PathHunter® Epoetin Alfa Bioassay Kit

For chemiluminescent detection of epoetin alfa activity

Simple Solutions for Complex Biology

DiscoveRx

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Read the entire product insert before beginning the assay
For additional information or Technical Support, contact support@discoverx.com or visit www.discoverx.com.

Overview

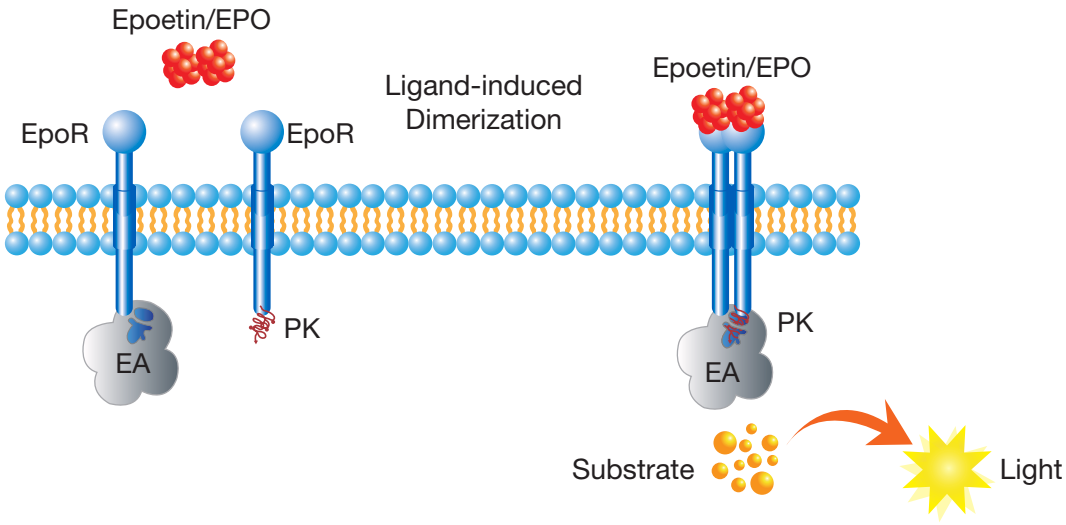
Intended Use

PathHunter® Epoetin Alfa Bioassay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study epoetin alfa 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® Epoetin Alfa 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 Epoetin Alfa 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 erythropoietin or epoetin alfa induced homodimeriziaton of the erythropoietin (EpoR) receptor. The cells have been engineered to co-express one EpoR fused to PK, and another EpoR construct fused to EA. Binding of agonist induces homodimerization, forcing complementation of the two enzyme fragments. This results in the formation of a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.



Materials Provided

List of Components	(93-0965Y3-00017)	(93-0965Y3-00018)
Description	**Contents**	**Contents**
PathHunter U2OS EpoR/EpoR 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 (Erythropoietin)	1 vial	1 vial
96-well Tissue Culture Treated Plates	2 plates	10 plates

Storage Conditions

PathHunter U2OS EpoR/EpoR 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 (2 weeks or less): Store vials at -80°C immediately upon arrival. (DO NOT store at –80°C for more than 2 weeks).
- Long term (greater than 2 weeks): Vials should ONLY be stored in the vapor phase of liquid nitrogen (LN_2).

Safety Warning: A face shield, gloves and 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 liquid N_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 liquid nitrogen 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 in 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 should be dispensed and frozen down. 4mL of Detection Reagent 2 per aliquot should 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 in 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 should 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 in 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, 10mL 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 Erythropoietin Control Agonist

Store at -20°C until ready to use (up to the expiration date listed in the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery. Reconstitute to a concentration of 40 µg/mL by adding 250 µL of Protein Dilution Buffer.

96-well Tissue Culture Treated Plates

Store at room temperature.

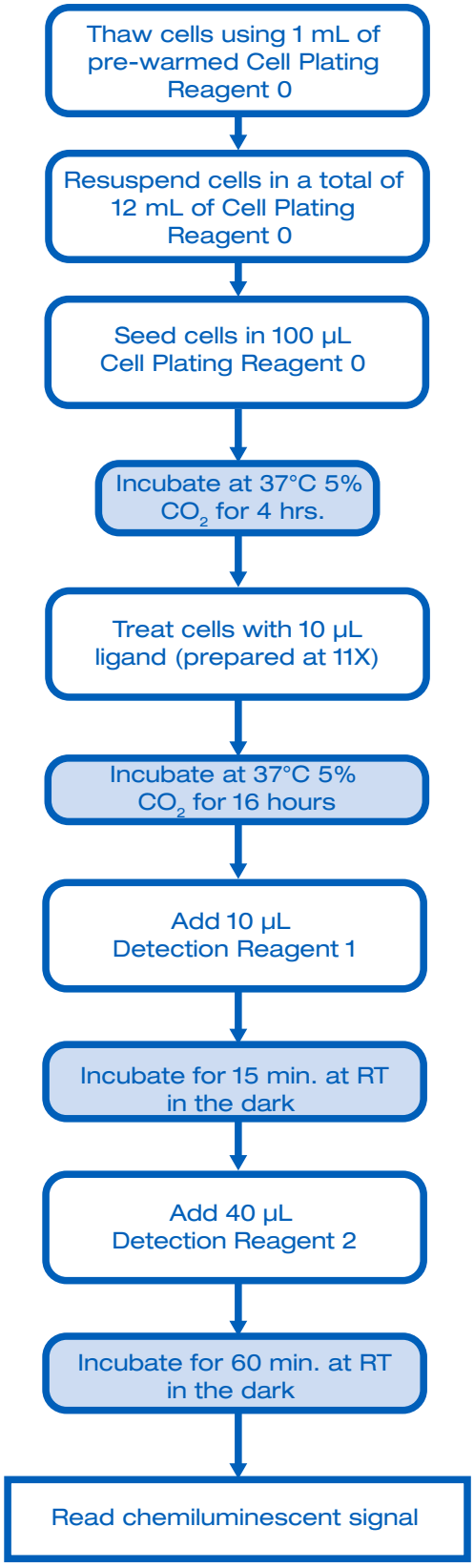
Additional Materials Required

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, Cat. #92-0011 or similar)
Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar)

Protocol Schematic

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



Detailed Protocols

PathHunter U2OS EpoR/EpoR Bioassay cell preparation:

The following protocol is for thawing and plating frozen PathHunter U2OS EpoR/EpoR 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 100 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, clear-bottom 96-well assay plate.
2. Dispense 12 mL of cell plating reagent 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.

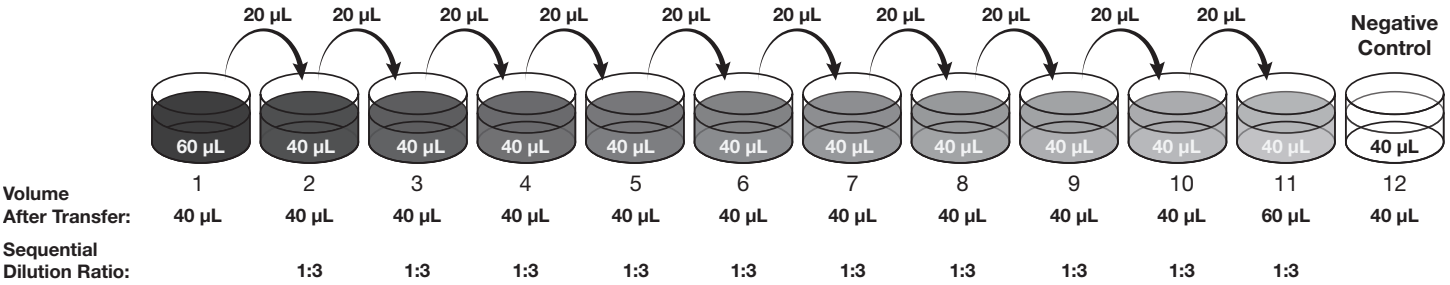
4. Immediately add 1 mL (using P1000) of pre-warmed Cell Plating Reagent from the 15 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 11 mL of Cell plating reagent. 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 reagent reservoir.
6. Add 100 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. Allow the cells to recover for 4 hours before continuing.

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.

Method 1

A 1:3 serial dilution. This method is simple to run and covers a broad concentration range, making it ideal for initial assay optimization. The volumes listed below are designed for running samples from one dose response curve in duplicate on the assay plate (as pictured in the assay plate map on page 9).



1. Prepare the reference agonist (erythropoietin) dose response curve, which will serve as a positive control in this assay. Agonist is prepared at 11x the desired final concentration as it will be diluted by adding to the 100 µL of media present in the assay plate.

   a. Add 40 µL of Protein Dilution Buffer (PDB) to columns 2-12, row A of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoveRx 92-0011 or similar).

   b. Add 250 µL of PDB to the erythropoietin vial containing 10 µg of lyophilized powder to make a 40 µg/mL stock solution.

   c. Add 53.4 µLof PDB to column one, row A of the master dilution plate. Add 6.6 µL of the 40 µg/mL erythropoietin stock to this well. Mix thouroughly by pipetting up and down several times. This results in an 4.4 µg/mL solution (11x the final 400 ng/mL curve top).

   d. Using a clean tip, transer 20 µL from column 1 into column 2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 20 µL from column 2 into column 3. Mix by pipetting up and down several times. Repeat this process iuntil column 11 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. Prepare epoetin alfa reference curve. Epoetin alfa is prepared at 11x the desired final concentration.

   a. Add 40 µL of Protein Dilution Buffer (PDB) to columns 2-12 in a new row of the master dilution plate.

   b. Add 60 µL of epoetin alfa prepared at 11x the desired final concentration to column 1 of this row on the master dilution plate.

   c. Using a clean tip, transfer 20 µL from column 1 into column 2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 15 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 ligand is transferred to column 12 as this will serve as a negative control.

3. Prepare biosimilar curves. Biosimilars are prepared at 11x the desired final concentration.

    a. Add 40 µL of Protein Dilution Buffer (PDB) to columns 2-12 in a new row of the master dilution plate.

    b. Add 60 µL of the highest concentration of biosimilar to column 1 of this row on the master dilution plate.

    c. Using a clean tip, transfer 20 µL from column 1 into column 2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 20 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 biosimilar is transferred to column 12 as this will serve as a negative control.

4. Remove the assay plate is removed from the 37C, 5% CO_2 incubator and bring into the tissue culture hood.

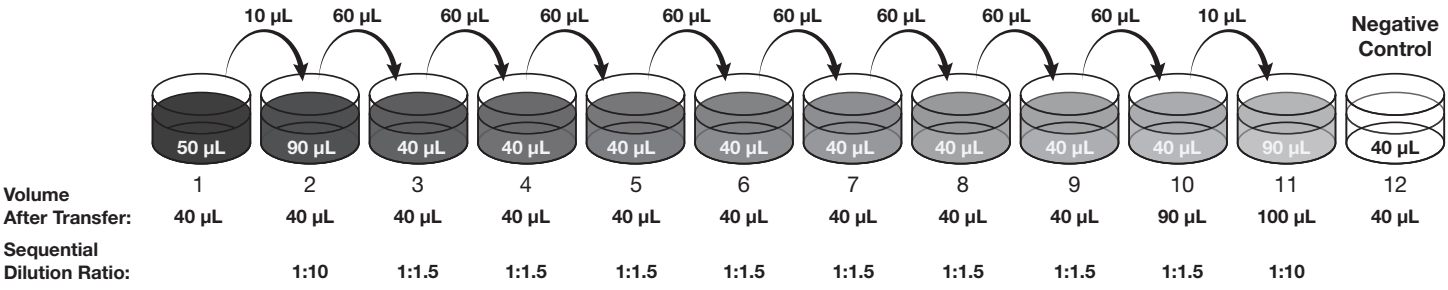
5. Add 10 µL from the agonist reference curve on the master dilution plate to the appropriate wells of the assay plate. Refer to the example plate map on page 9.

6. Add 10 µL from each well of the epoetin alfa and biosimilar curves from the master dilution plate to the appropriate wells of the assay plate. Refer to the example plate map on page 9.

7. Return the assay plate to the 37C, 5% CO_2 incubator and is incubate overnight (16 hours).

Method 2

This method covers a smaller concentration range in order to generate more data points within the linear range of the dose response curve, and is suitable for testing ligands with known pharmacology in this assay platform. The volumes listed below are designed for running samples from one dose response curve in duplicate on the assay plate (as pictured in the assay plate map on page 9).



1. Prepare the reference agonist (erythropoietin) dose response curve, which will serve as a positive control in this assay. Agonist is prepared at 11x the desired final concentration as it will be diluted by adding to the 100 µL of media present in the assay plate.

    a. Add 90 µL of Protein Dilution Buffer (PDB) to columns 2 and 11, row A of a master dilution plate (e.g. a V-bottom 96-well dilution plate, DiscoveRx 92-0011 or similar). Then add 40 µL of Protein Dilution Buffer (PDB) to the remaining wells in the same row.

    b. Add 250 µL of PDB to the erythropoietin vial containing 10 µg of lyophilized powder to make a 40 µg/mL stock solution.

    c. Add 53.4 µLof PDB to column one, row A of the master dilution plate. Add 6.6 µL of the 40 µg/mL erythropoietin stock to this well. Mix thouroughly by pipetting up and down several times. This results in an 4.4 µg/mL solution (11x the final 400 ng/mL curve top).

    d. Using a clean tip, transfer 10 µL from column 1 into column 2 and mix by pipetting up and down several times.

e. Replace the pipette tip, and transfer 60 µL from column 2 into column 3. Mix by pipetting up and down several times. Repeat this process until column 10 is reached.

f. Using a clean tip, transfer 10 µL from column 10 into column 11 and mix by pipetting up and down several times.

2. Prepare epoetin alfa reference curve. Epoetin alfa is prepared at 11x the desired final concentration.

   a. Add 90 µL of Protein Dilution Buffer (PDB) to columns 2 and 11 in a new row on the master dilution plate. Then add 40 µL of Protein Dilution Buffer (PDB) o the remaining wells in the same row.

   b. Prepare 50 µL of epoetin alfa at 11x the desired final concentration and add to column 1 of this row on the master dilution plate.

   c. Using a clean tip, transfer 10 µL from column 1 into column 2 and mix by pipetting up and down several times.

   d. Replace the pipette tip, and transfer 60 µL from column 2 into column 3. Mix by pipetting up and down several times. Repeat this process is repeated until column 10 is reached.

   e. Using a clean tip, transfer 10 µL from column 10 into column 11 and mix by pipetting up and down several times.

3. Prepare biosimilar curves. Biosimilars are prepared at 11x the desired final concentration.

   a. Add 90 µL of Protein Dilution Buffer (PDB) to columns 2 and 11 in a new row on the master dilution plate. Then add 40 µL of Protein Dilution Buffer (PDB) o the remaining wells in the same row.

   b. Add 50 µL of the highest concentration of biosimilar to column 1 of a new row on the master dilution plate.

   c. Using a clean tip, transfer 10 µL from column 1 into column 2 and mix by pipetting up and down several times.

   d. Replace the pipette tip, and transfer 60 µL from column 2 into column 3. Mix by pipetting up and down several times. Repeat this process until column 10 is reached.

   e. Using a clean tip, transfer 10 µL from column 10 into column 11 and mix by pipetting up and down several times.

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

5. Add 10 µL from the agonist reference curve on the master dilution plate to the appropriate wells of the assay plate. Refer to the example plate map on page 9.

6. Add 10 µL from each well of the epoetin alfa and biosimilar curves on the master dilution plate to the appropriate wells of the assay plate. Refer to the example plate map on page 9.

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

Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate.

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 1 hour in the dark.

5. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for PMT readers of 5-10 seconds for imager.

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 Ligand Dilution Curves

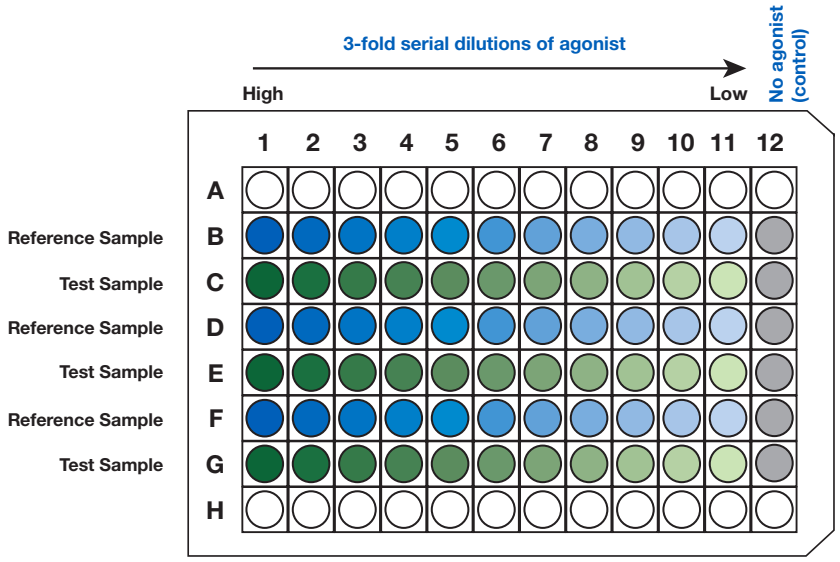


Figure 1. Serial dilution method 1. This plate map shows 11-point dose curves with 3 data points at each concentration for one reference and one test sample per plate, with a 1:3 serial dilution scheme.

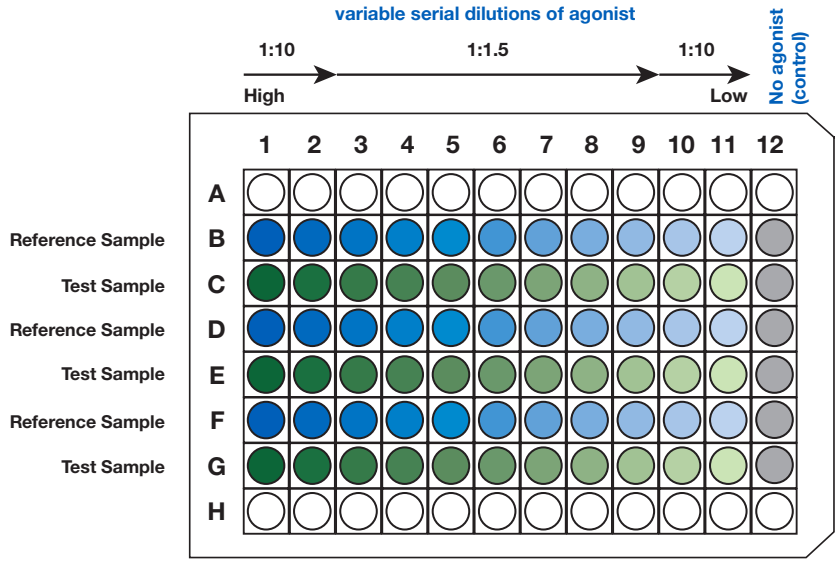


Figure 2. Serial dilution method 2. This plate map shows an 11 point dose curve with 3 data points at each concentration for one reference and one test sample per plate, with a variable serial dilution scheme.

Related Products

Description	Ordering Information
PathHunter Bioassay Detection Reagents	www.discoverx.com/detectionreagents
Cell Culture Kits, Reagents & Consumables	www.discoverx.com/cell-culture-kits-reagents-consumables
AssayComplete Cell Plating Reagents	www.discoverx.com/cellplatingreagents
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 **Hamamatzu** FDS6000, FDSS/RayCatcher **Thermo Scientific:** Luminoskan Ascent **Biotek:** Synergy 2	Luminescence

*For other instruments not listed here, please contact technical support at support@discoverx.com to ensure compatibility.

Troubleshooting Guide

Problem	Cause	Solution
No Response	Improper thawing procedure	Refer to thawing instructions on page 4 of this user manual.
	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 at support@discoverx.com.

Limited Use License Agreement

A. This product and/or its use is covered by U.S. patents #8,541,175 and #8,679,832 and/or related foreign patents and pending applications and trade secrets that are either owned by or licensed to DiscoveRx Corporation ("DiscoveRx"). The cells and detection reagents (collectively "Materials") purchased from DiscoveRx are expressly restricted in their use. DiscoveRx has developed a Cell-Based Dimerization assay ("Assay") that employs genetically modified cells and vectors (collectively, the “Cells”), and related detection reagents (the “Reagents”) (collectively referred to as “Materials”). By purchasing and using the Materials, the Purchaser agrees to comply with the following terms and conditions of this label license and recognizes and agrees to such restrictions:

1. Purchaser will not further propagate the Cells included in the Assay kit.
2. Purchaser is permitted to use the Cells only for use in the Assay and in connection with Reagents purchased from DiscoveRx or its authorized distributor.
3. The Materials are not transferable and will be used only at the site for which they were purchased. Transfer to another site owned by Purchaser will be permitted only upon written request by Purchaser followed by subsequent written approval by DiscoveRx.
4. The Reagents contain or are based upon the proprietary and valuable know-how developed by DiscoveRx, and the Reagents have been optimized by DiscoveRx to function more effectively with the Cells in performing the Assay. Purchaser will not analyze or reverse engineer the Materials nor have them analyzed on Purchaser’s behalf.
5. In performing the Assay, Purchaser will use only Reagents supplied by DiscoveRx or an authorized DiscoveRx distributor for the Materials.
6. Purchaser will not use the Cells with any other reagents or substrates, other than the Reagents that are provided by or purchased from DiscoveRx or an authorized DiscoveRx distributor, in connection with the Materials.
7. The number of Assays performed will not exceed the authorized number for which Materials were purchased.

B. The use of this cell line may require third party licenses. Purchaser shall have the sole responsibility for obtaining any such licenses prior to use of the cell lines.

If the purchaser has any further questions regarding the rights conferred with purchase of the Materials, please contact:

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