User Manual

PathHunter® IL-15 Bioassay Kit (Dimerization) User Manual

For Quantitation of IL-15 Mediated Dimerization of the IL-15 receptor (IL2Rβ/IL2Rγ)

For Bioassay Kits with ligand

93-0998Y3-00183: 2-Plate Kit

93-0998Y3-00184: 10-Plate Kit

For Bioassay Kit without ligand

93-0998Y3-00188: 10- Plate Kit

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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-15 Bioassay Kit (Dimerization) is a simple, robust, non-radioactive, dye free assay for quantitation of IL-15 -Mediated Dimerization of the IL-15 receptor (IL2Rβ/IL2Rγ). The PathHunter IL-15 bioassay kit with ligand 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-15 Bioassay Kit is also offered without ligand 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-15 Bioassay evaluates activity in the IL2Rβ/IL2Rɣ Dimerization assay, an application of the Eurofins DiscoverX Dimerization Assay platform. The assay is designed to detect the ligand-induced dimerization of the IL2Rβ and IL2Rɣ receptors, which comprise the receptor for IL-15. As shown in Figure 1, the bioassay cells have been engineered to co-express IL2Rβ fused to PK, and IL2Rɣ fused to EA. Binding of IL-15 to IL2Rβ causes dimerization of the two receptor chains, which brings the two β-gal fragments (PK and EA) into close proximity, forcing complementation. The result is formation of a functional β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal.

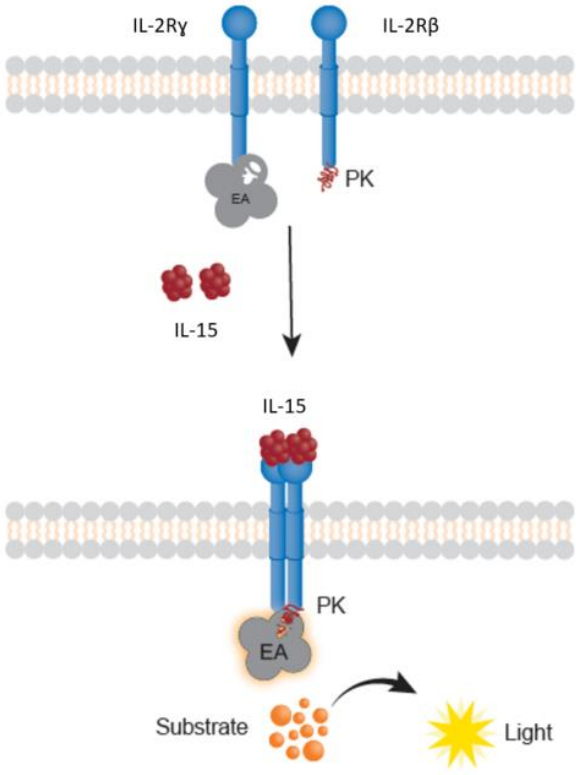


Figure 1 PathHunter IL-15 Bioassay (Dimerization) Assay Principle.

3. Materials Provided in PathHunter IL-15 Bioassay Kit (Dimerization)

Table 1: Materials Required

List of Components	93-0998Y3-00183 (2-Plate Kit)	93-0998Y3-00184 (10 Plate Kit)	93-0998Y3-00188 (10 Plate Kit without ligand)
PathHunter U2OS IL2RB/IL2RG Dimerization Bioassay Cells (0.6×10^6 cells in 0.1 mL per vial)	2 Vials	10 Vials	10 Vials
AssayComplete™ Cell Plating 5 Reagent (Bottle)	1 x 100 mL	2 x 100 mL	2 x 100 mL
Assay Complete Protein Dilution Buffer	1 x 50 mL	2 x 50 mL	2 x 50 mL
Recombinant Human IL-15 (10 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: F**or 93-0998Y3-00188 ligand not provided in the kit, would need to be obtained separately if needed

4. Storage Conditions

PathHunter U2OS IL2RB/IL2RG Dimerization 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 Detection Kit

Upon receipt, store reagents at -20°C or at -80°C for long-term storage, up to the indicated shelf life of that component. Thaw reagents at room temperature before use. Thawed reagents are stable for up to 4 days when stored at 2-8°C. For long-term storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be re-frozen in opaque containers 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 96-well 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)

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.

Protein Dilution Buffer (PDB)

Upon receipt, store at -20°C. Once thawed, the Protein Dilution Buffer can be stored at 2-8°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 can be dispensed and frozen down. This may vary depending on your stock sample concentrations and should be adjusted accordingly.

Recombinant Human IL-15 (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 100 µL of Protein Dilution Buffer to the 10 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 ligand is stable for 12 months at -20°C to -80°C, or 1 week at 2-8°C.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

5. Additional Materials and Equipment Recommended for Assay

The following equipment and additional materials are recommended to perform the assays. All materials should be stored at Room Temperature or under conditions specified by the manufacturer.

Table 2: Additional Materials and Equipments Recommended

Material	Ordering Information
Recombinant Human IL-15	DiscoverX (92-1265), or similar
A 96-well green, V-bottom, untreated, non-sterile dilution plate (Master Dilution Plate; for preparing serial dilutions of samples)	DiscoverX (92-0011), or similar
Disposable polystyrene reagent reservoirs (25mL), sterile	Thermo Fisher Scientific (Cat.#. 8094), or similar
15 mL LightSafe polypropylene tubes, sterile	Millipore Sigma (Cat # Z688320), or similar
50 mL and 15 mL Polypropylene tubes, sterile	
1.5 mL polypropylene microcentrifuge tubes, sterile	
Tissue culture disposable pipettes (1 mL - 25 mL), sterile	
Disposable pipet tips for P20, P100, P1000 pipetmans	
Sterile biosafety cabinet	
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	
Hemocytometer	
Luminescence Reader	discoverx.com/instrument-compatibility
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 IL2RB/IL2RG Dimerization 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 following section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

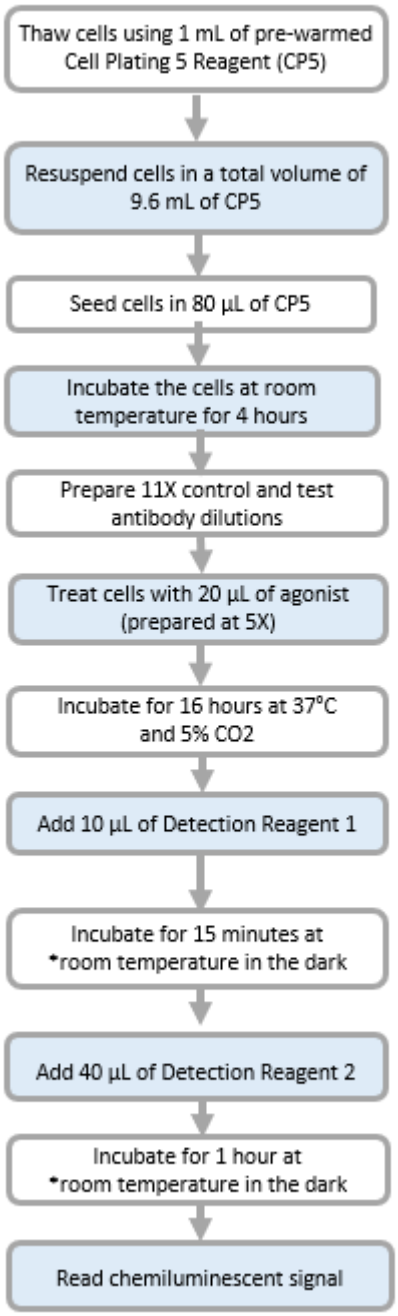
7. Protocol Schematic

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: _____

Quick-start Procedure: In a 96-well plate, perform the following steps.



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

8. Detailed Assay Protocol

This user manual provides a protocol for quantifying IL-15 mediated dimerization of the receptor for IL-15 (IL2Rβ/IL2Rγ). 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 ligand, IL-15 ligand can be sourced per the details in the table on Additional materials needed for the assay.

8.1 Bioassay Cell Preparation

Day 1

The following protocol is for thawing and plating the cryopreserved PathHunter U2OS IL2Rβ/IL2Rɣ 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:
    - One sterile 25 mL reagent reservoir
    - One sterile 10 mL conical tube
    - A micro pipet (P1000) set to dispense 1 mL
    - A multichannel pipette and tips set to dispense 80 µL
    - An aliquot of AssayComplete™ Cell Plating 5 Reagent (CP5), **pre-warmed in a 37˚C water bath for 15 minutes**
    - A 96-Well White, Clear, Flat-bottom Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
    - 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 sterile 10 mL conical tube.
3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.
4. Remove the cryovial from dry ice and ensure cap is tight. Holding vial by the cap, immediately thaw vial in 37°C water bath for 30 seconds (+/- 5 seconds), gently agitating the vial to thaw cells every 10 seconds.

   DO NOT LEAVE VIAL IN WATER BATH.
5. Visually inspect bottom of vial after 20 seconds. If pellet is thawed, remove vial from water bath, wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood. If ice is still visible, return vial to water bath for additional 10-15 seconds.
6. Pre-warm CP5 in 37°C water bath for 20 minutes.
7. Add 1 mL of pre-warmed CP5 from the 10 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 3 times to uniformly suspend the cells.
8. 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.
9. 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.

10. Using a manual 12-channel multichannel pipet, transfer 80 µL 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.
11. 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.
12. Incubate assay plate in humidified tissue culture incubator at 37°C, 5% CO_2 for 4 hours.

8.2 Sample Preparation

Day 1

The following protocol is an example for preparing a serial dilution of IL-15 reference control/ control ligand. IL-15 is reconstituted to a 100 µg/mL stock using the DiscoverX recommended IL-15. If IL-15 is obtained from a different source, the optimal dose range would need to be determined empirically.

1. Reconstitute IL-15 to 100 µg/mL stock solution by adding 100 µL of supplied reconstitution buffer.
2. On day of assay, prepare working stock (5X) of IL-15 in Protein Dilution Buffer (PDB) as detailed in Table 3 below. This will serve as top concentration in serial dilution curve.

Table 3. Example of preparation of IL-15 Working stock

Working Stock Solution	[IL-15], µg/mL (DiscoverX Part # 92-1265)	Volume 100 µg/mL IL-15, µL	Volume Protein Dilution Buffer, µL
IL-15, Dilution 1	5	7.5	142.5

Note: Working stocks of test samples should be prepared similarly.

3. On the day of assay, prepare serial dilutions of IL-15 reference control/ control ligand in row A of a fresh 96-well master dilution plate (MDP), at 5X the final concentrations of each dose, in Protein Dilution Buffer (PDB; i.e. the Ligand Diluent) as per Table 4 below. Sufficient volumes to run triplicate wells per dose, in the assay plate, are provided in the table.
4. Add volume of sample diluent (PDB) to Row A of the MDP (as indicated in column 6) of Table 4.
5. Transfer 150 µL of IL-15 Working stock (Dilution 1) prepared in Table 3 to well A2 of a fresh master dilution plate (MDP).
6. Prepare the dilution series by transferring the volume of IL-15 sample (indicated in column 5) from the source well (indicated in column 4) to the destination well (indicated in column 1). Pipet up and down several times to mix in destination wells. Replace pipet tips between each serial dilution. No sample is added to well 1 (vehicle only), as this serves as the negative control.

Table 4. Example of Preparation of IL-15 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 Row A, ng/mL**	**Dilution Ratio**	**Dilution (5X) Sample Source Well**	**Volume (5X) of sample added, µL**	**Volume Ligand Diluent, µL**	**Final Concentration (1X) of sample in Assay Plate, ng/mL**
Row A, Well 1	0	--	--	--	100	--
Row A, Well 2	5000	--	Dilution 1 from Table 1 (5 µg/mL)	150	-	1000
Row A, Well 3	1250	1:4	Row A, Well 2	50	150	250
Row A, Well 4	417	1:3	Row A, Well 3	50	100	83.3
Row A, Well 5	139	1:3	Row A, Well 4	50	100	27.8
Row A, Well 6	46.3	1:3	Row A, Well 5	50	100	9.3
Row A, Well 7	15.4	1:3	Row A, Well 6	50	100	3.1
Row A, Well 8	5.14	1:3	Row A, Well 7	50	100	1.03
Row A, Well 9	1.71	1:3	Row A, Well 8	50	100	0.34
Row A, Well 10	0.57	1:3	Row A, Well 9	50	100	0.11
Row A, Well 11	0.19	1:3	Row A, Well 10	50	100	0.04
Row A, Well 12	0.04	1:5	Row A, Well 11	20	80	0.013

7. In Row B of Master Dilution Plate, prepare test sample using same serial dilution scheme as in Table 4 above.
8. 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:
    1. Row A in MDP (Reference): transfer to Rows B, D and F in the assay plate
    2. Row B in MDP (Test Sample): transfer to Rows C, E and G in the assay plate
9. Incubate assay plate in a humidified tissue culture incubator at 37˚C and 5% CO_2 for 16-18 hours after sample addition.

Representative Plate Map

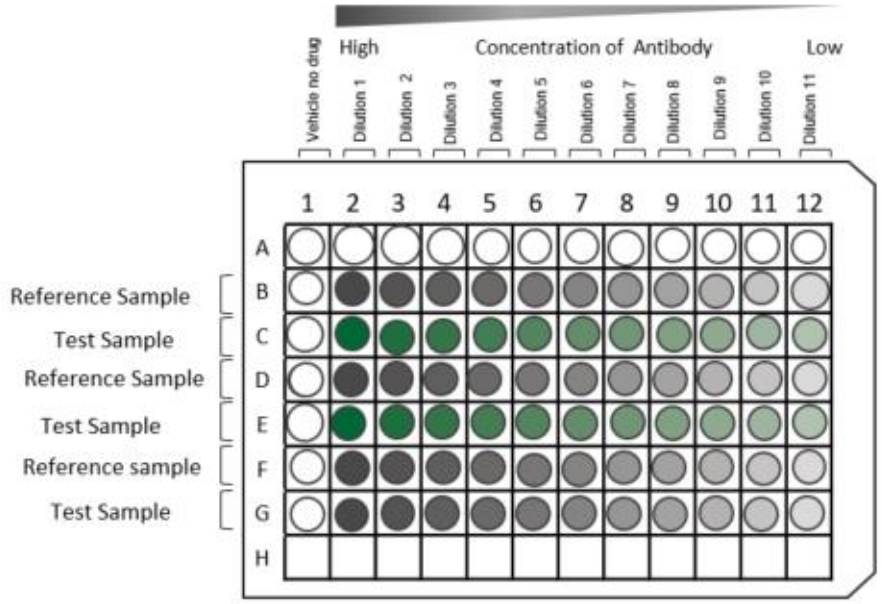


Figure 2 Representative Assay Plate Map

Assay Plate Map: This plate map shows 2 interdigitated 11-point dose curves, with three replicates per dose point, for one reference sample and one test sample tested using the same dilution scheme. Column 2 contains the highest dose of each sample, while column 12 contains the lowest dose. Column 1 contains no drug (vehicle only).

8.3 Addition of Detection Reagent

Day 2: Signal Detection

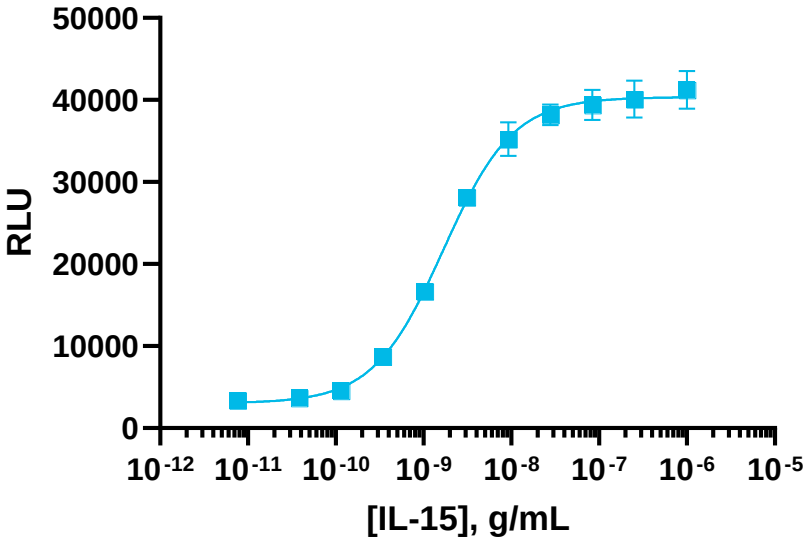
1. Thaw one aliquot of Bioassay Detection Reagent 1 from PathHunter Bioassay Detection kit, equilibrate to room temperature, and transfer 2.3 mL using a pipet into a sterile reservoir.
2. Remove assay plate from incubator and remove lid. Pipet 10 µL of the Bioassay Detection Reagent 1 from the reservoir 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).
5. Thaw one aliquot of Bioassay Detection Reagent 2 from PH 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. Place lid back on assay plate and incubate for at least 1 hour at room temperature in the dark. 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 imager.
    - A luminescence reader usually collects signal from all wavelengths. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.
10. Data analysis and plotting graph can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.)

9. Typical Results

The following graph displays representative data of a dose response of IL-15 (supplied by DiscoverX) in the PathHunter IL-15 Bioassay generated using the above protocol. Sample was read on a Perkin Elmer Envision plate reader, using a 0.2 sec/well integration time.

A



B

S/B	EC_{50}, ng/mL
13.3	1.67

Figure 3: Typical Results

Representative A, dose-response curve and B, the EC50, HillSlope and assay window for PathHunter IL-15 Bioassay Kit (Dimerization), as measured in this bioassay.

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