DiscoverX

User Manual

PathHunter®

FGFR4-β-Klotho Bioassay Kit

For chemiluminescent detection of receptor activity

Catalog No. 93-1060Y3- Series

Table of Contents

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

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Overview

Intended Use

PathHunter® FGFR4-β-Klotho Bioassay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study FGF-19 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, dilution buffer, and assay plates. The qualified, 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® FGFR4-β-Klotho 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.

In the PathHunter assay approach for tyrosine kinases, the ProLink tag is fused to the C-terminus of the receptor. The EA is fused to a phosphotyrosine SH2 domain containing protein that is able to bind the activated RTK. Ligand-induced activation of the receptor results in receptor phosphorylation. The SH2-EA fusion protein binds the phosphorylated receptor, forcing complementation of PK and EA to form an active β-gal enzyme. β-gal enzymatic activity is quantitatively measured using a chemiluminescent substrate in the PathHunter Detection Kit (Figure 1).

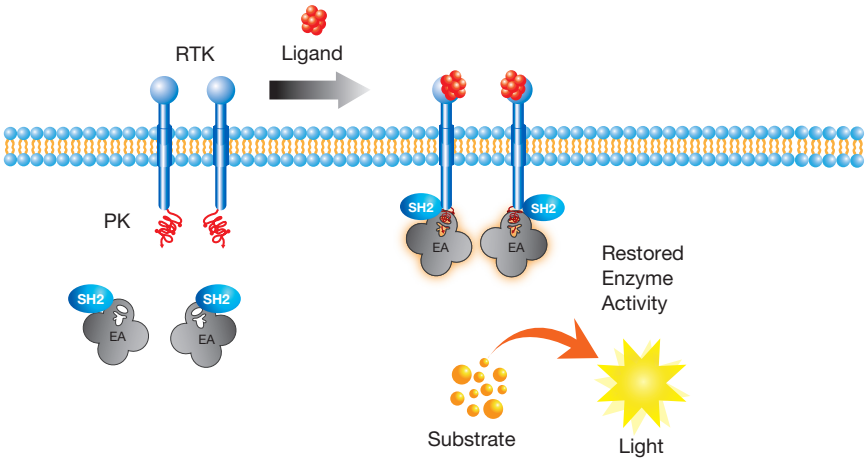


Figure 1. PathHunter FGFR4-β-Klotho Bioassay Principle

Materials Provided

List of Components	(93-1060Y3-00089)	(93-1060Y3-00090)
Description Kit Size	**Contents**	**Contents**
PathHunter U2OS FGFR4-β-Klotho Bioassay Cells	2 vials	10 vials
PathHunter Detection Kit	200 dp	1000 dp
• Cell Assay Buffer	11.4 mL	57 mL
• Substrate Reagent 1	3 mL	15 mL
• Substrate Reagent 2	0.6 mL	3 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 (FGF-19)	1 vial	1 vial
96-well Opaque-Bottom TC Treated, Sterile Plates w/lid	2 plates	10 plates

Storage Conditions

PathHunter U2OS FGFR4-β-Klotho Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state.

Immediately store the vials 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 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 Detection Kit

Store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 7 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. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance. The stability of the working solution once made is 24 hours at room temperature.

To make aliquots suitable for testing one assay plate each, 5.7 mL of Cell Assay Buffer per aliquot should be dispensed and frozen down. 1.5 mL of Substrate Reagent 1 per aliquot should be dispensed and frozen down separately. 0.3 mL of Substrate Reagent 2 per aliquot should be dispensed and frozen down separately. Do not mix the 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, 30 mL 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, 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 FGF-19 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. When ready to use, reconstitute to a concentration of 100 ug/mL by adding 50 µL of sterile PBS to the vial containing 5 ug of lyophilized powder. Reconstituted ligand is stable for 1 week at 2-8°C. For longer storage (up to the expiration date listed on the kit Certificate of Analysis), it is recommended to store in working aliquots at -20 to -80°C.

96-well Tissue Culture Treated Plates

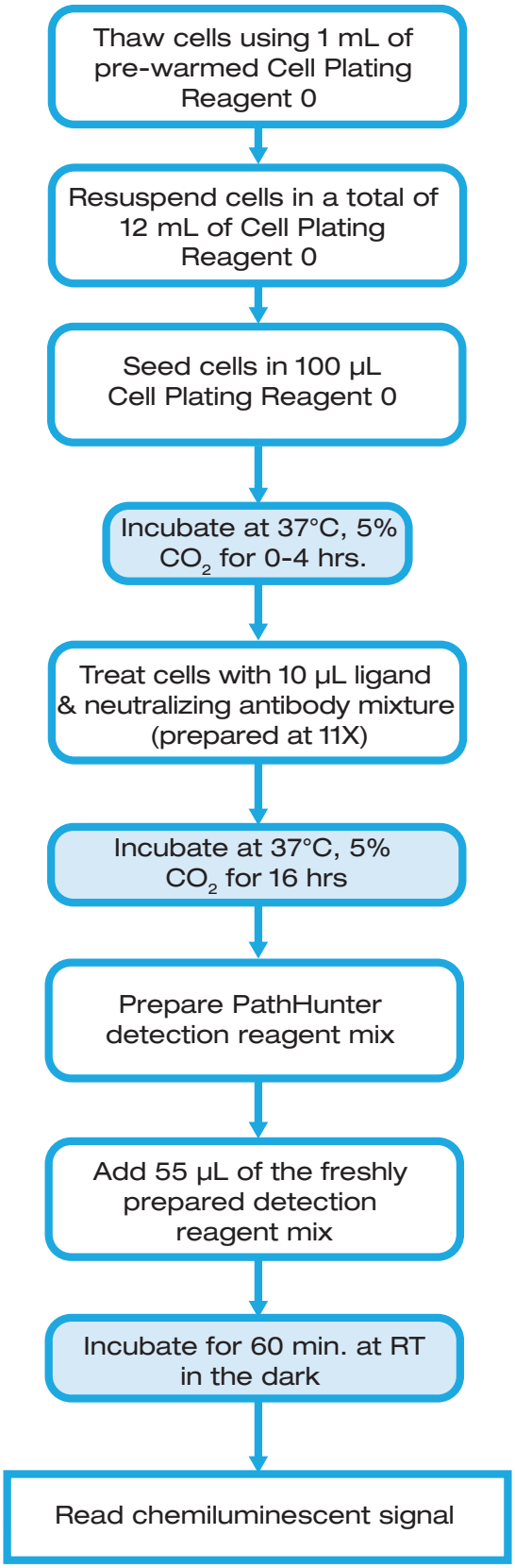
Store at room temperature.

Additional Materials Required

Equipment
The following equipment and additional materials are required to perform these assays:
Single and multichannel micro-pipettors and pipette tips
Multimode or luminescence plate reader
V-Bottom 96-well compound dilution plates (DiscoverX 92-0011 or similar)
Disposable Reagent Reservoir (Thermo Scientific, Cat. #8094 or similar)

Protocol Schematic

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



Protocol for Potency Assay Development

Day 1: PathHunter Bioassay Cell Preparation :

The following protocol is for thawing and plating frozen PathHunter U2OS FGFR4-β-Klotho Bioassay cells from cryovials.

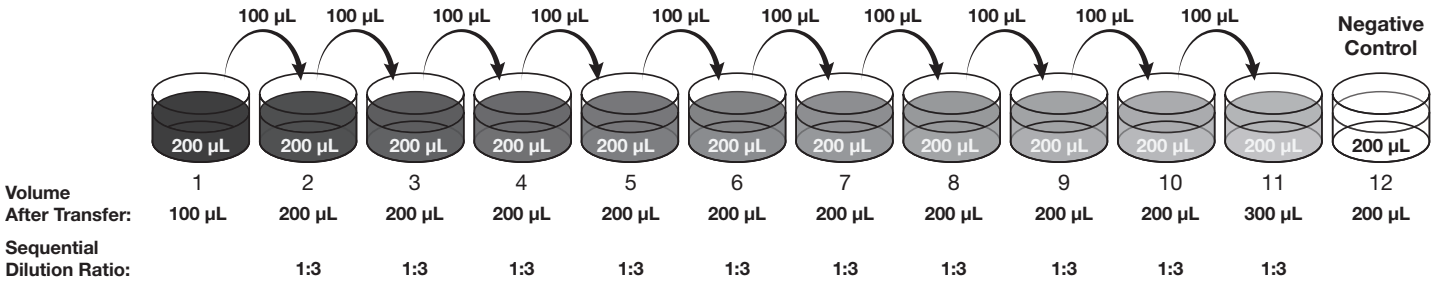
1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. This includes:
    a. One 25 mL reagent reservoir.
    b. One 15 mL conical tube.
    c. A micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 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, opaque-bottom 96-well assay plate (DiscoverX 92-0027 or similar).
    g. V-bottom 96 well compound dilution plates (DiscoverX 92-0011 or similar).
2. Dispense 12 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.
5. Transfer the cell suspension to the conical tube containing the remaining 11 mL of CP0. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
6. Mix the tube by gentle 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.
7. Add 100 µL of cells to each well of the 96 well assay plate using the multichannel pipette. Let the plate sit for 15 minutes at room temperature to allow the cells to settle and reduce potential edge effects.
8. Place the plate in a tissue culture incubator (37°C, 5% CO_2) for 0-4 hours before adding ligand.

Day 1: Ligand Preparation

The following protocol is designed for testing purified biologics to be run as a potency assay for QC lot release testing or stability testing of biologic molecules.



1. Prepare the agonist (rhFGF-19) dose response curve. 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 200 µL of Protein Dilution Buffer to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoveRx 92-0011 or similar).

   b. Add 200 µL of rhFGF-19 at 11 µg/mL (11x of 1 µg/mL, the final concentration of the top dose) to well A1 of the master dilution plate.

   c. 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. Add 10 µL from the agonist rhFGF-19 curve on the master dilution plate to the appropriate wells of the assay plate.

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

Day 2: Detection

1. Prepare PathHunter Detection Reagent by combining 1 part Substrate Reagent 2 with 5 parts Substrate Reagent 1, and 19 parts of Cell Assay Buffer respectively. Once prepared, the working solution is stable for at least 24 hours at RT with no impact on assay performance. Prepare the following quantity per 96-well plate needed.

Reagent Component	Volume
Cell Assay Buffer	5.7 mL
Substrate Reagent 1	1.5 mL
Substrate Reagent 2	0.3 mL
Total Volume	**7.5 mL**

2. Using a multichannel pipette add 55 µL of the PathHunter Detection Reagent mix to each well of the assay plate.

3. Incubate the plate at room temperature for 1 hour in the dark.

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

Protocol for Detecting Neutralizing Anti-Drug Antibodies

Day 1: PathHunter Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter U2OS FGFR4-β-Klotho 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, opaque-bottom 96-well assay plate (DiscoverX 92-0027 or similar).
    g. V-bottom 96 well compound dilution plates (DiscoverX 92-0011 or similar).
2. Dispense 12 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.
5. Transfer the cell suspension to the conical tube containing the remaining 11 mL of CP0. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
6. Mix the tube by gentle 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.
7. Add 100 µL of cells to each well of the 96 well assay plate using the multichannel pipette. Let the plate sit for 15 minutes at room temperature to allow the cells to settle and reduce potential edge effects.
8. Place the plate in a tissue culture incubator (37°C, 5% CO_2) for 0-4 hours before adding ligand.

Day 1: Ligand Preparation

The following protocol is designed for testing neutralizing anti-drug antibodies targeting FGF-19 and its analogs. The PathHunter assays can 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.

Prepare Protein Dilution buffer containing 10% Norman Human Serum (NHS): Equilibriate Protein Dilution buffer and reference pooled Normal Human Serum (NHS) sample at room temperature (RT) for 10-15 minutes. Then add 100 µL of NHS in 900 µL of Protein Dilution buffer to make a 10% solution. Adjust volumes as necessary to get desired final serum concentration. Note: when preparing patient samples for analysis, the diluent will not contain NHS.

1. Prepare the reference agonist (rhFGF-19) dose response curve, which will serve as a positive control in this assay. (Alternatively, an EC-80 dose of rhFGF-19 can be used as a positive control to allow more samples to be run on a single plate). Agonist is prepared at 11x the desired final concentration as it will be diluted by adding to the 100 µL of media (with cells) present in the wells to its final 1x concentration on the assay plate.

    a. Add 200 µL of Protein Dilution Buffer containing 10% NHS (as prepared above) to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoveRx 92-0011 or similar).

    b. Add 33 µL of rhFGF19 at 100 µg/ml stock concentration to 267 µL of Protein Dilution Buffer containing 10% NHS.

    c. Add 200 µL of rhFGF-19 at 11 µg/mL (11x of 1 µg/mL, final concentration of the top dose) to well A1 of the master dilution plate.

    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 positive control neutralizing antibody dose curves: The EC_{80} of rhFGF-19 (included in the kit) was determined to be approximately 0.011 µg/mL. It is recommended that the EC_{80} is empirically determined in each lab prior to running the assay to ensure more accurate results.

    a. Prepare 4 mls of Protein Dilution Buffer containing 10% NHS (serum-containing buffer) as described previously.

    b. Add 3.3 µL of 100 µg/mL stock of rhFGF-19 in 3 mL of serum-containing buffer to make the 11x desired final concentration of EC_{80} challenge at 0.11 µg/mL. This preparation is used as a diluent for the appropriate positive control neutralizing antibody dilutions.

3. Prepare neutralizing antibody curve with the positive control neutralizing antibody using the solution containing EC_{80} of rhFGF19, as prepared above in step 2. Neutralizing antibody is prepared at 11x the desired final concentration.

Note: Volumes given are sufficient to perform a dose curve in triplicate.

    a. The recommended top dose of the positive control neutralizing antibody is 10 µg/mL (this will need to be modified based on the potency of your positive control antibody).

    b. To prepare 11x concentration, add 178 µL of the FGF-19 containing diluent prepared in step 2 to well B1 of the dilution plate.

c. Add 22 µL of the 1 mg/mL stock concentration of positive control neutralizing antibody to well B1 on the dilution plate.

d. Add 200 µL of FGF-19 containing diluent prepared in step 2 to wells B2 to B12 of the dilution plate.

5. Using a clean tip, transfer 100 µL from well B1 into well B2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well B2 into well B3. Mix by pipetting up and down several times. Repeat this process until well B11 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. Cover the dilution plate with a lid.

6. The antibody and ligand is pre-incubated at RT for 30 minutes before adding to assay plate.

7. Add 10 µL from the agonist reference curve, row A on the dilution plate, to the appropriate wells of the assay plate (rows B, D & F if the assay is being run in triplicate).

8. Add 10 µL from each well of the positive control neutralizing antibody curve, row B on the dilution plate, to the appropriate wells of the assay plate (rows C, E & G if the assay is being run in triplicate).

9. Place the assay plate into the 37°C, 5% CO_2 incubator and incubate overnight (16-18 hours).

Day 2: Detection

1. Prepare PathHunter Detection Reagent by combining 1 part Substrate Reagent 2 with 5 parts Substrate Reagent 1, and 19 parts of Cell Assay Buffer respectively. Once prepared, the working solution is stable for at least 24 hours at RT with no impact on assay performance. Prepare the following quantity per 96-well plate needed.

Reagent Component	Volume
Cell Assay Buffer	5.7 mL
Substrate Reagent 1	1.5 mL
Substrate Reagent 2	0.3 mL
Total Volume	**7.5 mL**

2. Using a multichannel pipette add 55 µL of the PathHunter Detection Reagent mix to each well of the assay plate.

3. Incubate the plate at room temperature for 1 hour in the dark.

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

Supplemental Information

Representative Plate Maps for Agonist Curve

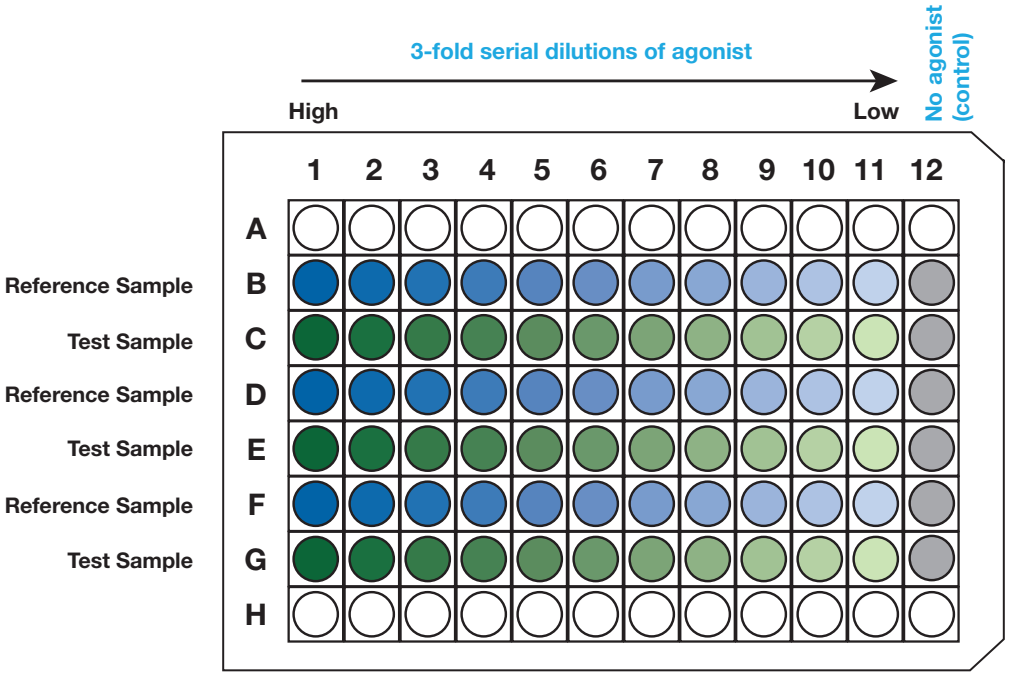


Figure 1. Serial dilution method 1. This plate map shows 11-point dose curves with 3 data points at each concentration for one reference and one test sample per plate.

Related Products

Description	Ordering Information
PathHunter 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 **Hamamatzu** 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 on page 5 of this user manual.
	Improper ligand used or improper ligand incubation time	Refer to the Detailed Protocols section of this manual for the 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 as provided on the certificate of analysis.
	Improper preparation of ligand (agonist or antagonist)	Check and repeat assay at correct incubation temperature as indicated on the assay datasheet.
		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 use the contact information below.

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