User Manual

PathHunter® U2OS MCHR1 Bioassay Kit

For Detection of β-Arrestin Recruitment by Ligand-Activated MCHR1

For Bioassay Kits:

93-0940Y3-00149: 2-Plate Kit

95-0940Y3-00150: 10-Plate Kit

Document Number 70-422 Rev 0

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

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For additional information or Technical Support, see contact information at the bottom of this page.

Overview

The PathHunter MCHR1 Bioassay kit provides a robust, highly sensitive, and easy-to-use functional cell-based assay to determine drug potency and detect neutralizing antibodies. The bioassay kit contains all the materials needed for a complete assay, including cryopreserved cells, detection reagents, cell plating reagent, positive control agonist, and assay plates. The ready-to-use cryopreserved cells have been manufactured to ensure assay reproducibility and faster implementation from characterization to lot release. This bioassay has been optimized for a 96-well plate format. The protocol can be optimized further for running the assay in a high-throughput 384-well plate format.

Assay Principle

The PathHunter MCHR1 bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, a small 42 amino acid fragment called ProLink™ (PK), and the larger, N-terminal deletion mutant of β-gal called 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.

This bioassay monitors MCHR1 (GPCR) activity by detecting β-Arrestin2 recruitment by this receptor as a result of ligand-mediated receptor activation. In this system, the MCHR1 is fused to the small β-gal fragment, PK and co-expressed in cells stably expressing β-Arrestin2 fused with the larger enzyme fragment, EA. Activation of MCHR1 stimulates the recruitment of β-Arrestin2 to the PK-tagged receptor and forces complementation of the two enzyme fragments. This results in the formation of an active β-gal enzyme, leading to an increase in enzymatic activity that can be measured using the chemiluminescent PathHunter Bioassay Detection Reagent. Since arrestin recruitment occurs independent of G-protein coupling, these assays provide a direct, universal platform for measuring receptor activation, thus providing an ideal tool to characterize functional biologics.

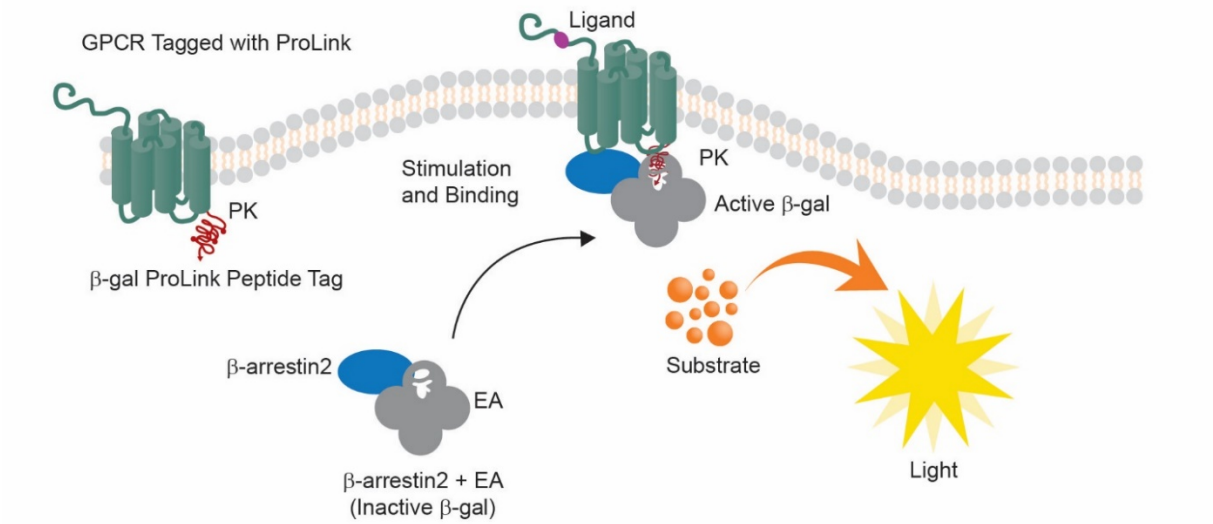


Figure 1. Assay Principle: Stimulation of the PK-tagged GPCR by its ligand enables the recruitment of β-arrestin2, which is tagged with EA. This results in the formation of an active β-gal enzyme, which hydrolyzes the substrate to generate a chemiluminescent signal.

Materials Provided

List of Components	93-0940Y3-00149	93-0940Y3-00150
PathHunter U2OS MCHR1 Bioassay Cells (1.2 x 10^6 cells in 1 mL per vial)	2	10
AssayComplete™ Cell Plating 5 Reagent* (100 mL per bottle)	1	4
Melanin Concentrating Hormone (6-17) (500 µg per vial)	1	2
PathHunter Bioassay Detection Kit Detection Reagent 1 (Bottle) Detection Reagent 2 (Bottle)	 1 x 3 mL 1 x 12 mL	 1 x 15 mL 1x 60 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

*Cell Plating 5 Reagent (CP5) is also used for diluting control agonist and antagonists in the bioassay.

Storage Conditions

PathHunter U2OS MCHR1 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 on dry ice in a covered container. Wait 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 5 Reagent (CP5)

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 on 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, 20 mL of reagent per aliquot can be dispensed and frozen down.

Melanin Concentrating Hormone (6-17) Control Agonist

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. Once prepared, the excess stock solution can be stored as suitable aliquots (e.g. 30 µL) at -20°C until needed. Do not freeze/thaw more than twice.

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 on 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 not using all the plates 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 volumes for each reagent component have been provided in the kit to make these aliquots.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

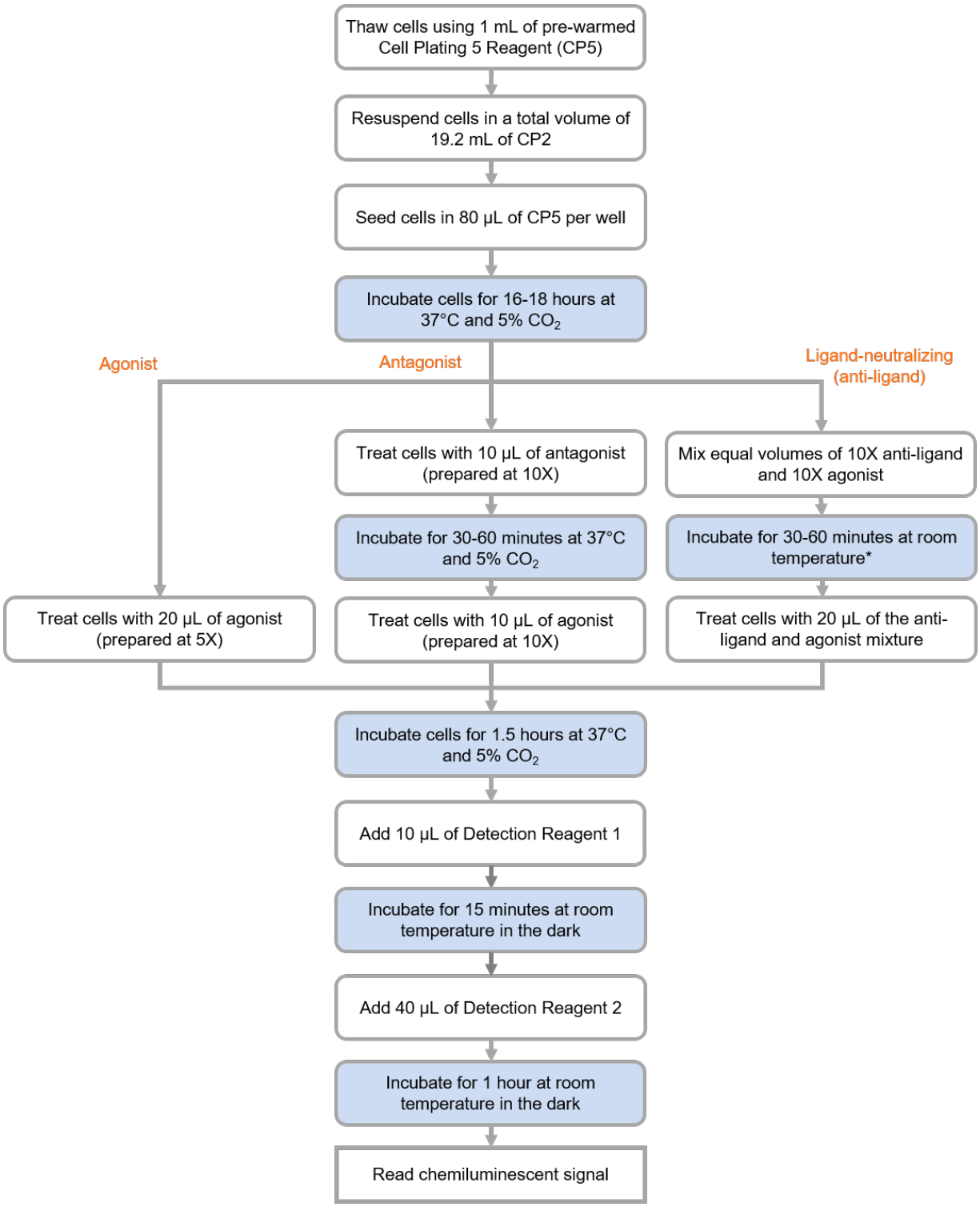
Additional Materials Required

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

Material	Ordering Information
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Multimode or luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Single and multichannel micropipettes and pipette tips (10 µL-1000 µL)	
Polypropylene tubes (50 mL and 15 mL)	
Microcentrifuge tubes (1.5 mL)	
Tissue culture disposable pipettes (1 mL-25 mL) and tissue culture flasks (T25 and T75 flasks, etc.)	

Protocol Schematic

Quick-start Procedure: In a 96-well plate, perform the following steps.



*Room temperature refers to a range of 23-25°C

Detailed Protocol

This user manual provides a protocol for determining potency in a 96-well format. To run this bioassay in a 384-well format, or to optimize the assay for the detection of neutralizing antibodies, contact Technical Support at DRX_SupportUS@eurofinsUS.com.

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating cryopreserved CHO-K1 MCHR1 bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the materials required are set up in the tissue culture hood. These include:
   a. One sterile 25 mL reagent reservoir
   b. One sterile 50 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 AssayComplete™ Cell Plating 5 Reagent (CP5), pre-warmed in a 37°C water bath for 15 minutes
   f. A 96-Well White, Clear Flat-Bottom, Tissue Culture-Treated Sterile Assay Plate (provided with the kit)
   g. A 96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plate, to be used as the master dilution plate
2. Dispense 19.2 mL of CP5 into the 50 mL conical tube.
3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.

DO NOT use a heated water bath to thaw the vial. Hold the cryovials by the cap; **DO NOT** touch the sides or bottom of the vial to avoid thawing of the cell pellet. Wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood.

4. Add 1 mL of pre-warmed CP5 from the 50 mL conical tube into the cryovial, to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet the cell suspension up and down, several times, to uniformly resuspend the cells.
5. Transfer the cell suspension to the conical tube containing the remaining 18.2 mL of CP5. Remove any remaining suspension from the cryovial to ensure maximum recovery of all the cells.
6. Replace the cap on the conical tube and gently invert it several times to ensure that the cells are uniformly resuspended in CP5, without creating any froth in the suspension. Immediately pour the suspension into the sterile 25 mL reagent reservoir.
7. Transfer 80 µL of the cell suspension to each well of the 96-well assay plate using a multichannel pipette. Replace the lid and leave the plate at room temperature for 15 minutes, to allow the cells to settle uniformly in the well and minimize potential edge effects.
8. Incubate the assay plate at 37°C and 5% CO_2 for 16-18 hours before proceeding with the assay.

Day 2: Sample 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. In some assays, standard curves of the control can be prepared in neat serum or plasma, and added directly to cells without further dilution. For best results, the optimized minimum required dilution of crude samples should be empirically determined.

A 1:3 serial dilution for the control agonist, MCH (6-17), has been used in this protocol, as shown in Figure 3. Agonist Serial Dilutions. The volumes listed below are designed for running samples from one dose-response curve in duplicate on the assay plate (Refer to Figure 3: Representative Assay Plate Map).

1. Add 80 µL of Cell Plating 5 Reagent (CP5) to Wells A2 to A12 of the master dilution plate.
2. Prepare the control agonist (MCH) dose-response curve:
   MCH (6-17) will serve as a positive control in this assay. The agonist is prepared at 5X the desired final concentration as it will be diluted by adding 20 µL to 80 µL of the medium present in the assay plate.
   - 2.1. Add 325 µL of the supplied reconstitution reagent to the vial containing 0.5 mg of the lyophilized MCH (6-17) powder to make a 1 mM stock solution. Mix by slowly pipetting up and down several times. Make suitable aliquots (e.g. 30 µL) and store at -20°C until needed.
   - 2.2. Add 190 µL of CP5 to Well A1 of the master dilution plate. Transfer 10 µL of the 1 mM MCH (6-17) stock prepared in Step 2.1 and mix well by slowly pipetting up and down several times. This results in a 50 µM solution (5X the final 10 µM dose).
   - 2.3. Using a clean pipette tip, transfer 40 µL from Well A1 to Well A2, and mix thoroughly by pipetting up and down several times.
   - 2.4. Replace the pipette tip and transfer 40 µL from Well A2 to A3. Repeat this process until Well A11 is reached, resulting in an 11-point, 1:3 dilution series. No sample is transferred to Well A12 as this is the negative control.
3. Prepare test samples in a similar manner in additional rows of the master dilution plate.
4. Remove the assay plate from the incubator and place it in the tissue culture hood.
5. Transfer 20 µL from each well of the agonist dilution series from the master dilution plate to the appropriate wells of the assay plate.
6. Incubate the assay plate in a humidified incubator at 37°C and 5% CO_2 for 1.5 hours.

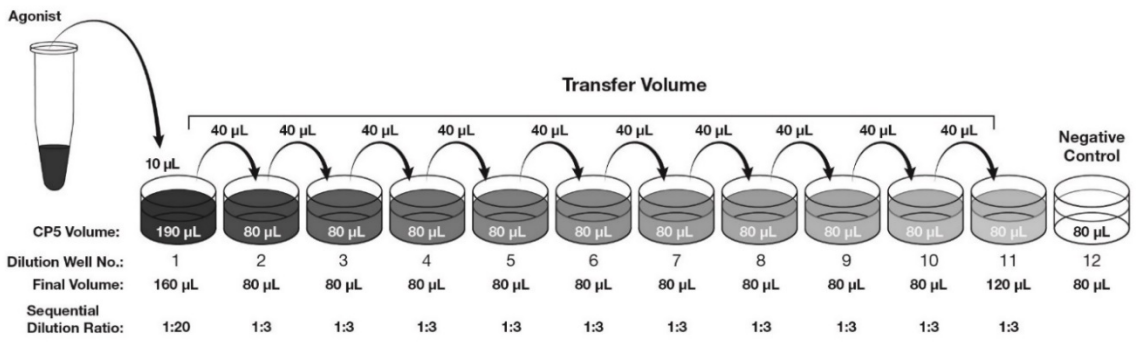


Figure 3. Agonist Serial Dilutions: Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

7. Prepare the antagonist dilution series using the following protocol. If antagonist samples are not being tested, then skip Steps 7-12 and proceed to Day 2: Ligand-Neutralizing Sample Preparation or Day 2: Detection.

    7.1. The antagonist is prepared at 10X the desired final concentration, in 11-point dilution series, similar to that described for MCH (6-17) in Steps 1 and 2. However, an appropriate top dose and dilution scheme (e.g. a 1:3 dilution scheme) must be determined empirically. No antagonist is added to any wells in Column 12, as these are the negative control wells.

    7.2. Remove the assay plate from the incubator and place it in the tissue culture hood.

    7.3. Transfer 10 µL from each well of the antagonist dilution series from the master dilution plate to the appropriate wells of the assay plate using a multichannel pipette.

    7.4. Incubate the assay plate at 37°C and 5% CO_2 for 30-60 minutes in a humidified incubator. The optimal incubation time for your antagonist must be determined empirically.

8. Prepare the agonist challenge for the antagonist dilution series at 10X the desired final concentration (e.g. at EC_{80} of the agonist).

9. Remove the assay plate from the incubator and place it in the tissue culture hood.

10. Transfer the 10X agonist challenge into a 25 mL reagent reservoir.

11. Transfer 10 µL of the 10X agonist challenge prepared in Step 8, to the appropriate wells (i.e. wells with antagonist dilution series) of the assay plate using a multichannel pipette.

12. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 1.5 hours.

Day 2: Ligand-Neutralizing Sample Preparation

The following protocol is used for quantitation of anti-ligand samples (e.g. antibodies or Fc-fusion proteins, etc.). The ligand is pre-incubated with the neutralizing sample for 15-30 minutes. However, the optimal incubation time should be determined empirically before addition to the assay.

1. Prepare the anti-ligand antibody dilution series at 10X the desired final concentration, in a new row of the master dilution plate.

2. Prepare the agonist challenge at 10X the desired final concentration, similar to that described in Step 8 in Sample Preparation.

3. For each dose-response curve, add 45 µL of the agonist challenge to the appropriate row of a pre-mixing plate (e.g. a V-bottom polypropylene 96-well plate). Transfer 45 µL of the 10X anti-ligand antibody dilution series to the appropriate row of the pre-mixing plate already containing 45 µL of the agonist challenge. Mix by gently pipetting up and down several times.

4. Incubate the plate at room temperature for 15-30 minutes. The optimal pre-incubation time for each antibody should be determined empirically.

5. Transfer 20 µL of the 10X anti-ligand antibody and 10X agonist mixture to the appropriate wells of the assay plate.

6. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 1.5 hours.

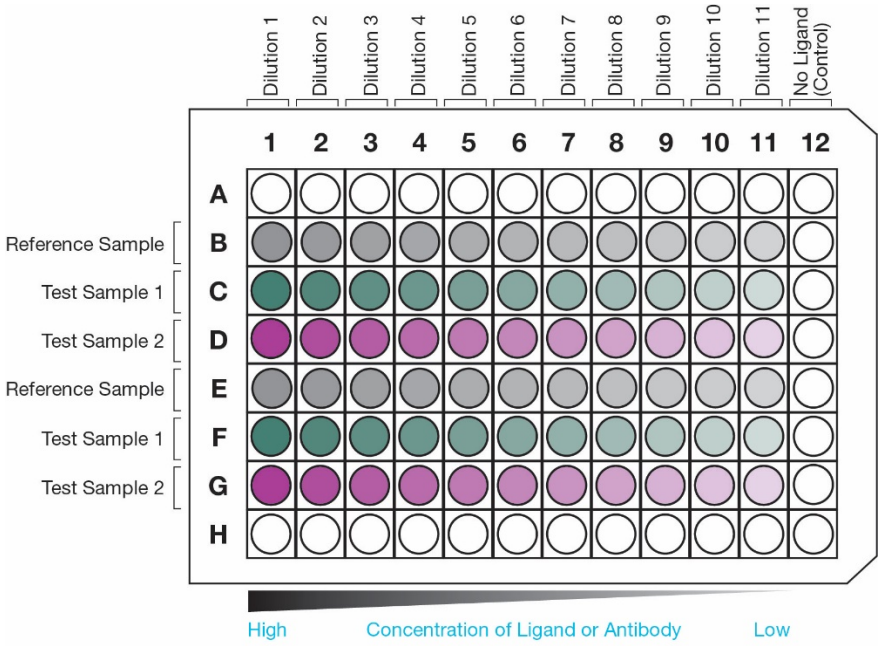


Figure 3. Representative Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with two replicates per dose point, for two test samples and one reference sample tested using the same dilution scheme.

Day 2: Detection

The following steps for adding detection reagents are applicable when testing purified biologics. For crude biologic samples, gently removing the liquid from all wells of the assay plate and replacing it with 100 µL of the Cell Plating Reagent before the addition of detection reagents can result in a higher signal. Additional Cell Plating Reagent will be required for this method.

1. Add 10 µL of the Detection Reagent 1 to each well of the assay plate.
2. Incubate the plate at room temperature for 15 minutes in the dark.

   Room temperature refers to a range of 23-25°C

3. Add 40 µL of the 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 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. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

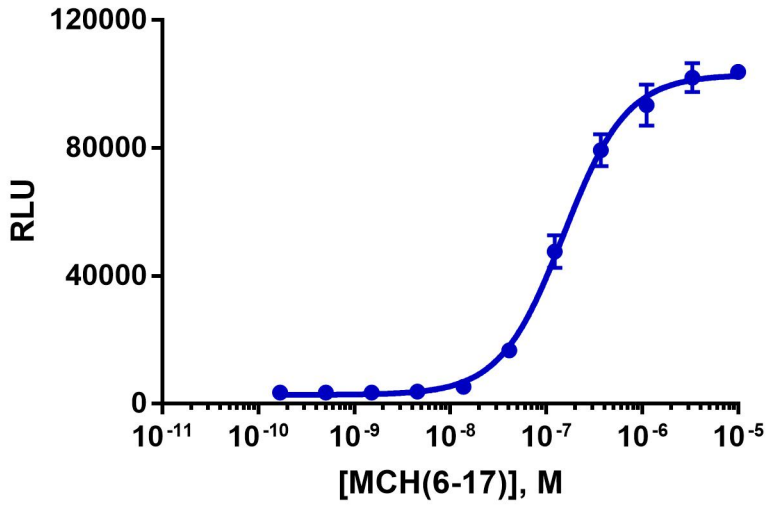
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel.

Typical Results

The following graph is an example of a typical dose-response curve for the MCHR1 Bioassay generated using the protocol outlined in this manual. The data shows potent, dose-dependent recruitment with β-arrestin, when treated with MCH (6-17).

The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.

A



B

EC_{50} (nM)	151
S/B	27.3

Figure 4. Typical Results: Representative **A,** dose-response curve and **B,** the EC_{50} and assay window for β-arrestin recruitment as a result of MCH (6-17)-mediated activation of MCHR1, as measured in this bioassay.

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 ligand used or improper ligand incubation time	Refer to the Certificate of Analysis for recommended ligand and assay conditions.
	Incorrect preparation of the ligand (agonist or antagonist)	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of the ligand.
	Sub-optimal time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased or no response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using a microscope.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
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 provided	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 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	Ensure that the 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.
	Problems with dynamic range or dilutions	Changing tips during dilution can help in avoiding carryover.
High variability between replicates	Instrument calibration	Ensure that the dispensing equipment is properly calibrated, and proper pipetting technique is used.

For questions on using this product, please contact Technical Support at 1.866.448.4864 or DRX_SupportUS@eurofinsUS.com

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