User Manual

PathHunter® Adalimumab Bioassay Kit

For Detection of Ligand-Induced IκB Degradation

For Bioassay Kits

2-Plate Kit: 93-0538B15-00131

10-Plate Kit: 93-0538B15-00132

Document Number 70-412 Revision 0

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

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For additional information or Technical Support, see contact information at the bottom of this page.

Overview

The PathHunter Adalimumab Bioassay Kit provides a robust, highly sensitive, and easy-to-use functional cell-based assay to determine drug potency and detect neutralizing antibodies. The bioassay kit contains all the materials needed to run the assay, including cryopreserved cells, cell plating reagent, control agonist, detection reagents, 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 and qualified with Humira® (not supplied in the bioassay kit). Humira® is a registered trademark of AbbVie, Inc.

Assay Principle

The PathHunter Adalimumab Bioassay Kit is designed to measure IκB degradation as a result of TNFα-mediated activation of the NF-κB signaling pathway. When bound to ligand-neutralizing agents such as Adalimumab, TNFα is unavailable to stimulate its cognate receptor. This prevents the activation of the NF-κB signaling pathway and consequently, TNFα-mediated IκB degradation.

This bioassay utilizes the Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, the Enzyme Donor or ProLabel™ (PL) and the Enzyme Acceptor (EA). Separately, these fragments have no β-gal enzymatic activity, however, when forced to complement, they form an active β-gal enzyme.

In this assay, cells have been engineered to overexpress IκB tagged with PL. The IκB levels can then be measured by the addition of the detection reagent containing EA, which forces complementation of the two enzyme fragments. The resulting active enzyme hydrolyzes the substrate present in the detection reagents to generate a chemiluminescent signal, which will be proportional to the degree of IκB stabilization.

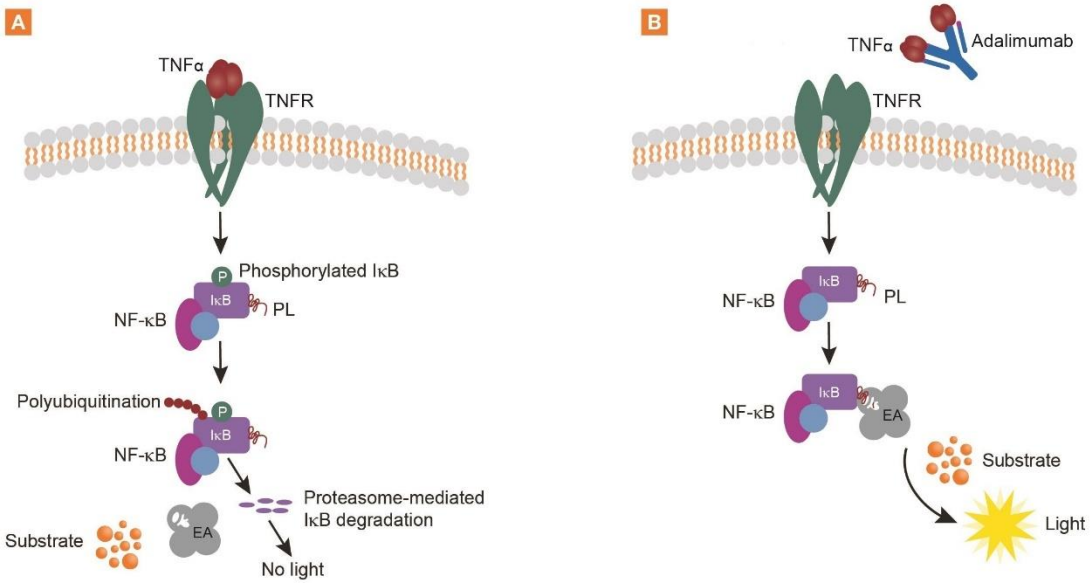


Figure 1. Assay Principle: This figure indicates the key steps that occur when, **A:** the NF-κB signaling pathway is activated by the ligand TNFα, resulting in proteasome-mediated IκB degradation, and **B:** when TNFα binds to adalimumab, thus preventing the activation of the signaling pathway. As a result, IκB does not undergo degradation, and the two enzyme fragments undergo complementation to form an active enzyme that hydrolyzes the substrate and generates a chemiluminescent signal.

Materials Provided

List of Components	93-0538B15-00131	93-0538B15-00132
PathHunter A549 IκB Bioassay Cells (1.2 x 10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 38 Reagent (100 mL per bottle)	1	2
Recombinant Human TNFα (10 µg per vial)	1	2
AssayComplete Protein Dilution Buffer (50 mL per bottle)	1	2
PathHunter Bioassay ED Detection Kit Detection Reagent 1 (Bottle) Detection Reagent 2 (Bottle) Detection Reagent 3 (Bottle)	 1 x 6 mL 1 x 20 mL 1 x 5 mL	 1 x 30 mL 1 x 100 mL 1 x 25 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

Storage Conditions

PathHunter A549 IκB 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 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 ED Detection Kit

Upon receipt, store at or below -20°C. Thaw reagents at room temperature before use. After thawing, reagents can be stored for up to 7 days at 4°C. The reagents can tolerate up to three freeze-thaw cycles without any impact on performance.

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 three detection reagents. Each aliquot will be adequate for two assay plates. Make five aliquots of 6 mL each for Detection Reagent 1, five aliquots of 20 mL each for Detection Reagent 2, and five aliquots of 5 mL each for Detection Reagent 3. Sufficient reagent volumes are provided in the kit to make these aliquots.

AssayComplete™ Cell Plating 38 Reagent (CP38)

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 TNFα

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. Once prepared, the excess stock solution can be stored as suitable aliquots (e.g. 30 µL) at -20°C until needed. Do not freeze-thaw more than twice.

AssayComplete™ Protein Dilution Buffer

Upon receipt, store at -20°C. 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.

96-Well Tissue Culture-Treated Plates

Store at room temperature.

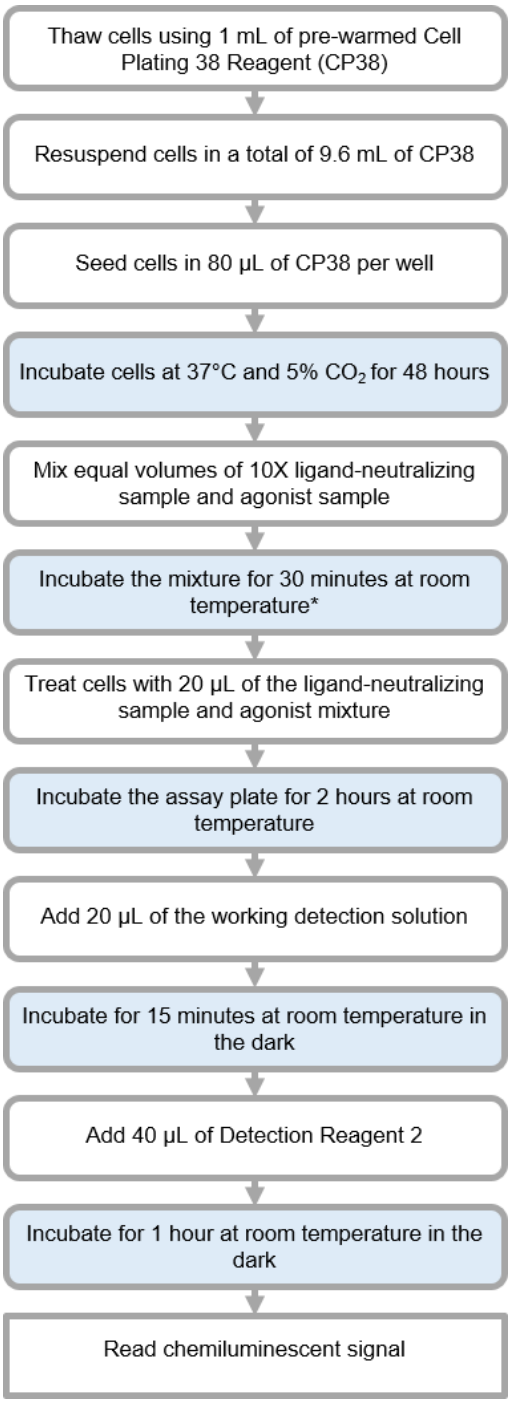
Additional Materials Required

The following equipment and additional materials are required to perform this assay:

Materials	Ordering Information
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Multimode or luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micropipettes and pipette tips (10 µL-1000 µL)	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	
Humidified tissue culture incubator (37°C and 5% CO_2)	

Protocol Schematic

Quick-start procedure: In a 96-well assay plate, perform the following steps:



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

Detailed Protocol

This user manual provides a protocol for determining potency in a 96-well format. To run this bioassay in a 384-well format, or to optimize the assay for the detection of neutralizing antibodies, contact Technical Support at DRX_SupportUS@eurofinsUS.com.

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and then plating cryopreserved A549 IκB 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 and tips (P1000) set to dispense 1 mL
    d. A multichannel pipette and tips set to dispense 80 mL
    e. A bottle of AssayComplete™ Cell Plating 38 Reagent (CP38), 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. Two 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates, one will be used as the master dilution plate, and the other will be used as the ligand-neutralizing sample plate.
2. Dispense 9.6 mL of CP38 into the 15 mL conical tube.
3. Remove the cryovial from liquid nitrogen and immediately place 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 cryovial quickly with 70% ethanol, and bring it into the tissue culture hood right away.

4. Add 1 mL of pre-warmed CP38 from the 15 mL conical tube to the cryovial of the A549 IκB bioassay cells, to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet the cell suspension up and down several times to uniformly resuspend the cells.
5. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP38. 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 uniformly resuspended in the reagent, without creating any froth in the suspension. Immediately pour the suspension into the sterile 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 keep 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 48 hours before proceeding with the assay.

Day 3: Control Agonist 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 can be added directly to cells without further dilution. For best results, the optimized minimum required dilution of crude samples should be determined empirically.

Recombinant Human TNFα (TNFα) will serve as a positive control (agonist) in this assay. Preparation of a 1:3 serial dilution of TNFα is described 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 Figure 3. Representative Assay Plate Map).

The control curve is used to confirm the EC_{80} of TNFα for the ligand-neutralizing assay.

1. Add 90 µL of the Protein Dilution Buffer (PDB) to Wells A2 to A12 of the master dilution plate.
2. Prepare the TNFα dose-response curve: TNFα 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 TNFα powder, to make a 100 µg/mL stock solution.
    2.2. Make a 1:100 dilution of the agonist, by adding 5 µL of the 100 µg/mL stock solution prepared in Step 2.1, to 495 µL of PDB in a sterile microcentrifuge tube. This results in a stock solution of 1 µg/mL (5X the top dose of 200 ng/mL).
    2.3. Transfer 120 µL of the 1 µg/mL stock solution prepared in Step 2.2 into Well A1 of the master dilution plate.
    2.4. Using a clean pipette tip, transfer 30 µL of the solution from Well A1 to Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 30 µL from Well A2 to Well A3, and mix well. Repeat this process through Well A11, resulting in an 11-point, 1:4 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 from the master dilution plate to the appropriate wells of the assay plate.
6. Incubate the assay plate at room temperature for 2 hours.

Day 3: Ligand-Neutralizing Sample Preparation

The following protocol is used for the quantitation of the anti-ligand antibody, adalimumab. TNFα is pre-incubated with adalimumab for 30 minutes. However, if using another anti-TNFα molecule in this assay, the optimal incubation time with TNFα and dilutions series for your sample should be determined empirically.

1. Prepare the adalimumab dilution series:
    1.1. Add 200 µL of PDB to wells B2 to B5 of the master dilution plate. Add 100 µL of PDB to Wells B6 to B9, and add 200 µL to Wells B10 to B12 of the master dilution plate.
    1.2. Prepare a 5 mg/mL working stock solution of adalimumab by adding 10 µL of the 50 mg/mL adalimumab stock solution to 90 µL of PDB.
    1.3. Add 6 µL of the working stock solution prepared in Step 1.2, to 294 µL. This results in a 100 µg/mL

solution (10X the final 10 µg/mL dose). Mix well.

1.4. Add 150 µL of the 100 µg/mL adalimumab solution to Well B1 of the master dilution plate.

1.5. Using a clean pipette tip, transfer 50 µL of solution from Well B1 to Well B2, resulting in a 1:5 dilution. Mix thoroughly by pipetting up and down several times.

1.6. Replace the pipette tip and transfer 100 µL of solution from Well B2 to Well B3, resulting in a 1:3 dilution. Mix thoroughly by pipetting up and down several times. Repeat this process until Well B5 is reached.

1.7. Using a clean pipette tip, transfer 100 µL of solution from Well B5 to Well B6, resulting in a 1:2 dilution. Mix thoroughly by pipetting up and down several times. Repeat this process until Well B9 is reached.

1.8. Using a clean pipette tip, transfer 100 µL of solution from Well B9 to Well B10, resulting in a 1:3 dilution. Repeat this process until Well B11 is reached. No sample is transferred to Well B12 as this is the negative control.

1.9. Prepare additional ligand-neutralizing test samples in a similar manner.

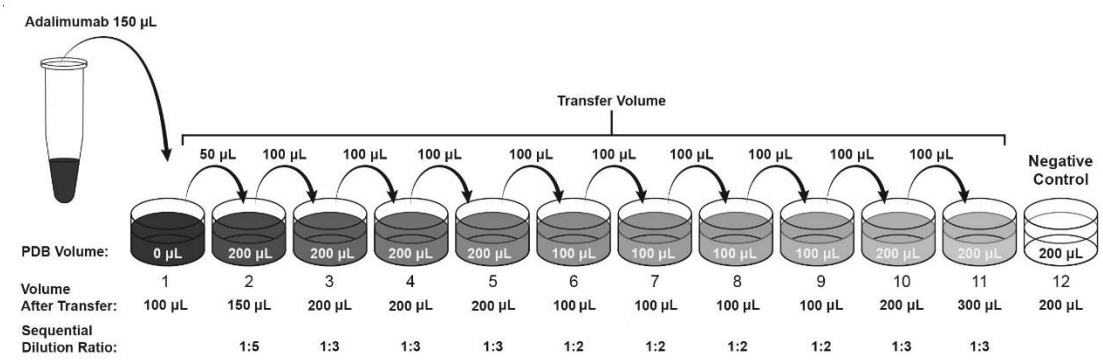


Figure 2. Ligand-Neutralizing Dilution Series: Make eleven serial dilutions, using the dilution ratios indicated in the figure.

2. Prepare the agonist challenge:

2.1. The optimal concentration of the supplied agonist, TNFα, required to stimulate the adalimumab bioassay was determined to be approximately 3 ng/mL. If TNFα from a different vendor is used, then the optimal agonist concentration should be determined empirically, prior to testing samples. Prepare the agonist challenge at 10X the desired final concentration.

2.2. Add 150 µL of the 1 µg/mL solution of TNFα (prepared in Step 2.2. in Control Agonist Preparation), into 4,850 µL of PDB in a 15 mL conical tube. This results in a 30 ng/mL stock solution of TNFα (10X the highest 3 ng/mL dose). Vortex the tube gently to mix the solution.

2.3. Pour the 10X TNFα stock solution into a sterile reagent reservoir. Transfer 50 µL of this solution into each well of Row B on the ligand-neutralizing sample dilution plate. Transfer this solution into additional rows of the plate, as needed for additional test samples.

2.4. Transfer 50 µL from each well of the adalimumab dilution series (Row B of the master dilution plate), to Row B of the ligand-neutralizing sample dilution plate. Mix well by pipetting up and down several times.

2.5. Prepare additional test sample(s) in a similar manner.

2.6. Incubate the ligand-neutralizing dilution plate for 30 minutes at room temperature inside the tissue culture hood.

3. Add 20 µL of solution from each row of samples in the dilution plate to the appropriate wells of the assay plate. Refer to Figure 3. Representative Assay Plate Map for the recommended sample layout.
4. Incubate the assay plate at room temperature for 2 hours.

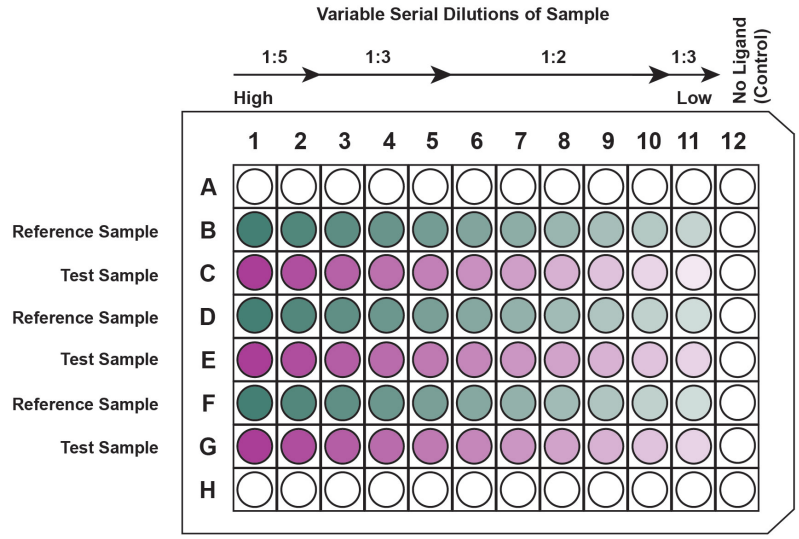


Figure 3. Representative Assay Plate Map: This assay plate map shows an 11-point dose curve with 3 data points at each concentration for one reference and one test sample per plate, with a variable serial dilution scheme.

Day 3: Detection

1. Prepare a working detection solution by mixing 1.25 mL of Detection Reagent 1 and 1.25 mL of Detection Reagent 3. Mix gently to avoid frothing of the solution. Do not vortex.
2. Immediately add 20 µL of the working detection solution to all wells of the assay plate.
3. Incubate the plate for 15 minutes at room temperature.

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

4. Add 40 µL of Detection Reagent 2 to all wells of the assay plate.
5. Incubate the plate for 1 hour at room temperature in the dark.
6. 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 an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

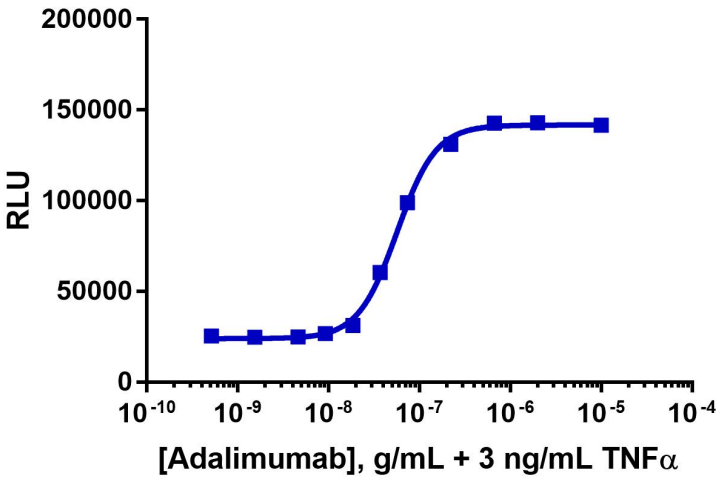
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel etc.

Typical Results

The following graph is an example of a typical dose-response curve for the adalimumab bioassay generated using the protocol outlined in this user manual. The data shows potent, dose-dependent stabilization of IκB levels, upon the addition of adalimumab to TNFα.

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

A



B

IC_{50} (ng/mL)	56.8
S/B	5.5

Figure 4. Typical Results: Representative **A,** dose-response curve and **B,** the IC_{50} and assay window for the stabilization of IκB levels, when adalimumab successfully binds to TNFα.

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 improper ligand incubation time	Refer to the datasheet for recommended ligand and assay conditions.
	Incorrect preparation of ligand (agonist or antagonist)	Refer to the 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 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 assay at 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 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_SupportUS@eurofinsUS.com

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