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User Manual

PathHunter® U2OS IL-10RA/IL-10RB Dimerization Bioassay Kit

For Detection of Ligand-Induced Stimulation of IL-10RA and IL-10RB Dimerization

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Table of Contents

Overview 1

Assay Principle 1

Materials Provided 2

Storage Conditions 2

Additional Materials Required 3

Protocol Schematic 4

Detailed Protocol 5

- Day 1: Bioassay Cell Preparation 5
- Day 1: Sample Preparation 5
- Day 2: Detection 7

Typical Results 8

Troubleshooting Guide 9

Limited Use License Agreement 9

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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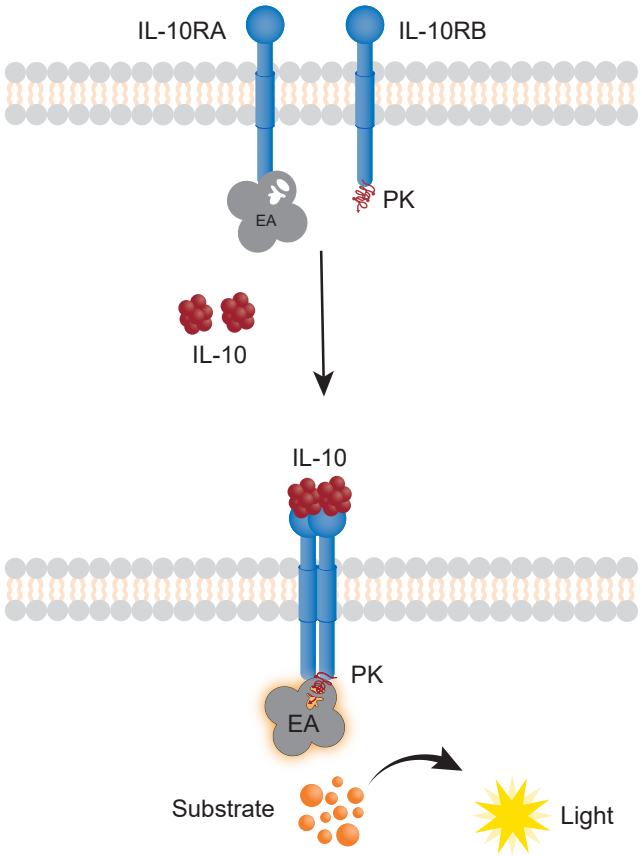
Overview

The PathHunter IL-10RA/IL-10RB 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 the materials needed for a complete assay, including 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-10RA/IL-10RB Bioassay is an application of Eurofins DiscoverX's Dimerization Assay platform. The assay is designed to detect IL-10-induced functional dimerization of the Interleukin 10 receptor alpha (IL-10RA) and the Interleukin 10 receptor beta (IL-10RB) chains. The cells have been engineered to co-express IL-10RA fused to PK, and IL-10RB fused to EA. Binding of an agonist, such as IL-10, to IL-10RA, induces it to dimerize with IL-10RB, bringing the two receptor chains into close proximity and forcing complementation of the two enzyme fragments. This results in the formation of a functional β-gal enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.



Materials Provided

List of Components	93-0985Y3-00135	93-0985Y3-00136
PathHunter U2OS IL-10RA/IL-10RB Bioassay Cells (1.2×10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 5 Reagent (100 mL per bottle)	1	2
Recombinant Human IL-10 (10 µg per vial)	1	2
AssayComplete Protein Dilution Buffer (Bottle)	1 x 50 mL	2 x 50 mL
PathHunter Bioassay Detection Kit		
Detection Reagent 1 (Bottle)	1 x 3 mL	1 x 15 mL
Detection Reagent 2 (Bottle)	1 x 12 mL	1 x 60 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

Storage Conditions

PathHunter U2OS IL10RA/IL10RB 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 5 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.

AssayComplete Protein Dilution Buffer

Once thawed, the 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 IL-10

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

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

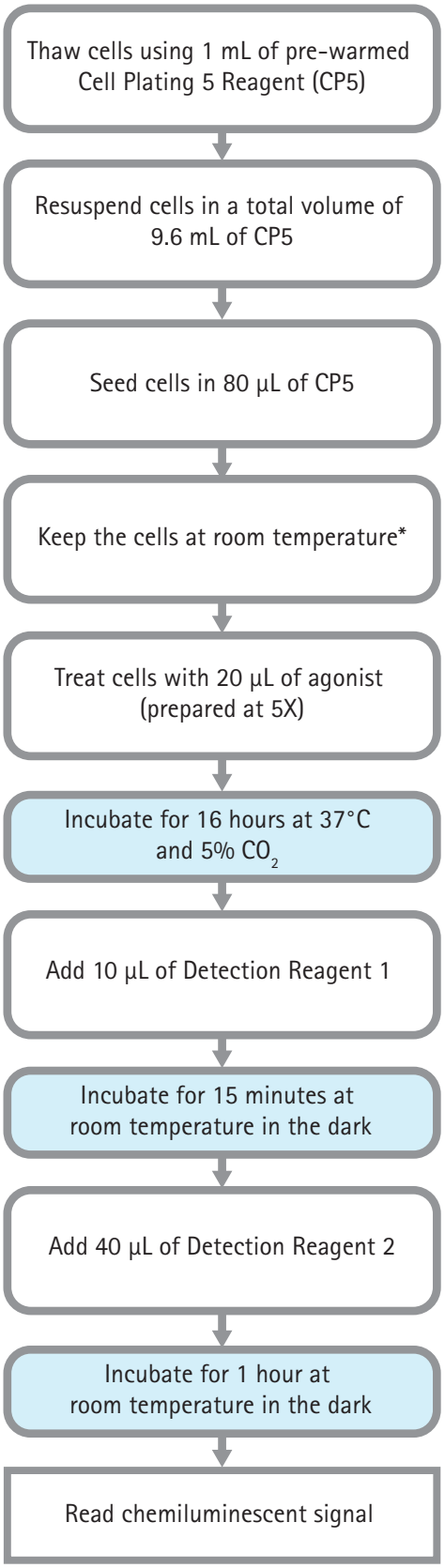
Additional Materials Required

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 the cryopreserved PathHunter U2OS IL-10RA/IL-10RB 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. 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 5 Reagent (CP5), 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 CP5 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 CP5 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 several times to uniformly resuspend the cells.
5. Transfer the cell suspension into the conical tube containing the remaining 8.6 mL of CP5. Remove any remaining liquid from the cryovial to ensure maximum recovery of all the cells.
6. Gently invert the conical tube several times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension. Immediately pour 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 in the tissue culture hood at room temperature while preparing samples for the assay.

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 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 for the control agonist, IL-10, 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 the Representative Assay Plate Map).

1. Add 80 µL of the Protein Dilution Buffer (PDB) to Wells A2 to A12 of the master dilution plate.

2. Prepare the IL-10 dose-response curve:
   The Recombinant Human IL-10 will serve as a positive control in this assay. The agonist is prepared at 5X the desired final concentration as it will be diluted 5-fold 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-10 powder, to make a 100 µg/mL stock solution. Gently shake the vial (do not vortex) for ten minutes to completely dissolve the powder.

   2.2. Make a 1:10 dilution of the 100 µg/mL stock solution prepared in Step 2.1. Add 108 µL of PDB to Well A1 of the master dilution plate. Add 12 µL of the 100 µg/mL IL-10 stock to Well A1, and mix thoroughly by pipetting up and down several times. This results in a 10 µg/mL solution (5X the final 2 µg/mL top dose).

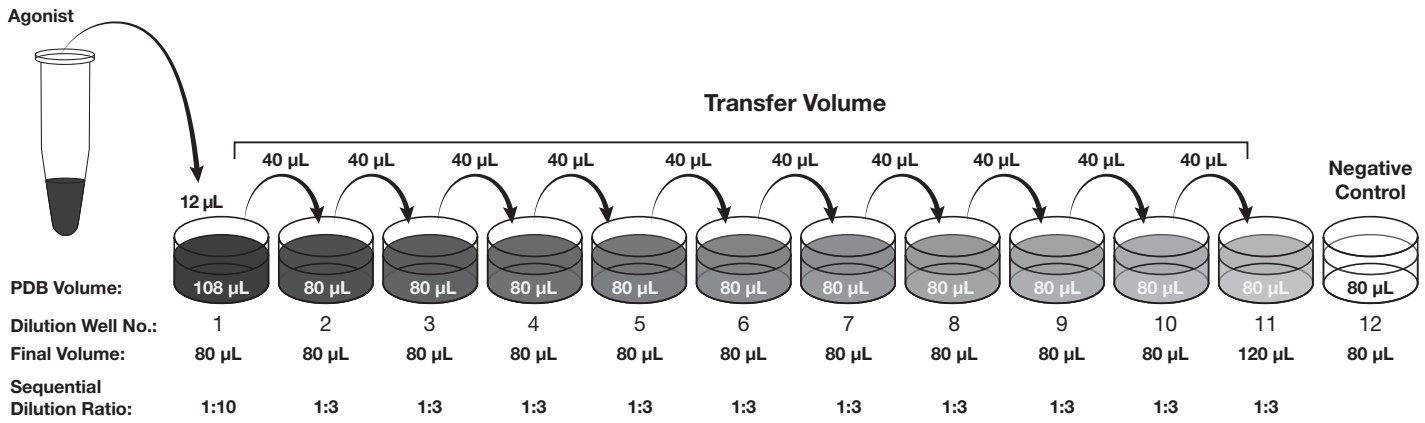
   2.3. Using a clean pipette tip, transfer 40 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 40 µL from Well A2 into Well A3, and mix well. Repeat this process through Well A11, resulting in an eleven-point, 1:3 dilution series. No sample is transferred to Well A12, as this will serve as a negative control.

3. Prepare any additional agonist test samples in a similar manner.

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

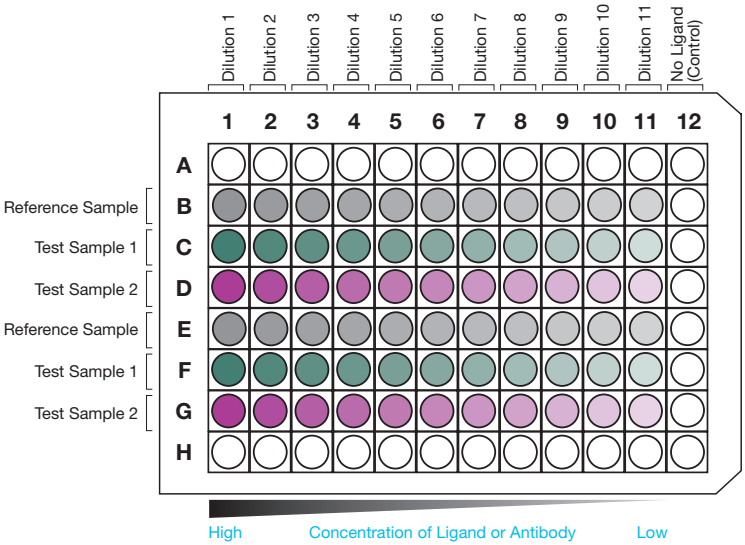
5. Transfer 20 µL from each well of the agonist dilution series (or prepared test samples) from the master dilution plate to the appropriate wells of the assay plate.

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



Agonist dilution series: Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

Representative Assay Plate Map



Assay Plate Map: This plate map shows 11-point dilution with 2 data points at each concentration for one reference and two test samples per plate, each prepared with a 1:3 dilution scheme.

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 it 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.

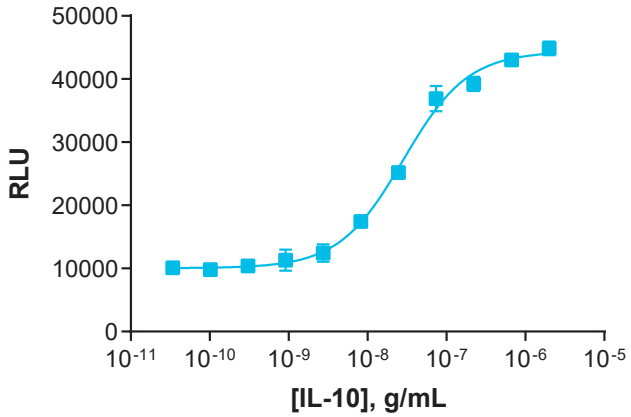
1. Add 10 µL of Detection Reagent 1 to each well of the assay plate.
2. Incubate the plate at room temperature for 15 minutes in the dark.
3. Add 40 µL of Detection Reagent 2 to each well of the assay plate.
4. Incubate the plate at room temperature for 1 hour in the dark.
5. Read the sample on a standard 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-10RA/IL-10RB 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-10.

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



EC_{50} (ng/mL)	28.4
S/B	4.6

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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 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 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 ligands are difficult to handle. Confirm the final concentration of ligands.
	Sub-optimal agonist challenge concentration	Perform the 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 the ligand handling requirements.
	Difference in agonist binding affinity	Refer to the Certificate of Analysis 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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