# User Manual

## PathHunter® IGF-1 Bioassay Kit

### For Chemiluminescent Detection of Receptor Activity

For Bioassay Kits with control:

93-0505Y1-00069: 2-Plate Kit

93-0505Y1-00070: 10-Plate Kit

For Bioassay Kit without control

93-0505Y1-00195: 10-Plate Kit

Document Number 70-379 Rev 2

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



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# 1. Overview

The PathHunter IGF-1 Bioassay Kit is a robust, highly sensitive, and easy-to-use, cell-based functional assay to quantify potency of drugs targeting IGF-1, IGF-2, or the IGF-1 receptor, or to detect neutralizing antibodies. The PathHunter IGF-1 Bioassay Kit with control contains all the materials needed to perform a complete assay, including cryopreserved, single-use cells, detection reagents, cell plating reagent, control agonist for stimulating the cells, and assay plates. A 10-Plate PathHunter IGF-1 Bioassay Kit 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 IGF-1 Bioassay cells, as shown in Figure 1, overexpress the ProLink-tagged IGF-1 receptor and an EA-tagged SH2 domain protein. Upon activation of the receptor by its control or an agonist antibody or ligand, recruitment of SH2-EA protein to the phosphorylated receptor tail forces complementation of PK and EA to form an active β-gal enzyme. β-gal enzymatic activity is quantitatively measured using a chemiluminescent substrate that leads to production of EFC signal.

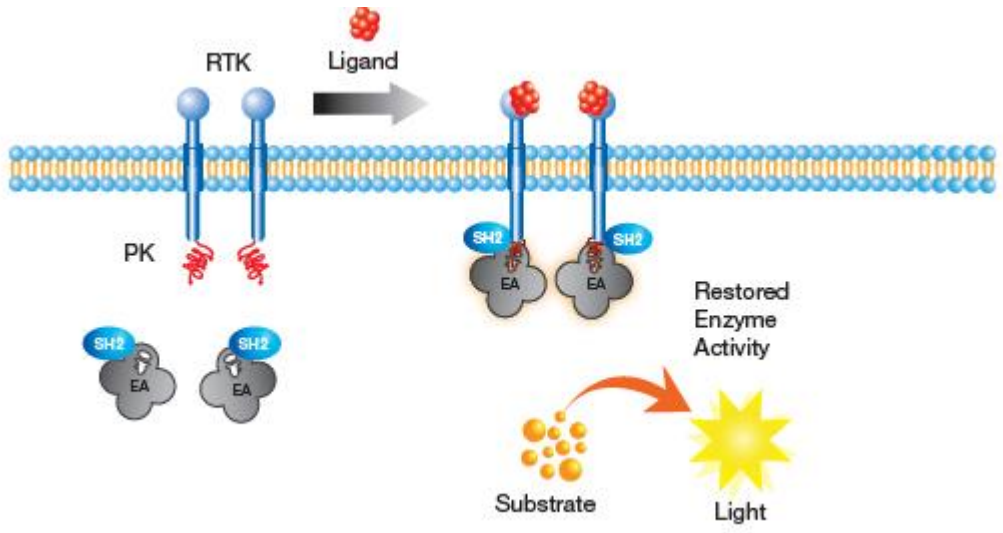



**Figure 1. PathHunter IGF-1 Bioassay Principle.**

# 3. Materials Provided in PathHunter IGF-1 Bioassay Kit

| List of Components | 93-0505Y1-00069 (2-Plate Kit) | 93-0505Y1-00070 (10-Plate Kit) | 93-0505Y1-00195 (10-Plate Kit without control) |
|---|---|---|---|
| PathHunter HEK 293 IGF1R Bioassay Cells ($1.2 \times 10^6$ cells in 0.1 mL per vial) | 2 | 10 | 10 |
| AssayComplete™ Cell Plating 17 Reagent (100 mL per bottle) | 1 x 100 mL | 3 x 100 mL | 3 x 100 mL |
| AssayComplete Protein Dilution Buffer (Bottle) | 1 x 50 mL | 2 x 50 mL | 2 x 50 mL |
| *Control Agonist Recombinant Human (IGF-1) (100 µg per vial) | 1 | 1 | N/A* |
| PathHunter Bioassay Detection Kit<br>Detection Reagent 1 (Bottle)<br>Detection Reagent 2 (Bottle) | <br>1 x 3 mL<br>1 x 12 mL | <br>1 x 15 mL<br>1 x 60 mL | <br>1 x 15 mL<br>1 x 60 mL |
| 96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid | 2 Plates | 10 Plates | 10 Plates |

***Note-**For 93-0505Y1-00195 control not provided in the kit, would need to be obtained separately if needed

# 4. Storage Conditions

## PathHunter HEK 293 IGF-1 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 17 Reagent (CP17)

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 IGF-1R, 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 100 µL of supplied reconstitution 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 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 IGF-1 | Eurofins DiscoverX (Part # 92-1030), 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 | Thermo 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 HEK 293 IGF-1 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.

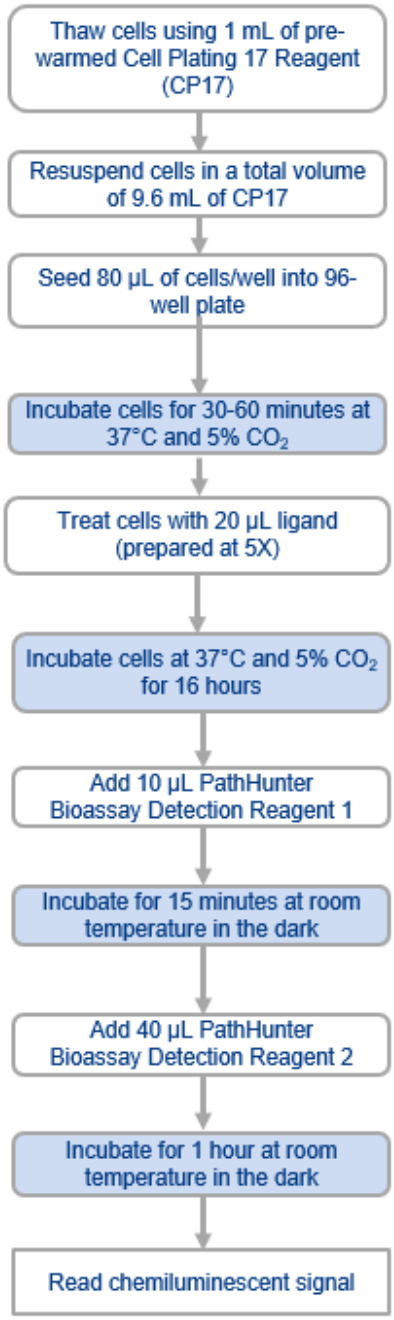
# 7. Protocol Schematic

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 Eurofins DiscoverX provided control recombinant human IGF-1 (Part # 92-1030) induced activation of the receptor, subsequently leading to recruitment of SH2 domain protein to the phosphorylated receptor tail further leading to production of chemiluminescent signal. 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, control 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 HEK 293 IGF-1 Bioassay Cells from cryovials.

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 80 µL.

   e. A bottle of Cell Plating Reagent 17 (CP17, pre-warmed in a 37˚C water bath for 15 minutes).

   f. A white-walled, clear-bottom 96-well assay plate.

2. Dispense 9.6 mL of CP17 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. Add 1 mL of pre-warmed CP17 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 3 times to uniformly suspend the cells.

5. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP17. 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 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.

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. Proceed immediately to sample preparation. Note: if sample preparation will take longer than 30 minutes, return assay plate to humidified tissue culture incubator at 37°C, 5% $CO_2$ during preparation.

## 8.2 Sample Preparation (Day 1)

The following protocol is an example for preparing intermediate dilutions, working stocks and serial dilutions of reference, test sample and control agonist (optional).

On day of assay, prepare intermediate and working stocks of the control agonist (IGF-1) in Protein Dilution Buffer (PDB) as detailed in Table 1 below. The working stock will serve as the top concentration in the IGF-1 serial dilution curve, prepared as 5X stocks, as shown in Table 2.

a. Reconstitution of recombinant human IGF-1 (Eurofins DiscoverX Part # 92-1030): Quickly spin the vial prior to reconstituting to ensure control is at bottom of tube. Add 1 mL of Protein Dilution Buffer (PDB) to 0.1 mg of lyophilized powder in the vial to prepare a stock concentration of 0.1 mg/mL.

Table 1. Example of Preparation of IGF-1 Intermediate and Working Stocks

| Working Stock Solution | [IGF-1], µg/mL | Volume IGF-1, µL | Volume PDB, µL |
|---|---|---|---|
| IGF-1, Intermediate Dilution 1 | 10 | 10 (of 0.1 mg/mL stock) | 90 |
| IGF-1, Working Stock | 2.5 | 57.5 (of Dilution 1) | 172.5 |

10. On the day of assay, prepare serial dilutions of control agonist IGF-1 in Row A of the MDP at 5X the final concentration for each dose, 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 200 µL of IGF-1 working stock (prepared in Table 1) to Well 1 of Row A of the MDP.

c. Prepare serial dilutions by transferring the volume of IGF-1 (indicated in column 5 of Table 2) from the source well (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 Serial Dilutions for IGF-1 Control Agonist

| Master Dilution Plate (MDP) Location | [Sample] in MDP (5X), ng/mL | Dilution Factor | 5X Sample Source Location | Volume 5X Sample, µL | Volume Control Diluent, µL | Final [Sample] in Assay (1X), ng/mL |
|---|---|---|---|---|---|---|
| Row A, Well 1 | 2,500 | -- | Working Stock from Table 1 (2.5 µg/mL) | 200 | - | 500 |
| Row A, Well 2 | 833.3 | 3 | Row A, Well 1 | 100 | 200 | 166.7 |
| Row A, Well 3 | 277.8 | 3 | Row A, Well 2 | 100 | 200 | 55.6 |
| Row A, Well 4 | 92.6 | 3 | Row A, Well 3 | 100 | 200 | 18.5 |
| Row A, Well 5 | 30.9 | 3 | Row A, Well 4 | 100 | 200 | 6.2 |
| Row A, Well 6 | 10.3 | 3 | Row A, Well 5 | 100 | 200 | 2.1 |
| Row A, Well 7 | 3.4 | 3 | Row A, Well 6 | 100 | 200 | 0.69 |
| Row A, Well 8 | 1.14 | 3 | Row A, Well 7 | 100 | 200 | 0.23 |
| Row A, Well 9 | 0.38 | 3 | Row A, Well 8 | 100 | 200 | 0.08 |
| Row A, Well 10 | 0.13 | 3 | Row A, Well 9 | 100 | 200 | 0.025 |
| Row A, Well 11 | 0.04 | 3 | Row A, Well 10 | 100 | 200 | 0.008 |
| Row A, Well 12 | 0 | -- | -- | -- | 100 | 0 |

11. Prepare reference and test samples in Rows B and C of Master Dilution Plate (MDP), using appropriate

serial dilution scheme for the samples being tested.

a. The appropriate serial dilution scheme should be established empirically for reference standard and then applied to test samples. Volumes shown in Table 2 as examples may be scaled up/down as appropriate.

b. When preparing serial dilutions, pipet up and down three times to mix in destination wells on the MDP. Replace pipet tips between each serial dilution. No sample is added to well 12, as this serves as the negative control in each DRC.

12. Add 20 µL from each well of the IGF-1 agonist dose curve on the MDP to the appropriate wells of the assay plate (e.g. Rows A and H) as shown in the Representative Assay Plate Map

13. Add 20 µL from the reference curve from Row B on the MDP to the appropriate wells of the assay plate (e.g. Rows B, D and F) as shown in the Representative Assay Plate Map.

14. Add 20 µL from the test sample curve from Row C on the MDP to the appropriate wells of the assay plate (e.g. Rows C, E and G) as shown in the Representative Assay Plate Map.

15. Incubate the assay plate at 37°C and 5% $CO_2$, in a humidified incubator, for 16-18 hours.

## 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. Using a multichannel pipette, 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.

   a) Optional: place the plate onto an orbital shaker at 350 rpm for 1 minute to promote even mixing.

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 PH Bioassay Detection kit, equilibrate to room temperature, and transfer 9.2 mL into a fresh sterile reservoir.

6. Using a multichannel pipette, add 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 plate at room temperature for 1 hour in the dark.

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.

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

## Representative Assay Plate Map

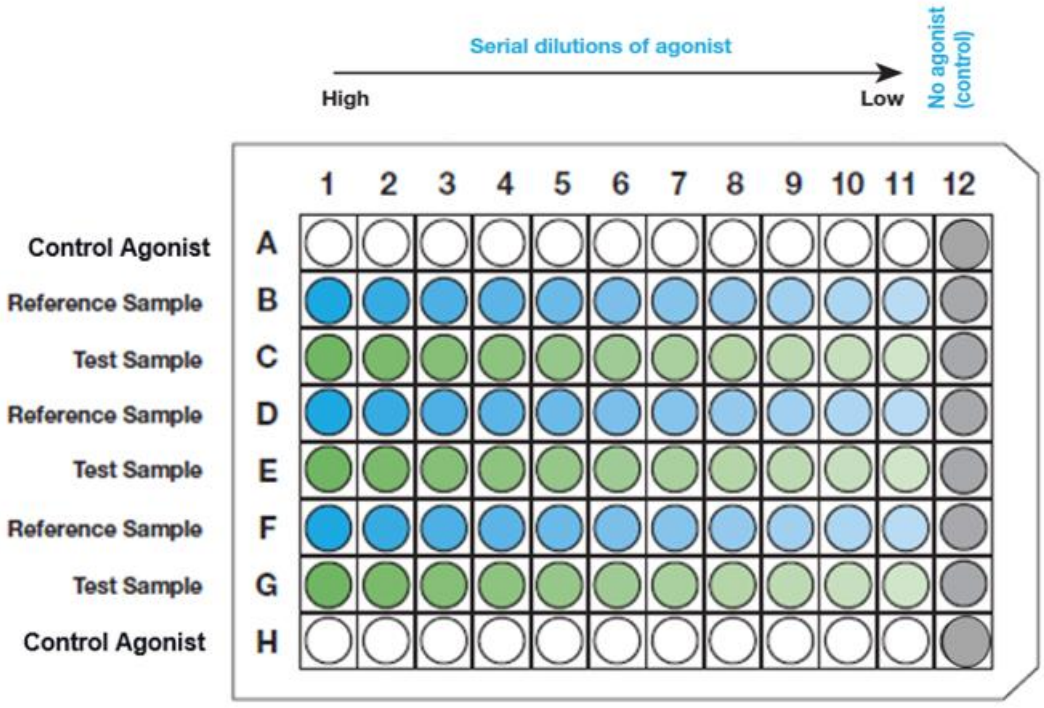



**Figure 3. Representative Assay Plate Map**

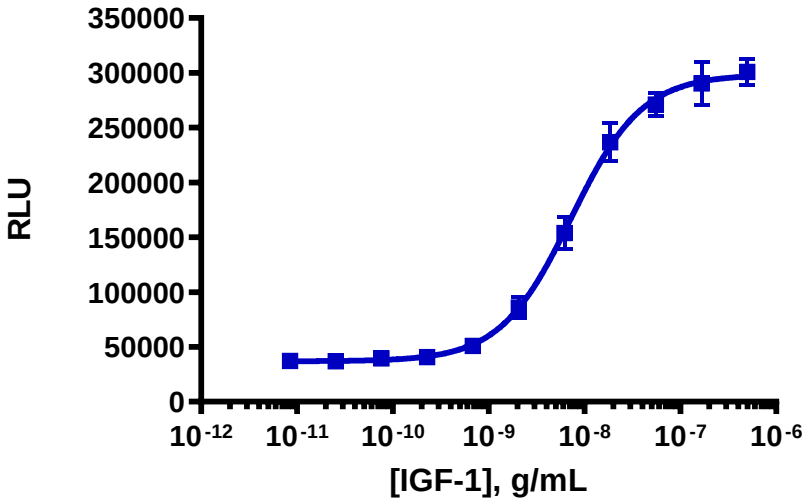
This plate map shows an 11-point dose response curves with 3 data points at each concentration for one reference and one test sample per plate. Note: the optional control agonist is run in rows A and H and is prepared using a 1:3 dilution series.

# 9. Typical Results

The following graph is an example of a typical dose-response curve for the PathHunter IGF-1 Bioassay Kit generated using the protocol outlined in this user manual. The data shows potent, dose-dependent control induced activation of the receptor, subsequently leading to recruitment of SH2 domain protein to the phosphorylated receptor tail, leading to production of chemiluminescent signal.

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

**A**




**B**

| S/B | $EC_{50}$ (ng/mL) |
|---|---|
| 8.0 | 7.3 |

**Figure 4. Typical Results:**

Representative **A**, dose-response curve with IGF-1 and **B**, $EC_{50}$ and assay window for dose-dependent stimulation that enables assessment of control-based activation of IGF-1 receptor via detection of SH2 domain protein recruitment.

## 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 control (agonist or antagonist) | Some controls are difficult to handle. Confirm the final concentration of controls. |
| | Sub-optimal agonist challenge concentration | Perform the agonist curve to reassess $EC_{80}$ with the control 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 control handling or storage | Check the control handling requirements. |
| | Difference in agonist binding affinity | Refer to the Certificate of Analysis for the control provided in the kit to confirm that the control 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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