DiscoverX

User Manual

PathHunter®

U2OS MC4R Bioassay Kit

For Chemiluminescent Detection of αMSH Activity

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Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

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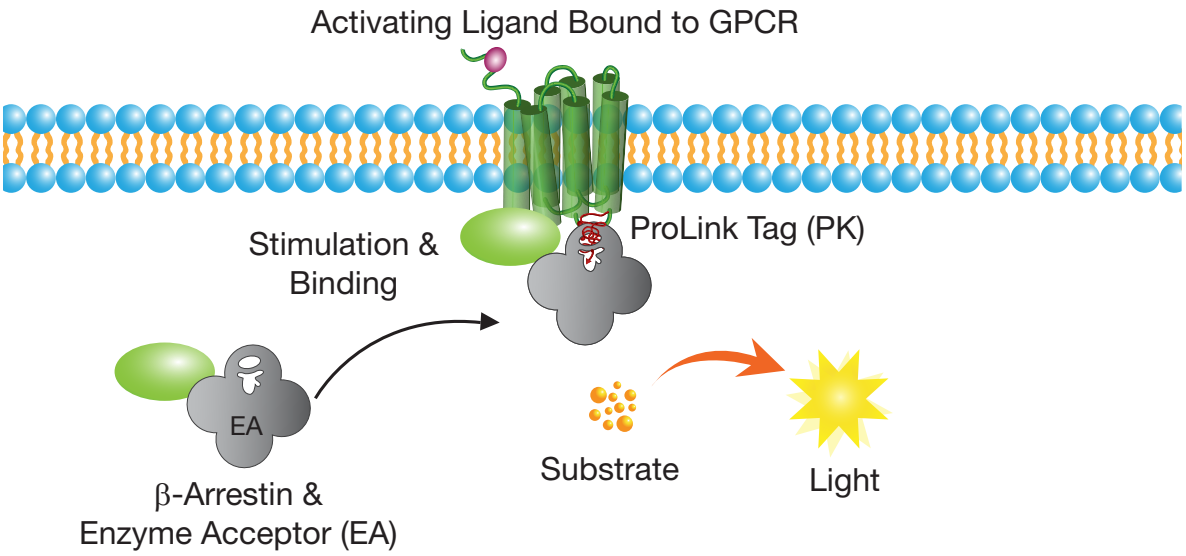
Overview

PathHunter U2OS MC4R Bioassay Kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study drug 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-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 U2OS MC4R 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 U2OS MC4R Bioassay monitors GPCR functional activation by detecting the interaction of β-Arrestin with the activated GPCR using EFC. The GPCR is fused with the PK tag and co-expressed in cells stably expressing β-Arrestin-EA fusion protein. Functional activation of the GPCR stimulates recruitment of β-Arrestin to its intracellular surface and forces complementation of the two EFC fragments. The resulting active enzyme hydrolyzes a substrate to generate a chemiluminescent signal. These assays are independent of G-protein coupling and directly measure functional activation of GPCRs.



Materials Provided

List of Components	93-0211Y3-00085	93-0211Y3-00086
PathHunter U2OS MC4R Bioassay Cells	2 vials	10 vials
PathHunter Bioassay Detection Kit Detection Reagent 1 (mL) Detection Reagent 2 (mL)	 2 8	 10 40
AssayComplete™ Cell Plating Reagent 4	1 X 100 mL	3 X 100 mL
Protein Dilution Buffer	1 X 50 mL	2 X 50 mL
Control Agonist (αMSH)	1 vial	1 vial
96-Well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

PathHunter U2OS MC4R 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.

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 liquid nitrogen. Upon thawing, if liquid nitrogen 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-thaws.

To make aliquots suitable for testing one assay plate each, 1 mL of Detection Reagent 1 per aliquot can be dispensed and frozen down. 4 mL 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 4 (CP4)

Once thawed, the CP4 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, 30 mL of reagent per aliquot can be dispensed and frozen down.

Protein Dilution Buffer (PDB)

Once thawed, the PDB 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 αMSH 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 and reconstitute as noted in the ligand datasheet. 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

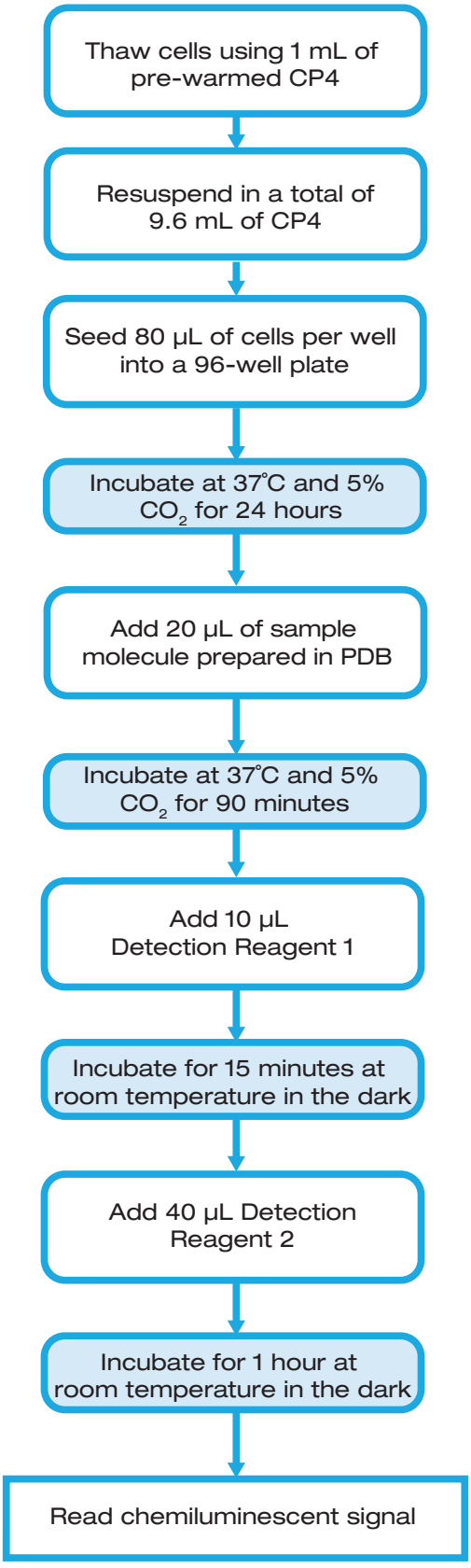
Store at room temperature.

Additional Materials Required

Material	Ordering Information
V-Bottom 96-well ligand dilution plates	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Single and multichannel micro-pipettors and pipette tips	
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar

Protocol Schematic

Quick-Start Procedure: In a 96-well tissue culture treated plate provided in the kit, perform the following steps:



Detailed Protocols

Day 1: PathHunter Bioassay Cell Preparation

The following protocol is for thawing and plating frozen U2OS MC4R 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 4 (CP4, 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 pre-warmed CP4 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 CP4 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 8.6 mL of CP4. 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.
7. Let the plate sit for 15 minutes at room temperature to allow cells to settle, reducing the potential for edge effects.
8. Incubate the cells in a humidified tissue culture incubator at 37°C and 5% CO_2 for 24 hours.

Day 2: Sample Preparation

The following protocol is designed for testing purified biologics. 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.

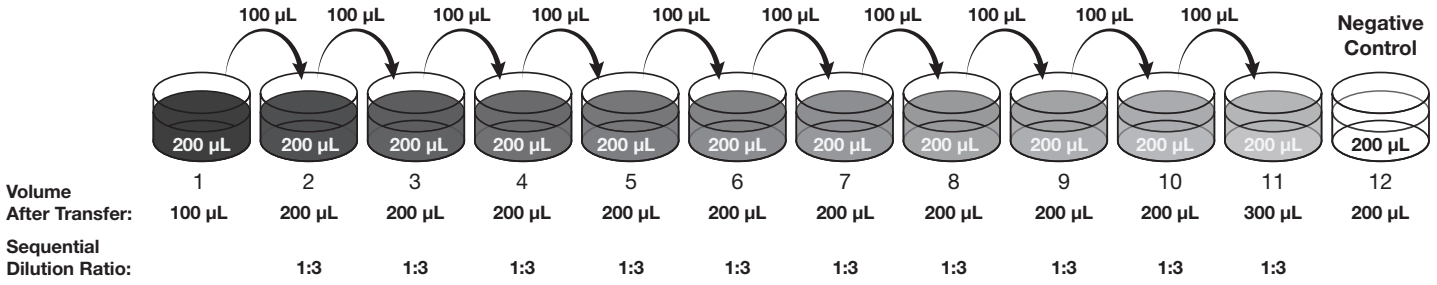
1. Prepare α-Melanocyte Stimulating Hormone (αMSH) curve. αMSH dilutions are prepared at 5X the desired final concentration. Top dose for the DiscoverX ligand is 10 µg/mL at final assay concentration.

   a. Add 1000 µL of reconstitution solvent (DI water) to 1 mg of αMSH lyophilized powder to make a stock concentration of 1 mg/mL.

   b. Add 10 µL of αMSH from the stock (1000 µg/mL) in 190 µL of PDB in well A1. This creates a working stock of 50 µg/mL of αMSH (5X the final concentration).

   c. Add 200 µL of PDB to wells A2 to A12 of the master dilution plate.

   d. Using a clean tip, transfer 100 µL from well A1 into well A2 for a 1:3 dilution and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 to A3 for a 1:3 dilution. Repeat this process until well A11 is reached. No sample is transferred to A12 as this will serve as a negative control.



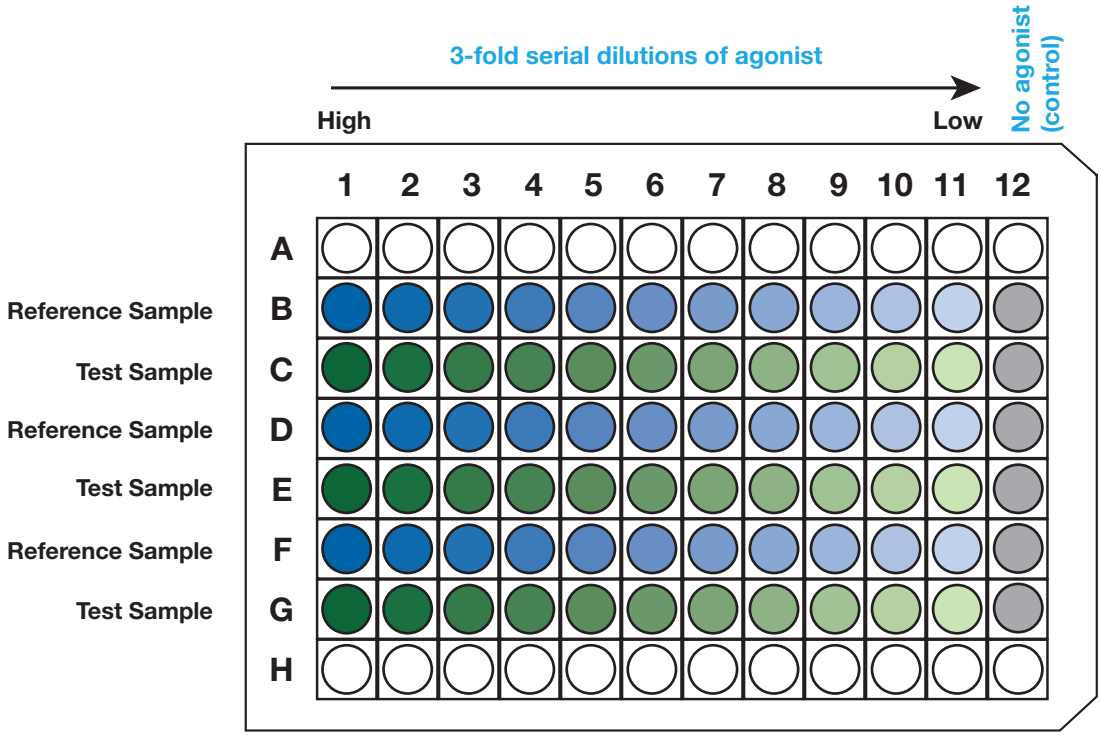
2. Add 20 µL from the αMSH dilutions on the master dilution plate to the appropriate wells of the assay plate.

3. Incubate assay plate at 37°C and 5% CO_2 in a humidified incubator for 90 minutes.

Day 2: Detection

1. Using a multichannel pipette, add 10 µL of Bioassay 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 Bioassay 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 photomultiplier tube (PMT) readers or 5-10 seconds for imager.

Representative Plate Maps for Agonist Curve



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

Troubleshooting Guide

Problem	Cause	Solution
No response	Improper thawing procedure	Refer to thawing instructions in this user manual. Thawing process can have a significant effect on cell viability.
	Improper ligand used or improper ligand incubation time	See certificate of analysis for recommended ligand and assay conditions.
	Improper preparation of ligand (agonist or antagonist)	Refer to ligand 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 seconds/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	Make sure ligands are stored and incubated at the proper temperature.
	Difference in agonist binding affinity	Confirm that the ligand used is comparable to the ligand in the certificate of analysis.
	Contamination from tips	Changing tips during serial dilutions can help to avoid carryover.
High variability between replicates	Instrument calibration	Ensure dispensing equipment is properly calibrated, and proper pipetting technique is used.

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

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