User Manual

PathHunter® IL-17A/IL-17F Bioassay Kit (Dimerization)

For Quantitation of IL-17A Mediated Dimerization of the IL-17 receptor (IL17RA/IL17RC)

For Bioassay Kits with control:

93-0999Y3-00053: 2-Plate Kit

93-0999Y3-00054: 10-Plate Kit

For Bioassay Kit without control

93-0999Y3-00194: 10-Plate Kit

Document Number 70-402 Rev 1

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

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1. Overview

The PathHunter IL-17A/IL-17F Bioassay Kit (Dimerization) is a simple, robust, non-radioactive, dye free assay for quantitation of control-induced functional dimerization of the two subunits of the IL-17 receptor. Specifically, this assay detects IL-17A induced heterodimerization of the IL-17RA and IL-17RC receptor subunits. The PathHunter IL-17 Bioassay Kit (Dimerization) with control contains all the materials needed to perform a complete assay, including cryopreserved, single-use cells, detection reagents, cell plating reagent, agonist for stimulating the cells, and assay plates. A 10-Plate PathHunter IL-17A/IL-17F Bioassay Kit (Dimerization) is also offered without control but contains all other components listed above to run the assay. This bioassay has been optimized for a 96-well plate format.

2. 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-17A/IL-17F Bioassay quantifies control-mediated dimerization of the IL-17RA and IL-17RC subunits of the IL-17 receptor, utilizing the Eurofins DiscoverX Dimerization Assay platform. The assay is designed to detect the IL-17A induced dimerization of the IL-17RA and IL-17RC receptor subunits. As shown in Figure 1, the bioassay cells have been engineered to co-express IL-17RC fused to PK, and IL-17RA fused to EA. Binding of IL-17A to the IL-17RC subunit induces dimerization with IL-17RA, forcing the two β-gal enzyme fragments (PK and EA) into proximity. The result is formation of a functional β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal. Control induced receptor dimerization results in a dose-dependent gain of signal, while therapeutics that block control-mediated dimerization result in loss of assay signal.

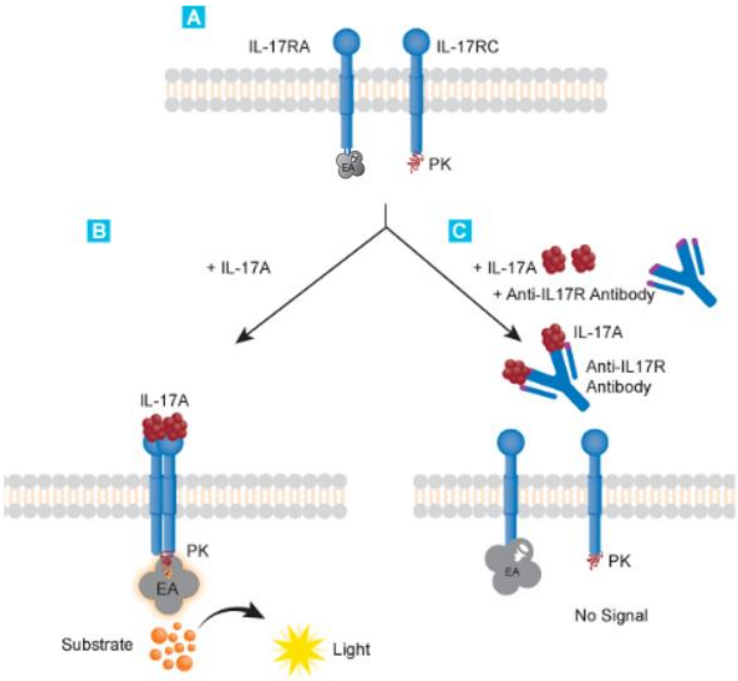


Figure 1. PathHunter IL-17A/IL-17F Bioassay (Dimerization) Assay Principle.

3. Materials Provided in PathHunter IL-17A/IL-17F Bioassay Kit (Dimerization)

List of Components	93-0999Y3-00053 (2-Plate Kit)	93-0999Y3-00054 (10-Plate Kit)	93-0999Y3-00194 (10-Plate Kit without control)
PathHunter U2OS IL17RA/IL17RC Bioassay Cells (1.2×10^6 cells in 0.1 mL per vial)	2	10	10
AssayComplete™ Cell Plating 5 Reagent (100 mL per bottle)	1 x 100 mL	2 x 100 mL	2 x 100 mL
AssayComplete Protein Dilution Buffer (Bottle)	1 x 50 mL	2 x 50 mL	2 x 50 mL
*Control Agonist (Recombinant Human IL-17A) (25 µg per vial)	1	1	N/A*
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	 1 x 15 mL 1 x 60 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

***Note:** For 93-0999Y3-00194 control not provided in the kit, would need to be obtained separately if needed.

4. Storage Conditions

PathHunter U2OS IL17RA/IL17RC 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.

AssayComplete™ Cell Plating 5 (CP5) 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), aliquot the reagent and store at -20°C until needed. Do not freeze-thaw more than three times. 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 (PDB)

Once thawed, 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-17A control agonist (If supplied in the kit)

Upon receipt, store at -20°C, until ready to use (up to the date listed in the kit's Certificate of Analysis). Centrifuge the vial before opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 250 µL of supplied reconstitution buffer to the 25 µg vial. Once prepared, the stock solution should be stored as suitable aliquots (e.g. 30 µL) at -20°C until needed. Do not freeze/thaw more than twice. Reconstituted control is stable for 12 months at -20°C to -80°C, or 1 week at 2-8°C.

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.

96-Well Tissue Culture-Treated Plates

Store at room temperature.

5. Additional Materials and Equipment Recommended for Assay

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

Materials	Ordering Information
Recombinant Human IL-17 (IL-17A)	Eurofins DiscoverX (Part # 92-1229), or similar
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	Eurofins DiscoverX (Part # 92-0011), or similar
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Disposable reagent reservoir	Thermofor Fisher Scientific, (Part # 8094) or similar
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettes and pipet tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	
Single and multichannel pipettors (e.g. P20, P100, P1000)	

6. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.1 mL of AssayComplete™ Freezing Reagent (refer to the CoA for cell number provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.


Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

PathHunter U2OS IL17RA/ IL17RC Bioassay Cells must arrive in a frozen state on dry ice.


Contact technical support immediately, if cells received were already thawed.

1. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, place vials in the vapor phase of liquid nitrogen storage.


Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

2. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.
3. Proceed with the thawing protocol in the detailed assay protocol section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

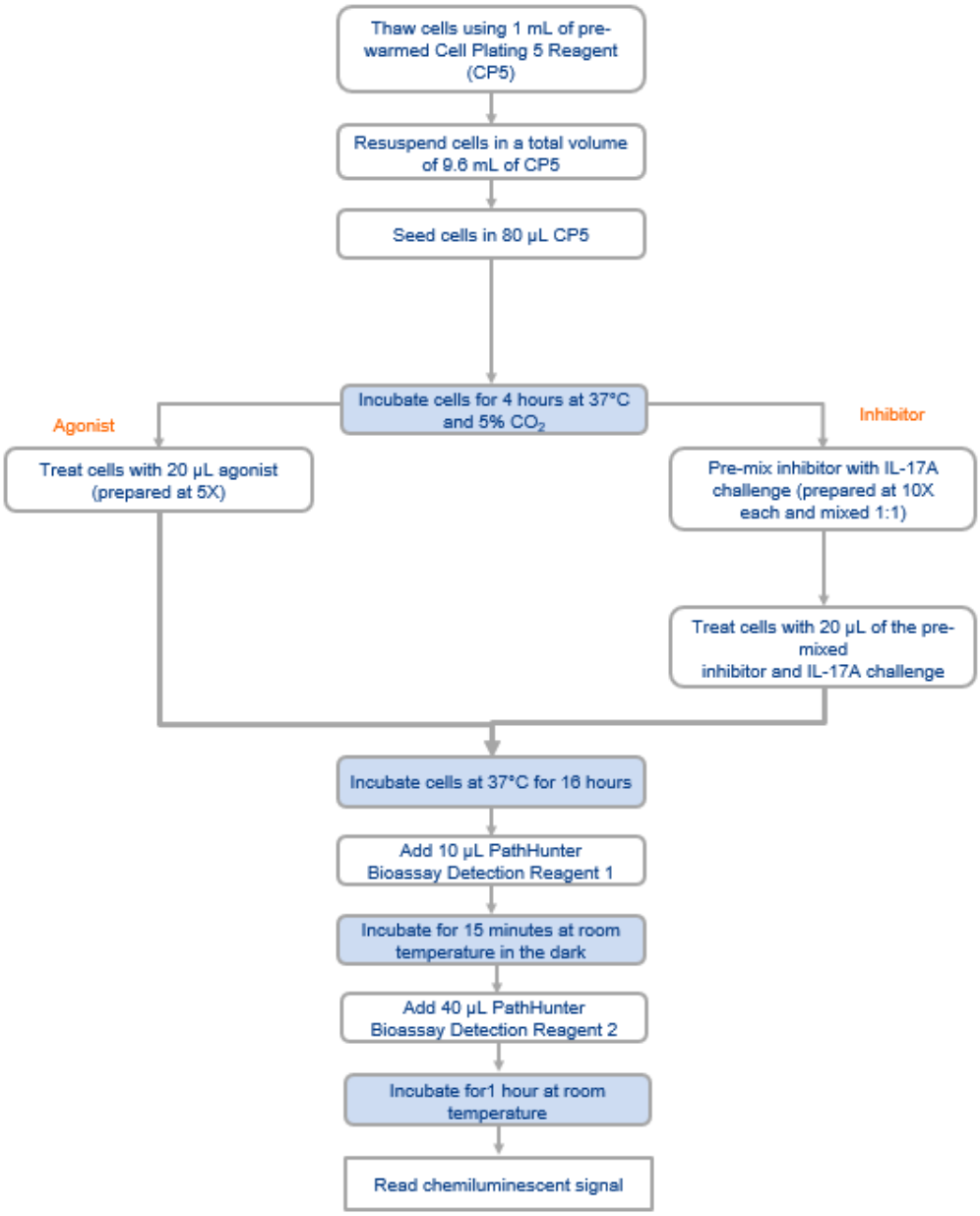
7. Protocol Schematic

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

Tip: *Use this sheet to note your assay specific conditions. Post it on your bench to use as a quick reference guide.*

Assay Name: _____Date: _____

Product Details: _____



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

8. Detailed Assay Protocol

This user manual provides a protocol for quantifying control mediated dimerization of the IL-17 receptor (IL17RA and IL17RC) subunits. This assay is performed under aseptic conditions. Preparation of cells, Reference Standards/Test Samples are performed in a Biological Safety Cabinet following good aseptic technique.

All appropriate materials are either certified sterile or prepared aseptically.

If purchasing the bioassay kit without control, it can be sourced per the details in the Additional Materials and Equipment Recommended for Assay table.

8.1 Bioassay Cell Preparation

Day 1

The following protocol is for thawing and plating frozen PathHunter U2OS IL17RA/ IL17RC Bioassay Cells from cryovials (one cryovial per 96-well assay plate).

1. Prior to thawing the cells, ensure that all the required materials are set up in the tissue culture hood. These include:

   a. One 25 mL reagent reservoir.

   b. One 15 mL conical tube.

   c. A micropipette (P1000) set to dispense 1 mL.

   d. A multichannel pipette and tips set to dispense 100 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, TC-Treated Sterile Plate (provided with bioassay kit).

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 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 pre-warmed CP5 from the 15 mL conical tube. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet up and down 3 times to uniformly suspend the cells.

5. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP5. Remove all the suspension from the cryovial tube to ensure maximum recovery of all the cells.

6. Replace the cap on the conical tube and mix by gentle inversion 2-3 times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension. Pour it immediately into the sterile 25 mL reservoir.

7. Using a multichannel pipet, transfer 80µL of the cell suspension to each well of the 96-well assay plate, one

row at a time, using reverse pipetting. Mix cells in trough by pipetting up/down 2-3 times before aspirating and pipetting cells into each subsequent row in the assay plate.

8. Replace the lid on assay plate and leave the plate at room temperature in biosafety cabinet for 15 minutes (but no more than 30 minutes) to allow the cells to settle uniformly in the well, to minimize potential for edge effects.

9. Gently place the assay plate in a humidified tissue culture incubator set to 37˚C and 5% CO_2 for 4 hours before proceeding.

8.2 Agonist Sample Preparation

Day 1

The following protocol is an example for preparing serial dilutions of IL-17A control agonist and agonist test samples.

1. On day of assay, prepare IL-17A working stock of control agonist, IL-17A.

   a. Reconstitute IL-17A (25 µg lyophilized powder) to 100 µg/mL stock solution by adding 250µL of supplied reconstitution buffer.

   b. Prepare working stock of IL-17A in Protein Dilution Buffer (PDB) as shown in Table 1 below. This will serve as the top concentration in the serial dilution curve, prepared as a 5X stock.

Table 1. Example of Preparation of IL-17A Working Stock

Working Stock Solution	[IL-17A], µg/mL	Volume IL-17A, µL	Volume PDB, µL
IL-17A, Working Stock	10	12 (100 µg/mL stock)	108

2. On the day of assay, prepare serial dilutions of IL-17A control agonist in Row A of a fresh 96-well master dilution plate (MDP) as 5X stocks in PDB as per Table 2 below. Sufficient volumes to run triplicate wells per dose in the assay plate are provided in the table. Volumes may be scaled up/down as appropriate.

   a. Add appropriate volume of sample diluent (PDB) to Row A of the MDP, (as indicated in column 6) of Table 2.

   b. Transfer 120 µL of working stock prepared in Table 1 to well 1 in Row A of MDP

   c. Prepare serial dilutions by transferring the volume of IL-17A indicated in column 5 (of Table 2) from the source well, (as indicated in column 4) to the destination well (indicated in column 1).

   d. Pipet up and down three times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 12, as this serves as the negative control.

Table 2. Example of Preparation of IL-17A Control Serial Dilutions

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
Well Location on Master Dilution Plate (MDP)	Concentration (5X) of sample on MDP, ng/mL	Dilution Ratio	Dilution (5X) Sample Source Well	Volume (5X) of Sample Added, µL	Volume Control Diluent, µL	Final Concentration (1X) of sample in Assay Plate, ng/mL
Row A, Well 1	10,000	--	Working Stock from Table 1 (10 µg/mL)	120	-	2,000
Row A, Well 2	3,333	1:3	Row A, Well 1	40	80	667
Row A, Well 3	1,111	1:3	Row A, Well 2	40	80	222
Row A, Well 4	370	1:3	Row A, Well 3	40	80	74.1
Row A, Well 5	123	1:3	Row A, Well 4	40	80	24.7
Row A, Well 6	41.2	1:3	Row A, Well 5	40	80	8.2
Row A, Well 7	13.7	1:3	Row A, Well 6	40	80	2.7
Row A, Well 8	4.6	1:3	Row A, Well 7	40	80	0.91
Row A, Well 9	1.5	1:3	Row A, Well 8	40	80	0.30
Row A, Well 10	0.51	1:3	Row A, Well 9	40	80	0.10
Row A, Well 11	0.17	1:3	Row A, Well 10	40	80	0.034
Row A, Well 12	0	--	--	--	100	0

3. In Row B of MDP, prepare test sample using same serial dilution scheme as in Table 2 above.

4. Transfer 20 µL of 5X sample serial dilutions from the MDP to the appropriate wells containing cells in the assay plate, as shown in the Representative Plate Map:

    a. Row A in MDP (Reference): transfer to Rows B, D and F in the assay plate

    b. Row B in MDP (Test Sample): transfer to Rows C, E and G in the assay plate

5. Incubate assay plate in humidified tissue culture incubator 37°C and 5% CO_2 incubator for 16 hours. Proceed to section 8.4 for signal detection procedure.

8.3 Ligand Neutralizing Sample Preparation

Day 1

The following protocol is used for the quantitation of anti-ligand antibody (e.g. anti-IL-17A) samples. IL-17A is

pre-incubated with the ligand-neutralizing sample(s) for 15 - 60 minutes before addition to the assay plate. The optimal incubation time (and temperature) for your antibody should be determined empirically.

1. On the day of the assay, prepare anti-ligand antibody dilution series at 10X the desired final concentration. Preparation of a 1:3 dilution series of antibody is given below as an example, but the dilution series for your antibody should be optimized empirically; volumes may be scaled up or down as needed.

    a. Add 120 µL of 10X anti-ligand antibody sample to column 1 of Row A of a new master dilution plate (MDP).

    b. Add 80 µL of Protein Dilution Buffer (PDB) to columns 2 through 12 of this row of the MDP.

    c. Using a clean tip, transfer 40 µL from column 1 into column 2 and mix by pipetting up and down several times. Replace the pipette tip and transfer 40 µL from column 2 into column 3. Mix by pipetting up and down several times. Repeat this process until column 11 is reached, resulting in an eleven point, 1:3 dilution series. No antibody is transferred to column 12 as this will serve as a negative control.

    d. Prepare an additional anti-ligand test sample in a similar manner in a new row of the MDP (e.g. Row C), leaving an empty row between each sample.

2. Prepare the agonist challenge and ligand neutralizing samples.

    a. Determine the EC_{80} of IL-17A and prepare the agonist challenge at 10X the desired final concentration.

    b. Pour the 10X EC_{80} agonist stock solution into a sterile reagent reservoir. Transfer 50 µL of this solution into each well of Row B on the MDP. Transfer this solution into additional rows of the MDP as needed for additional test samples (e.g. into Row D if sample is prepared in Row C).

    c. Transfer 50 µL from each well of the first anti-ligand dilution series (e.g. Row A of the MDP), to Row B of the MDP. Mix well by pipetting up and down several times.

    d. Prepare additional test samples in a similar manner.

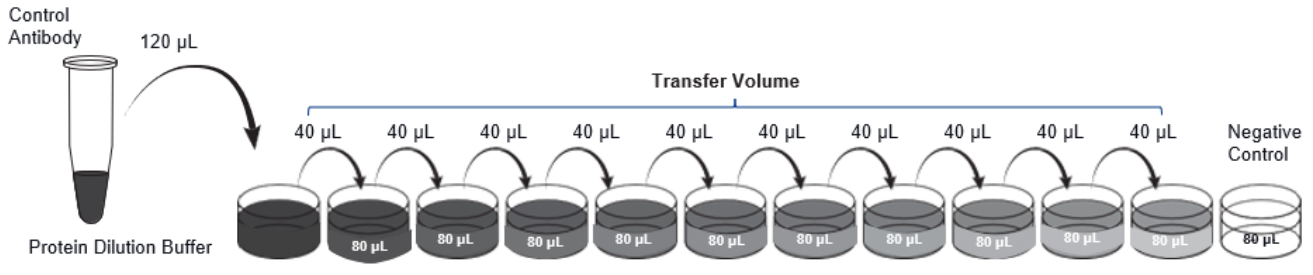
    e. Incubate the MDP for 30 minutes at room temperature inside the tissue culture hood.

    Note: The optimal pre-incubation time and temperature for your antibody should be determined empirically. Typical ligand neutralizing assay conditions are 15-30 minutes at room temperature.

3. After the antibody and ligand pre-incubation step, remove the assay plate from the 37°C and 5% CO_2 incubator and add 20 µL from each row of ligand neutralizing samples in the MDP (e.g. Row B and Row D) to the appropriate wells of the assay plate. See Representative Assay Plate Map for recommended sample layout.

4. Place the assay plate in the 37°C and 5% CO_2 incubator and incubate for 16 hours.

Figure 2. Ligand Neutralizing Dilution Series: Make three sequential series of dilution of antibody in a separate master dilution plate.



Dilution vial no.	1	2	3	4	5	6	7	8	9	10	11	12
Final Volume	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	120 µL	80 µL
Sequential dilution ratio		1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	

8.4 Addition of Detection Reagent

Day 2: Signal Detection

1. Thaw one aliquot of Bioassay Detection Reagent 1 from PathHunter Bioassay Detection kit, equilibrate to room temperature, and transfer 2.3 mL using a pipet into a sterile reservoir.

2. Remove assay plate from incubator and remove lid. Add 10 µL of the Bioassay Detection Reagent 1 from the reservoir into each row of the assay plate.

3. Replace lid on assay plate and gently mix the contents by slowly moving the plate back and forth 3-4 times on the surface of the tissue culture hood, in a criss-cross pattern.

4. Incubate the assay plate for 15 minutes (+/- 5 minutes) at room temperature (22°C - 25°C) in the dark (e.g. in a drawer or covered location on the bench top).


Detection reagents are light sensitive, thus incubation in the dark is necessary.

5. Thaw one aliquot of Bioassay Detection Reagent 2 from PathHunter Bioassay Detection kit, equilibrate to room temperature, and transfer 9.2 mL into a fresh sterile reservoir.

6. Remove assay plate from incubator and remove lid. Pipet 40 µL of the Bioassay Detection Reagent 2 from the reservoir into each row of the assay plate.

7. Replace lid on assay plate and gently mix the contents by slowly moving the plate back and forth 3-4 times on the surface of the tissue culture hood, in a criss-cross pattern.

8. Incubate the assay plate for 1 hour (+/- 15 minutes) at room temperature (22°C - 25°C) in the dark (e.g. in a drawer or covered location on the bench top).

9. 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.

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

Representative Assay Plate Map

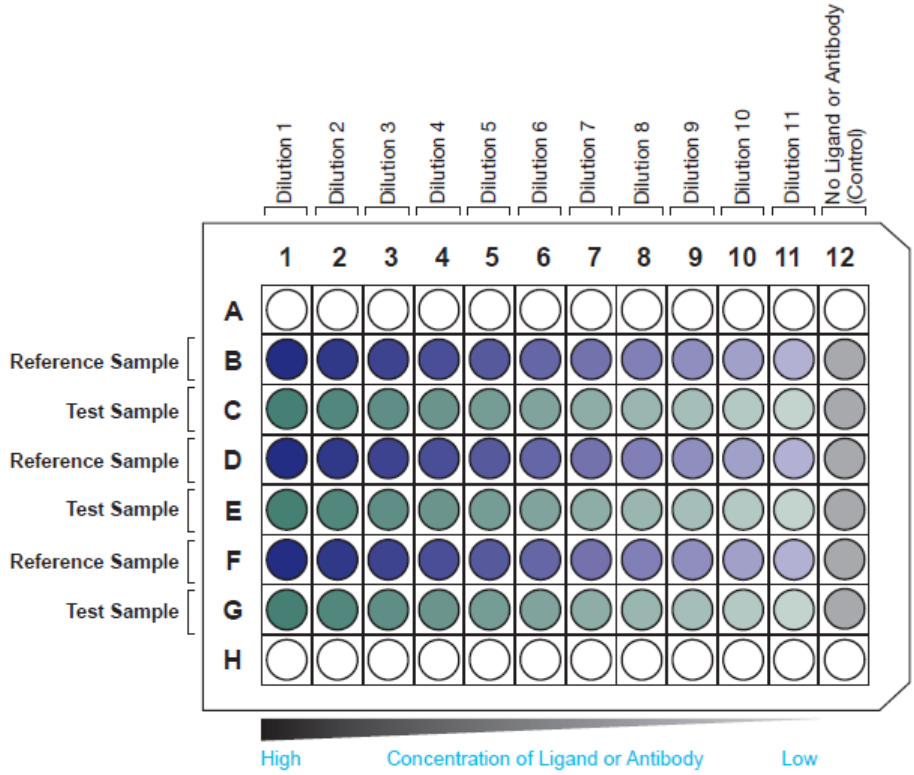


Figure 3. Representative Assay Plate Map

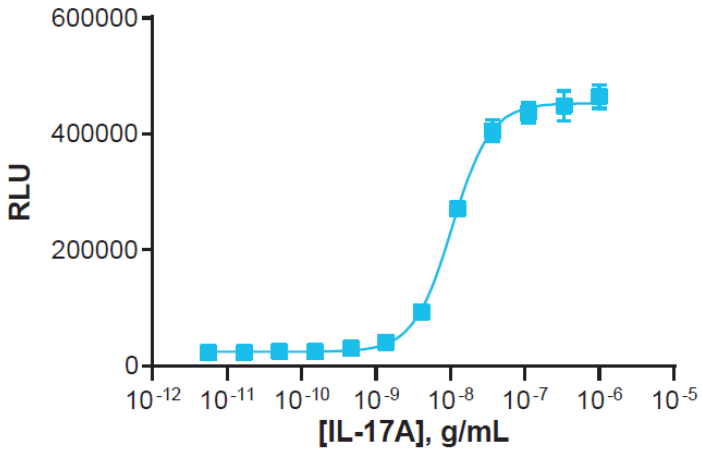
This plate map shows a 11-point dilution series with 3 data points at each concentration for one reference and one test sample per plate, with a 1:3 serial dilution scheme.

9. Typical Results

The following graph is an example of a typical dose-response curve for the PathHunter IL-17A/IL-17F Bioassay Kit (Dimerization) generated using the protocol outlined in this user manual. The data show potent, dose-dependent dimerization of the IL-17 receptor (comprised of the IL-17RA and IL-17RC) subunits, when treated with IL-17A (Eurofins DiscoverX; Part #: 92-1229).

The plate was read on an EnVision® Multimode Plate Reader (with a 0.2 sec / well integration time) and data analysis was conducted using GraphPad Prism.

A



B

S/B	EC_{50} (ng/mL)
20.8	10.5

Figure 4. Typical Results:

Representative **A**, dose-response curve and **B**, EC_{50} and assay window for dose-dependent stimulation of dimerization of the subunits of the IL-17 receptor (IL-17RA and IL-17RC) in U2OS cells when treated with a dose response of IL-17A.

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 control used or incorrect control incubation time	Refer to the datasheet for recommended control 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	Hydrophobic compounds should be tested for solubility and may require evaluation in different dilution buffers.
	Problems with compound stability	Non-binding surface plates may be necessary for hydrophobic compounds.

For questions on using this product, please contact Technical Support at discoverx.com/support/.

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Contact Information

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Technical Support: discoverx.com/support/

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