DiscoverX

User Manual

PathHunter®

Ranibizumab Bioassay Kit

For Chemiluminescent Detection of Ranibizumab Activity

Table of Contents

Overview **1**

Intended Use 1

Technology Principle: PathHunter® Ranibizumab Bioassay 1

Materials Provided **2**

Storage Conditions **2**

Additional Materials Required **3**

Protocol Schematic **4**

Quickstart Guide 4

Detailed Protocols

Day 1: PathHunter Bioassay Cell Preparation 5

Day 1: Ligand Preparation 6

Day 2: Detection 7

Supplemental Information **8-9**

Representative Plate Maps for Agonist/Inhibitor Curves 8

Instrument Compatibility 9

Troubleshooting Guide **10**

Limited Use License Agreement **10**

Please read entire booklet before proceeding with the assay.
For additional information or Technical Support see contact information below.

w discoverx.com usa 1.866.448.4864 e SupportUS@discoverx.com eu +44.121.260.6142 e SupportEurope@discoverx.com

Overview

Intended Use

PathHunter Ranibizumab Bioassay kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study ranibizumab potency and 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: PathHunter Ranibizumab Bioassay

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 Ranibizumab Bioassay is an application of the DiscoverX Dimerization Assay platform, which can be used to detect ligand-induced dimerization of two subunits of a receptor-dimer pair. This assay detects VEGF-A-induced homodimeriziaton of the kinase insert domain (KDR), also known as VEGFR2 receptor. The cells have been engineered to co-express KDR fused to PK, and another KDR construct fused to EA. Activation of the KDR receptor through VEGF-A leads to receptor dimerization, which is an essential event in the receptor's signaling cascade. Receptor dimerization forces the two enzyme fragments to complement, resulting in the formation of a functional β-gal enzyme. The enzyme hydrolyzes a substrate to generate a chemiluminescent signal. Ranibizumab binds to and inactivates the VEGF-A protein, preventing VEGF from activating the VEGFR2 receptors. This leads to an inhibition of the signaling event and therefore a reduction in signal.

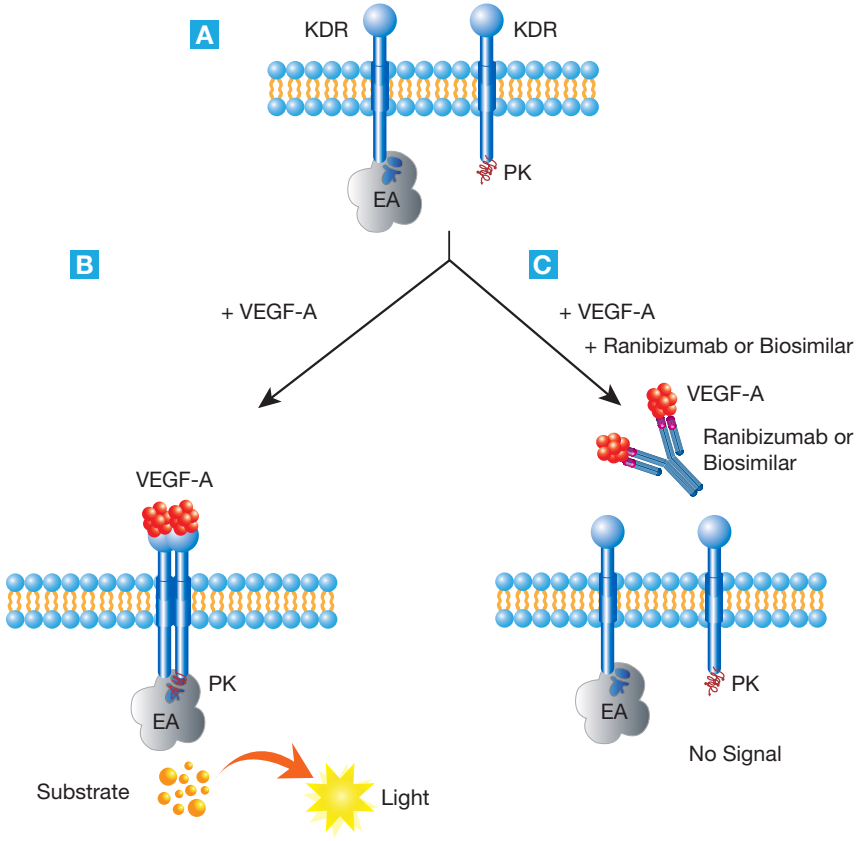


Figure 1. PathHunter Ranibizumab Bioassay Principle

Materials Provided

List of Components	93-0996Y1-00003	93-0996Y1-00004
PathHunter HEK 293 KDR/KDR Bioassay Cells	2 vials	10 vials
PathHunter Bioassay Detection Kit	200 dp	1,000 dp
Detection Reagent 1	2 mL	10 mL
Detection Reagent 2	8 mL	40 mL
AssayComplete Cell Plating Reagent 0	1 X 100 mL	3 X 100 mL
Protein Dilution Buffer	1 X 50 mL	2 X 50 mL
Control Agonist ($VEGF_{165}$)	1 vial	1 vial
96-Well Opaque-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

PathHunter HEK 293 KDR/KDR Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vials as soon as possible upon receipt.

If continued storage of the frozen vials is necessary, store as follows:

- Short term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for more than 24 hours).
- Long term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen (LN_2).

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the LN_2. Upon thawing, if LN_2 is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the LN_2 tank.

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 longer storage (up to the expiration date listed on the kit 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, 1mL of Detection Reagent 1 per aliquot can be dispensed and frozen down. 4mL of Detection Reagent 2 per aliquot can be dispensed and frozen down separately. Do not mix the two reagents during aliquoting.

AssayComplete Cell Plating Reagent 0

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 on the kit 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, 30mL of reagent per aliquot can be dispensed and frozen down.

Protein Dilution Buffer

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 on the kit 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 your stock sample concentrations and should be adjusted accordingly.

Recombinant Human $VEGF_{165}$ Control Agonist

Store at -20°C until ready to use (up to the expiration date listed on the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 100 µL of Protein Dilution Buffer. Reconstituted ligand is stable for 12 months at -20 to -80°C, or 1 week at 2-8 °C.

96-well Tissue Culture Treated Plates

Store at room temperature.

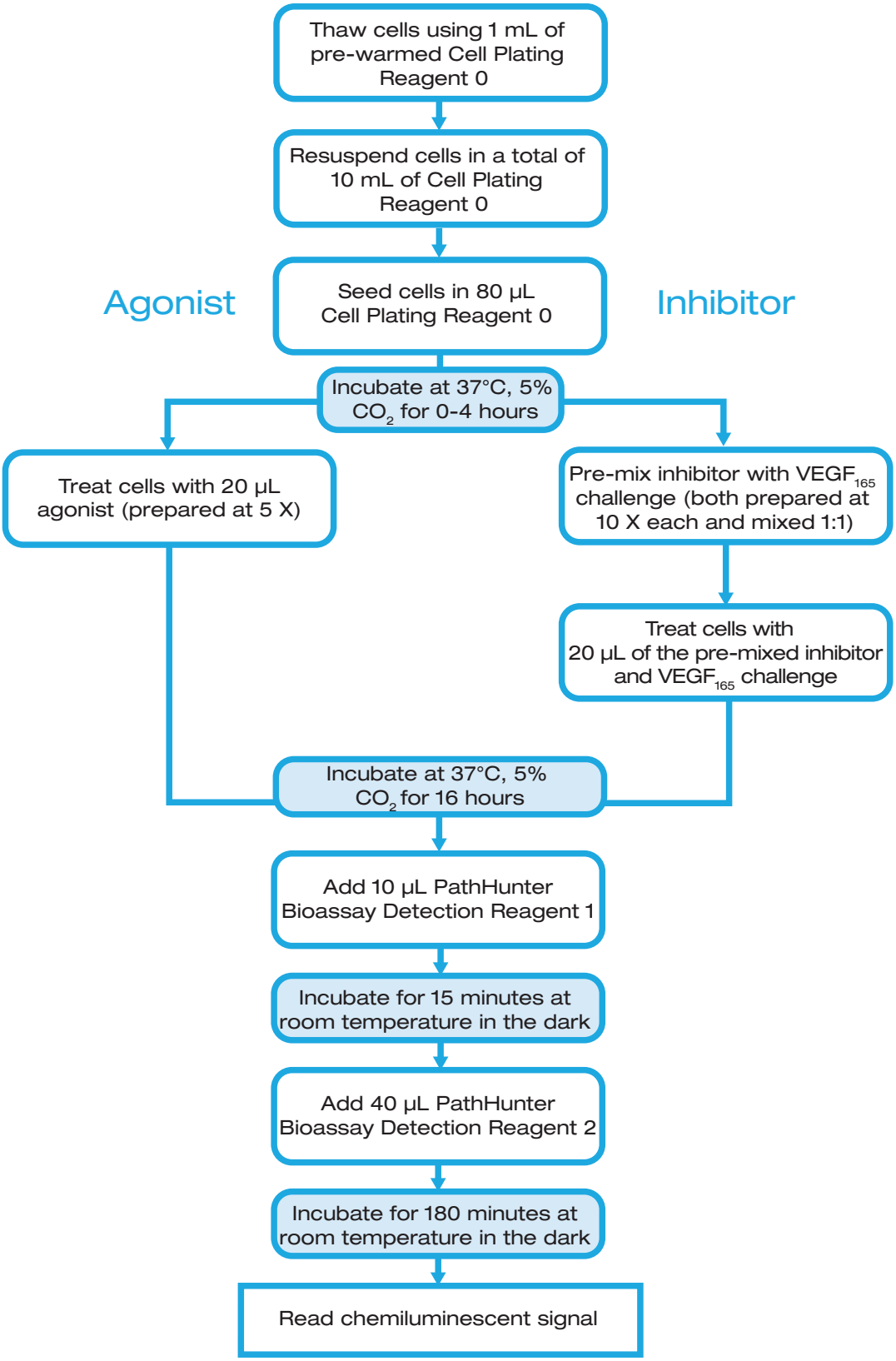
Additional Materials Required

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

Equipment
Single and Multichannel Micro-Pipettors and Pipette Tips
Multimode or Luminescence Plate Reader
V-Bottom 96-Well Compound Dilution Plates (DiscoverX, Catalog Number 92-0011 or Similar)
Disposable Reagent Reservoir (Thermo Scientific, Catalog Number 8094 or Similar)

Protocol Schematic

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



Detailed Protocols

Day 1: PathHunter Bioassay Cell Preparation:

The following protocol is for thawing and plating frozen PathHunter HEK 293 KDR/KDR Bioassay cells from cryovials.

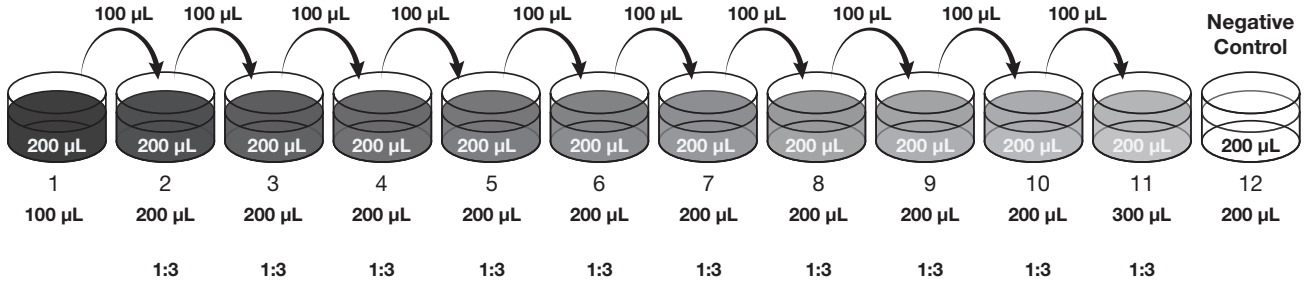
1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir.
    b. One 15 mL conical tube.
    c. A micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 80 µL.
    e. A bottle of Cell Plating Reagent 0 (CP0, pre-warmed in a 37˚C water bath for 15 minutes).
    f. A white-walled, opaque-bottom 96-well assay plate.
2. Dispense 10 mL of CP0 into the 15 mL conical tube.
3. Remove the cryovial from liquid nitrogen and immediately place in dry ice.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% EtOH, 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 (using P1000) of pre-warmed CP0 from the 15 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 9 mL of CP0. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Mix the tube by inversion to ensure the cells are properly mixed in the medium without creating any froth in the suspension and immediately pour the suspension into the 25 mL reservoir.
6. Add 80 µL of cells to each well of the 96-well assay plate using the multichannel pipette. Gently place the assay plate in a tissue culture incubator set to 37˚C, 5% CO_2. Ligand may be added to the cells immediately (0-4 hours after plating).

Day 1: Ligand 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 significantly impacting assay performance. Therefore, standard curves of control can typically be prepared in neat serum or plasma and added directly to cells without further dilution. For the best results, the optimized minimum required dilution (MRD) of crude samples should be empirically determined.



	1	2	3	4	5	6	7	8	9	10	11	12
Volume After Transfer:	100 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL	300 µL	200 µL
Sequential Dilution Ratio:		1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	

1. Prepare the reference agonist (DiscoverX $VEGF_{165}$) dose response curve, which will serve as a positive control in this assay. Agonist is prepared at 5 X the desired final concentration as it will be diluted by adding 20 µL to 80 µL of media present in the assay plate.

   a. Add 200 µL of Protein Dilution Buffer (PDB) to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX Catalog Number 92-0011 or similar).

   b. Add 100 µL of PDB to the $VEGF_{165}$ vial containing 10 µg of lyophilized powder to make a 100 µg/mL stock solution.

   c. Add 190 µL of PDB to well A1 of the master dilution plate. Add 10 µL of the 100 µg/mL $VEGF_{165}$ stock to this well. Mix thoroughly by pipetting up and down several times. This results in an 5 µg/mL solution (5 X the final 1 µg/mL curve top).

   d. Using a clean tip, transfer 100 µL from well A1 into well A2 and mix by pipetting up and down several times. Replace the pipette tip and transfer 100 µL from well A2 into well A3. Mix by pipetting up and down several times. Repeat this process until well A11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to column 12 as this will serve as a negative control.

2. Agonist challenge for biosimilar curves: The EC_{80} of the DiscoverX $VEGF_{165}$ was determined to be approximately 50 ng/mL. If $VEGF_{165}$ from a different vendor is used, the EC_{80} should be determined empirically prior to running samples.

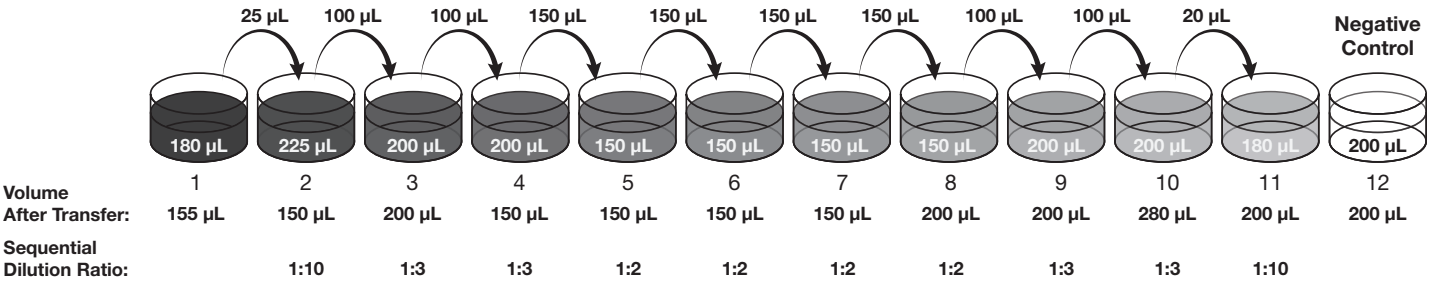
   Prepare the agonist challenge at 10 X the desired final concentration. For enough agonist challenge for a single biosimilar curve run in triplicate, dilute 7.5 µL of the 100 µg/mL stock with 1.5 mL of PDB in an Eppendorf tube.

3. Prepare ranibizumab reference curve. Ranibizumab is prepared at 10 X the desired final concentration. The suggested final concentration of the top dose for ranibizumab is 10 µg/mL.

   a. Add 225 µL of PDB to well B2, 200 µL to wells B3, B4, B9, B10 & B12, 150 µL to wells B5 to B8, and finally 180 µL to wells B11.

   b. Add 180 µL of ranibizumab prepared at 10 X the desired final concentration (10 µg/mL) to well B1 of this row on the master dilution plate. To make this, add 3 µL of ranibizumab from a stock of 6 mg/mL to 177 µL of PDB.

c. Using a clean tip, transfer 25 µL from well B1 into well B2 for a 1:10 dilution, and mix by pipetting up and down several times. Replace the pipette tip and transfer 100 µL from well B2 into well B3 for a 1:3 dilution. Mix by pipetting up and down several times. Repeat this process for well B4. Then transfer 150 µL from well B4 to well B5 and mix for a 1:2 dilution. Continue transferring 150 µL with a clean pipette and mix until well B8 is reached. Transfer 100 µL from well B8 to well B9 for a 1:3 dilution and repeat for well B10. Then transfer 20 µL from well B10 to B11 for a 1:10 dilution. No antibody is transferred to well B12 as this will serve as a negative control.



4. Transfer 100 µL from the ranibizumab curve prepared in step 3, to a new row on the master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX Catalog Number 92-0011 or similar).

5. Add 100 µL of $VEGF_{165}$ agonist challenge prepared above in step 2, to columns 1-11 of the ranibizumab row of the pre-mixing plate. 100 µL of PDB can be added to the negative control well of column 12 to maintain equal volumes in all wells.

6. Mix the contents of the pre-mixing plate wells thoroughly by pipetting up and down with a clean tip for each well, or with a multi-channel pipette.

7. Remove the assay plate from the 37°C, 5% CO_2 incubator and bring into the tissue culture hood.

8. Add 20 µL from the agonist reference curve prepared in step 1 on the master dilution plate, to the appropriate wells of the assay plate.

9. Add 20 µL from each well of the ranibizumab plus $VEGF_{165}$ pre-mixed curve from the master dilution plate to the appropriate wells of the assay plate.

10. Return the assay plate to the 37°C, 5% CO_2 incubator and incubate overnight (16-18 hours).

Day 2: Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate. Place the plate onto an orbital shaker at 350 rpm for 1 minute to cause even mixing.

2. Incubate the plate at room temperature for 15 minutes in the dark.

3. Using a multichannel pipette add 40 µL of Detection Reagent 2 to each well of the assay plate.

4. Incubate the plate at room temperature for 3 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.

PathHunter Bioassay Detection Reagents are light sensitive, thus incubation in the dark is necessary.

Note: For crude biologic samples, gently removing the liquid from all wells 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 is necessary for this method.

Representative Plate Maps for Agonist/Inhibitor Curve

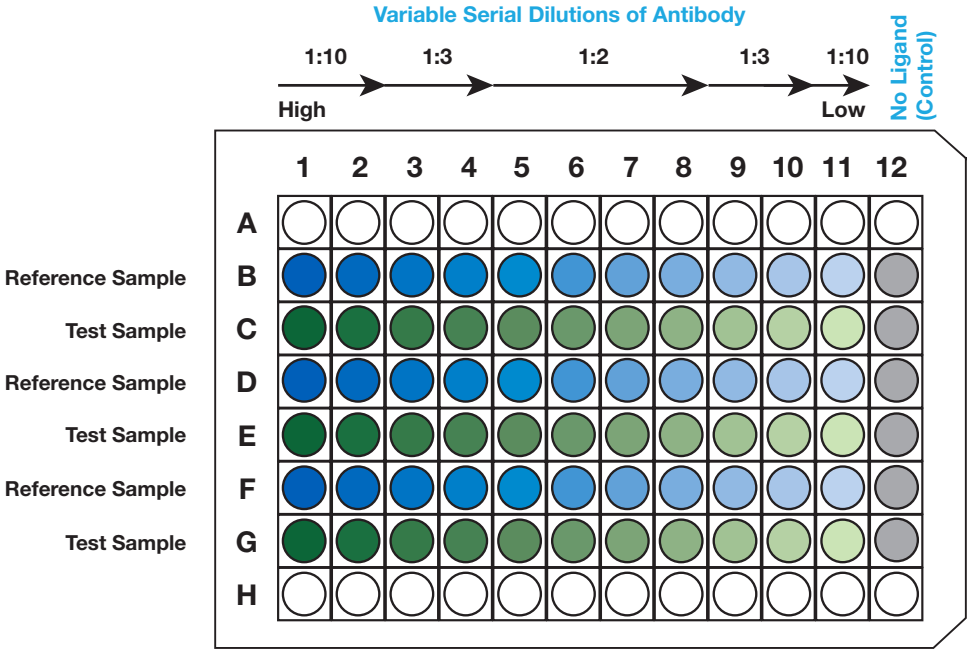


Figure 1. This plate map shows an 11-point dose curve with 3 data points at each concentration for one reference and one test sample per plate, with a variable serial dilution scheme.

Related Products	
PathHunter Bioassay Detection Reagents	www.discoverx.com/detectionreagents
Cell Culture Kits, Reagents & Consumables	www.discoverx.com/cell-culture-kits-reagents-consumables
Control Ligands	www.discoverx.com/controlligands

Instrument Compatibility Chart

Assay	Instrument	Read-Out
All PathHunter® Assays HitHunter® cAMP HitHunter® cGMP	**COMPATIBLE WITH ANY LUMINOMETER** **BMG:** PheraStar, Cytostar, LumiStar **Perkin Elmer:** TopCount, Victor 2 or V, Fusion, LumiCount, Envision, Micro-beta (Trilux), Viewlux, Northstar **GE:** LEAD seeker, Farcyte **Molecular Devices:** FLIPR, SpectraMax M3/M4/M5/M5e, FlexStation 3, SpectraMax L **Tecan:** Ultra, Evolution **Turner BioSystems:** Modulus Microplate **Caliper** LabChip 3000 & EZ Reader **Berthold Technologies:** Mithras LB940, CentroLIApc **Hamamatsu** FDS6000, FDSS/RayCatcher **Thermo Scientific:** Luminoskan Ascent **Biotek:** Synergy 2	Luminescence

*For other instruments not listed here, please use the information below to contact Technical Support.

Troubleshooting Guide

Problem	Cause	Solution
No Response	Improper thawing procedure	Refer to thawing instructions in this user manual as these cells are sensitive to proper thawing process
	Improper ligand used or improper ligand incubation time	See datasheet for recommended ligand and assay conditions
	Improper preparation of ligand (agonist or antagonist)	Refer to vendor specific datasheet to ensure proper handling, dilution and storage of ligand
	Improper time course for induction	Optimize time course of induction with agonist and antagonist
Decreased Response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope
Low or No Signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 sec/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)	Some ligands are difficult to handle. Confirm the final concentration of ligands
EC_{50} is Right-Shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the Product Insert
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may be diluted in Protein Dilution Buffer
		Non-binding surface plates may be necessary for hydrophobic compounds

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

Limited Use License Agreement

These products may be covered by issued US and/or foreign patents, patent application and subject to Limited Use Label License.

Please visit www.discoverx.com/license for limited use label license and trademark information for this product.