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User Manual

PathHunter® Tocilizumab Bioassay Kit

For Chemiluminescent Detection of IL6R/IL6ST Dimerization

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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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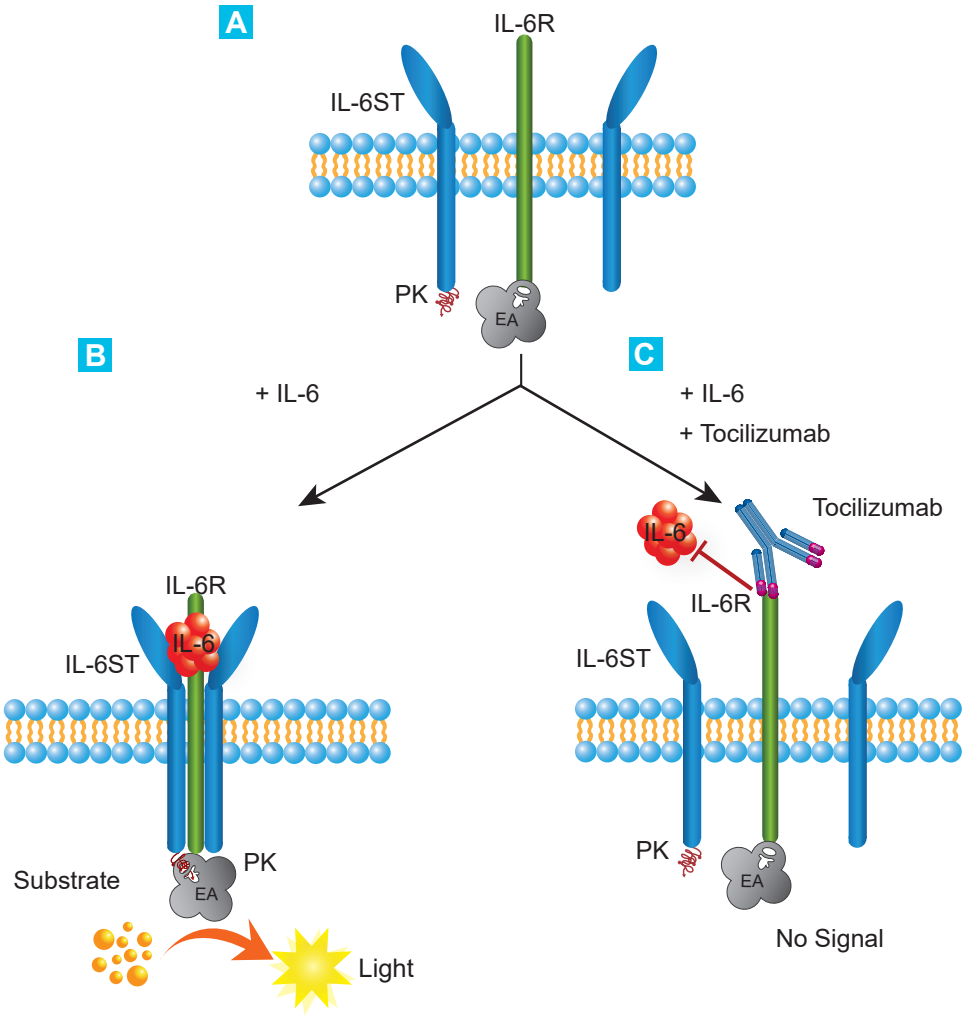
Overview

PathHunter Tocilizumab Bioassay kits provide a robust, highly sensitive, and easy-to-use cell-based functional assay to study potency of anti-IL-6R or anti-IL-6 antibodies and their 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-validated, 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

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 Tocilizumab Bioassay is an application of the DiscoverX Dimerization Assay platform. The assay is designed to detect the ligand-induced interaction of the interleukin-6 receptor (IL-6R) with the interleukin-6 signal transducer protein (IL-6ST). The cells have been engineered to co-express IL-6ST fused to PK, and IL-6R fused to EA. Binding of an agonist to the IL-6R causes multimerization of the IL-6R and IL-6ST receptors, resulting in activation of downstream signaling events. This brings the two β-gal fragments into close proximity, forcing complementation. Heterodimerization of the two receptor chains results in the formation of a functional β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal. Blocking IL-6 binding with an anti-ligand or anti-receptor antibody can prevent this interaction, resulting in a loss of signal.



Materials Provided

List of Components	93-1045B3-00109	93-1045B3-00110
PathHunter U2OS IL6R/IL6ST Bioassay Cells (No. of vials)	2	10
PathHunter Bioassay Detection Kit (No. of datapoints) Detection Reagent 1 (mL) Detection Reagent 2 (mL)	200 2 8	1,000 10 40
AssayComplete™ Cell Plating 5 Reagent*	1 x 100 mL	2 x 100 mL
Control Agonist, IL-6 (No. of vials)	1	1
96-well White, Clear Flat-bottom Tissue Culture-treated Sterile Plates with Lid	2	10

* Cell Plating 5 Reagent is also used for diluting control agonist and tocilizumab in the bioassay.

Storage Conditions

PathHunter U2OS Tocilizumab 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 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 (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. 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.

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 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 2.3 mL each for Detection Reagent 1, and five aliquots of 9.2 mL each for Detection Reagent 2. Sufficient reagent volumes are provided in the kit to make these aliquots.

If 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 (CP5)

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-6 Control Agonist

Store at -20°C 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. Reconstitute to a concentration of 100 µg/mL by adding 200 µL of Protein Dilution Buffer and gently shake (do not vortex) for ten minutes to increase solubility. Reconstituted ligand is stable for 12 months at -20 to -80°C, or for 1 week at 2-8 °C.

96-Well Tissue Culture-Treated Plates

Store at room temperature.

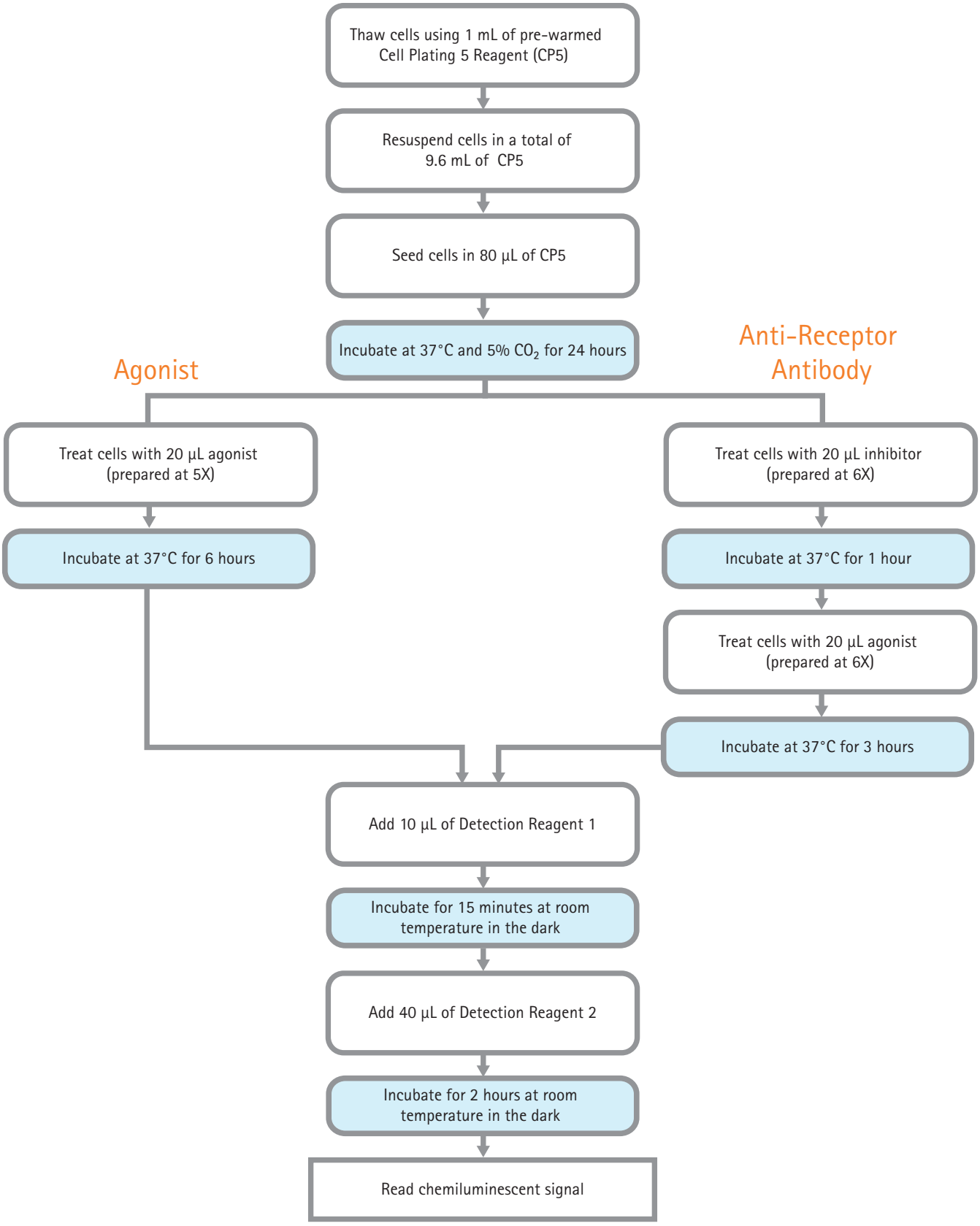
Additional Materials Required

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

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
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micro-pipettors and pipette tips	

Protocol Schematic

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



Detailed Protocols

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter U2OS IL6R/IL6ST Bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the necessary materials are set up in the tissue culture hood. These includes:
    a. One 25 mL reagent reservoir
    b. One 15 mL conical tube
    c. A pipette set to dispense 1 mL
    d. A multichannel pipette and tips set to dispense 80 µL
    e. A bottle of Cell Plating Reagent 5 (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)
2. Dispense 9.6 mL of CP5 into the 15 mL conical tube
3. Remove the cryovial from liquid nitrogen, and immediately place it in dry ice.

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

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 side of the wall of the cryovial tube. Mix the cells gently by pipetting up and down several times to uniformly resuspend the cells. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP5. Remove all medium/suspension in the tube to ensure maximum recovery of all the cells from the vial.
5. Gently invert the tube several times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension, and immediately pour the suspension into the 25 mL reagent reservoir.
6. Add 80 µL of cell suspension to each well of the 96-well assay plate using the multichannel pipette. Replace 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. Gently place the assay plate in a tissue culture incubator set to 37˚C and 5% CO_2 for 24 hours before proceeding with the assay.

Day 2: 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 empirically determined.

A 1:3 serial dilution for the Agonist/Control Ligand, IL-6 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 Plate Map).

1. Add 200 µL of Cell Plating 5 Reagent (CP5) to wells A2 to A12 of a dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX, Cat. No. 92-0011 or similar).

2. Prepare the reference agonist (IL-6) dose-response curve:
   IL-6 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 the 80 µL of medium present in the assay plate.

   a. Add 200 µL of the IL-6 Reconstitution Buffer to the vial containing 20 µg of lyophilized IL-6 powder, to make a 100 µg/mL stock solution. Gently shake (do not vortex) for ten minutes to completely dissolve the powder.

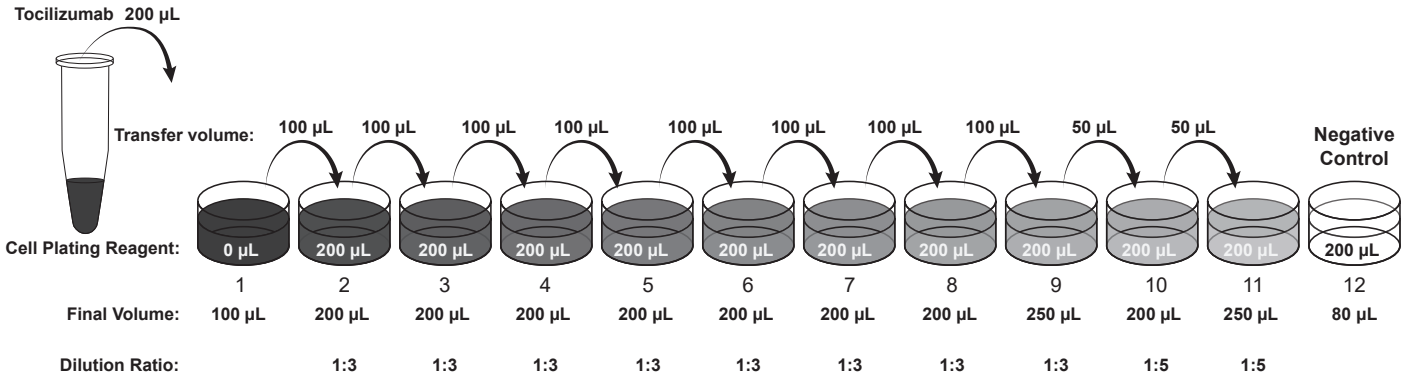
   b. Make a 2 µg/mL working stock for IL-6, by adding 10 µL of the 100 µg/mL IL-6 solution into 490 µL of CP5 in a sterile microcentrifuge tube.

   c. Add 190 µL of CP5 to well A1 of the master dilution plate. Add 10 µL of the working stock to well A1. Mix thoroughly by pipetting up and down several times. This results in a 100 ng/mL solution (5X the final 20 ng/mL highest dose).

   d. Using a clean pipet 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 until well A11, resulting in an eleven-point, 1:3 dilution series. No ligand is transferred to any well in the column 12, as this will serve as a negative control.

3. Prepare the Tocilizumab Curve:

   a. Tocilizumab is prepared at 6X the desired final concentration, in 11-point dilution series, with a top dose of 120 µg/mL. Note: No antibody is added to any wells in column 12, as these will be the negative control wells.



Antibody Serial Dilution: Make eleven 3-fold serial dilutions of the antibody in a dilution plate.

b. Add 200 µL of Tocilizumab prepared at 6X the desired final concentration (i.e. 720 µg/mL) to well B1 of the master dilution plate.

c. Add 200 µL of CP5 to wells B2 to B12 of the master dilution plate.

d. Using a clean pipet tip, transfer 100 µL from well B1 into well B2 and mix thoroughly by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well B2 into well B3. Mix well by pipetting up and down several times. Repeat this process until well B9 is reached. Then transfer 50 µL from well B9 into well B10, and mix thoroughly by pipetting up and down several times. Repeat the process by transferring 50 µL from well B10 into well B11. No ligand is transferred to Well B12, as this will serve as a negative control.

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

5. Add 20 µL from the agonist reference curve (IL-6) on the master dilution plate (wells A1-A12) to the appropriate wells of the assay plate.

6. Add 20 µL from each well of the Tocilizumab curve on the master dilution plate (wells B1-B12) to the respective wells of the assay plate.

7. Place the assay plate in a humidified 37°C, 5% CO_2 incubator for 1 hour.

8. Prepare agonist challenge for the biosimilar curves: The EC_{80} of the supplied IL-6 was determined to be approximately 6 ng/mL. If IL-6 from a different vendor is used, the EC_{80} should be determined empirically, prior to testing samples. Prepare the agonist challenge at 6X the desired final concentration. The following steps will provide enough agonist challenge for a single antagonist curve run in triplicate:

   a. Prepare a 1:20 dilution by transferring 10 µL of the 2 µg/mL working stock of IL-6, prepared in Step 1b above, into 190 µL of CP5 in a non-binding eppendorf tube, to generate a 100 ng/mL IL-6 stock solution. Mix well.

   b. Transfer 108 µL from the 100 ng/mL IL-6 stock solution into 192 µL of CP5 to prepare a 36 ng/mL agonist solution.

9. Add 20 µL from each well of the agonist challenge prepared in Step 2c, to the appropriate wells of the assay plate containing the anti-IL-6 antibody.

10. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 3 hours.

Day 2: Detection

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.

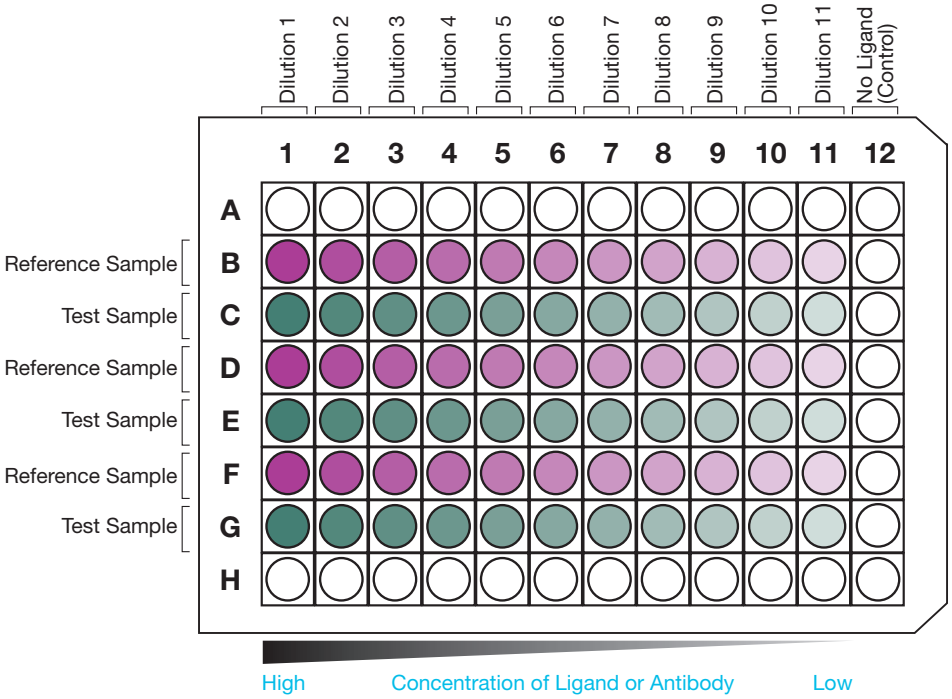
3. Add 40 µL of Detection Reagent 2 to each well of the assay plate.

4. Incubate the plate at room temperature for two hours in the dark.

5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for imager.

Note: 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 higher signal. Additional Cell Plating Reagent will be required for this method.

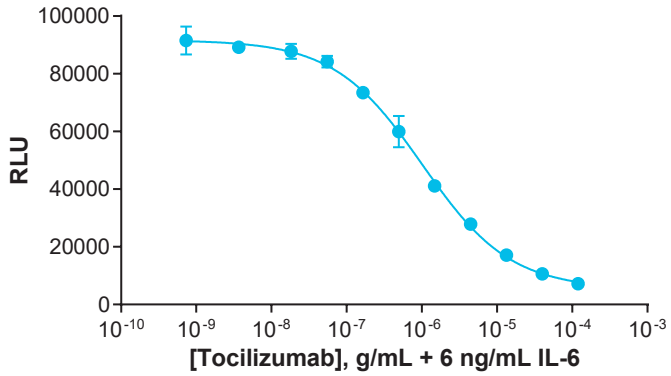
Representative Plate Map for Agonist/Inhibitor Dose-Response Curve



Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with 3 replicates per dose point, for a test and reference sample tested with their appropriate dilution schemes.

Typical Results

The following graph is an example of a typical dose-response curve for the Tocilizumab Bioassay, generated using the protocol outlined in this user manual.



IC_{50}	1.01 µg/mL
S/B	13.4

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to thawing instructions in Bioassay Cell Preparation section of of this user manual.
	Incorrect ligand used or incorrect ligand incubation time	See datasheet for recommended ligand and assay conditions.
	Incorrect preparation of ligand (agonist or antagonist)	Refer to vendor specific datasheet to ensure proper handling, dilution, and storage of ligand.
	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 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) Suboptimal 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 assay window.
EC_{50} is right-shifted	Improper ligand handling or storage	Check 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 may require evaluation in different dilution buffers.
		Non-binding surface plates may be necessary for hydrophobic compounds.

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

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