µL of the 15 µg/mL intermediate solution (prepared in Step 2.2 in Sample Preparation), to 495 µL of CP0, resulting in a 150 ng/mL solution. This is enough agonist challenge for a single, 11-point antibody curve run in triplicate.

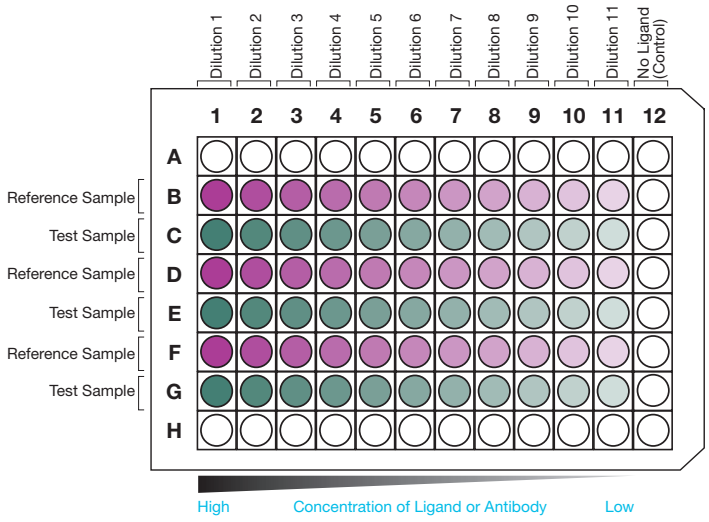
3. For each anti-ligand dose-response curve, add 45 µL of the agonist challenge to the appropriate row of a pre-mixing plate (e.g. a V-bottom polypropylene 96-well plate). Transfer 45 µL of the 10X anti-ligand antibody dilution series to the row of the pre-mixing plate already containing 45 µL of agonist challenge. Mix by slowly pipetting up and down several times.

4. Incubate the plate at room temperature for 15-30 minutes. The optimal pre-incubation time for each antibody should be determined empirically.

5. Transfer 20 µL from each well of the anti-ligand antibody and agonist mixture prepared in Step 3, to the appropriate wells of the assay plate.

6. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 16 hours.

Representative Assay Plate Map



Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test and reference sample tested with their respective dilution schemes.

Day 2: Detection

The following steps for adding detection reagents are applicable when testing purified biologics. For crude biologic samples, gently removing the liquid from all wells of the assay plate and replacing with 100 µL of Cell Plating Reagent, before the addition of the detection reagents, can result in a higher signal. Additional Cell Plating Reagent will be required for this method.

1. 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. 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.

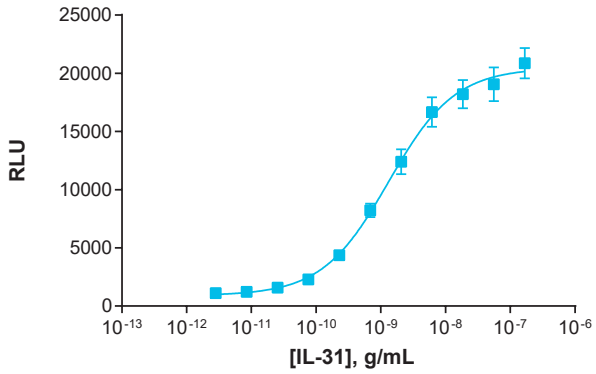
5. Read the sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility

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

Typical Results

The following graph is an example of a typical dose-response curve for the IL-31RA/OSMRβ Bioassay generated using the protocol outlined in this user manual. The data shows potent, dose-dependent heterodimerization of the two receptors, when treated with IL-31.

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



EC_{50} (ng/mL)	1.32
S/B	18.6

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Troubleshooting Guide

Problem	Cause	Solution
No response	Incorrect thawing procedure	Refer to the thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or incorrect ligand incubation time	Refer to the datasheet for recommended ligand and assay conditions.
	Sub-optimal time course for induction	Optimize time course of induction with 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	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) Sub-optimal agonist challenge concentration	Some ligands are difficult to handle. Confirm the final concentration of ligands. Perform agonist curve to reassess EC_{80} with the ligand 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 ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Refer to the datasheet for the ligand provided in the kit to confirm that the ligand used is comparable.
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may require evaluation in different dilution buffers.
		Non-binding surface plates may be necessary for hydrophobic compounds.

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

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