User Manual

PathHunter® CHO-K1 MRGPRX2 Bioassay Kit

For Detection of β-Arrestin Recruitment by Ligand-Activated MRGPRX2

For Bioassay Kits:

2-Plate Kit: 93-0309Y2-00143

10-Plate Kit: 93-0309Y2-00144

Document Number 70-419 Revision 0

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

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

Overview

The PathHunter MRGPRX2 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 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 format.

Assay Principle

This bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, the ProLink™ (PK), and the Enzyme Acceptor (EA). Independently, these fragments have no β-gal enzymatic activity; however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

The PathHunter MRGPRX2 Bioassay monitors MRGPRX2 (GPCR) activity by detecting β-Arrestin2 recruitment by this receptor as a result of ligand-mediated receptor activation. In this system, the MRGPRX2 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 MRGPRX2 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.

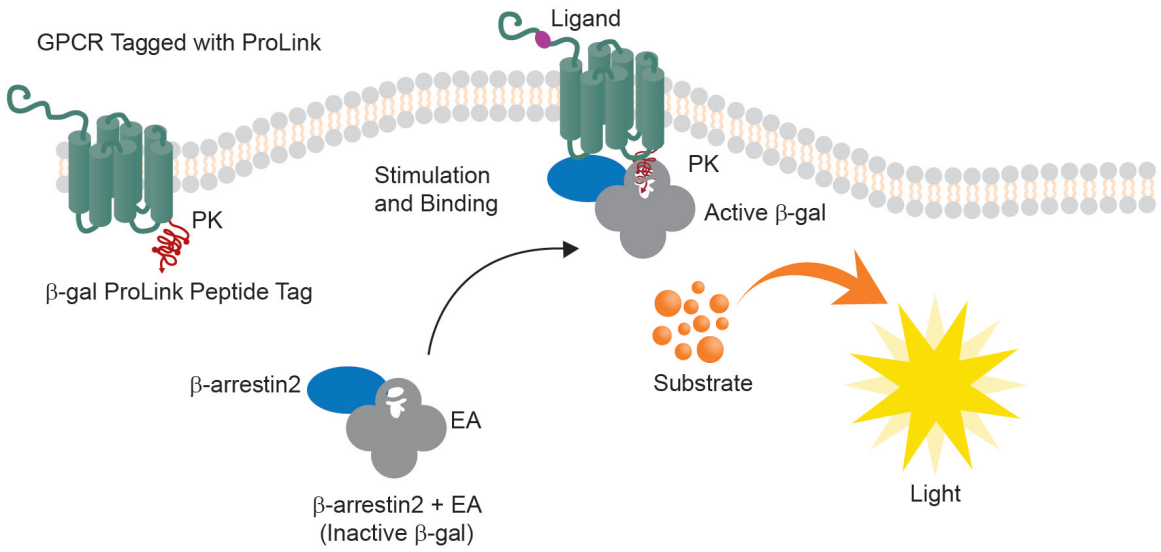


Figure 1. Assay Principle Ligand-mediated activation of PK-tagged MRGPRX2 leads to the recruitment of β-arrestin2, which is tagged with EA. The resulting complementation of the two β-gal fragments forms an active enzyme, which hydrolyzes the substrate and generates a chemiluminescent signal.

Materials Provided

List of Components	93-0309Y2-00143	93-0309Y2-00144
PathHunter CHO-K1 MRGPRX2 Bioassay Cells (0.6×10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 2 Reagent* (100 mL per bottle)	1	3
Cortistatin-14 (0.5 mg per vial)	1	2
PathHunter Detection Kit (mL per bottle)		
Cell Assay Buffer	1 x 11.4	1 x 57
Substrate Reagent 1	1 x 3	1 x 15
Substrate Reagent 2	1 x 0.6	1 x 3
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

*Cell Plating 2 Reagent is used for diluting the control agonist and antagonists in this bioassay.

Storage Conditions

PathHunter CHO-K1 MRGPRX2 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 2 Reagent (CP2)

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 in 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, 30 mL of reagent per aliquot can be dispensed and frozen down.

Cortistatin-14

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 Detection Kit

Upon receipt, store reagents at -20°C. The detection kit is stable until the expiration date listed on the outer label of the kit box. Thaw reagents at room temperature before use. Once thawed, the reagents can be stored for up to 1 month at 2-8°C. For long-term storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be re-frozen in opaque containers at -20°C until needed. Avoid multiple freeze-thaw cycles.

For the ten-plate kit: If all the plates will not be used at the same time, we recommend making five aliquots for the Cell Assay Buffer and Substrate Reagent 1. Each aliquot will be adequate for two assay plates. Make five aliquots of 11.4 mL each for Cell Assay Buffer, and five aliquots of 3 mL each for Substrate Reagent 1. Sufficient reagent volumes are provided in the kit to make these aliquots. We do not recommend making aliquots of Substrate Reagent 2, as it does not freeze well and results in low reagent recovery while pipetting.

96-Well Tissue Culture-Treated Plates

Upon receipt, store at room temperature.

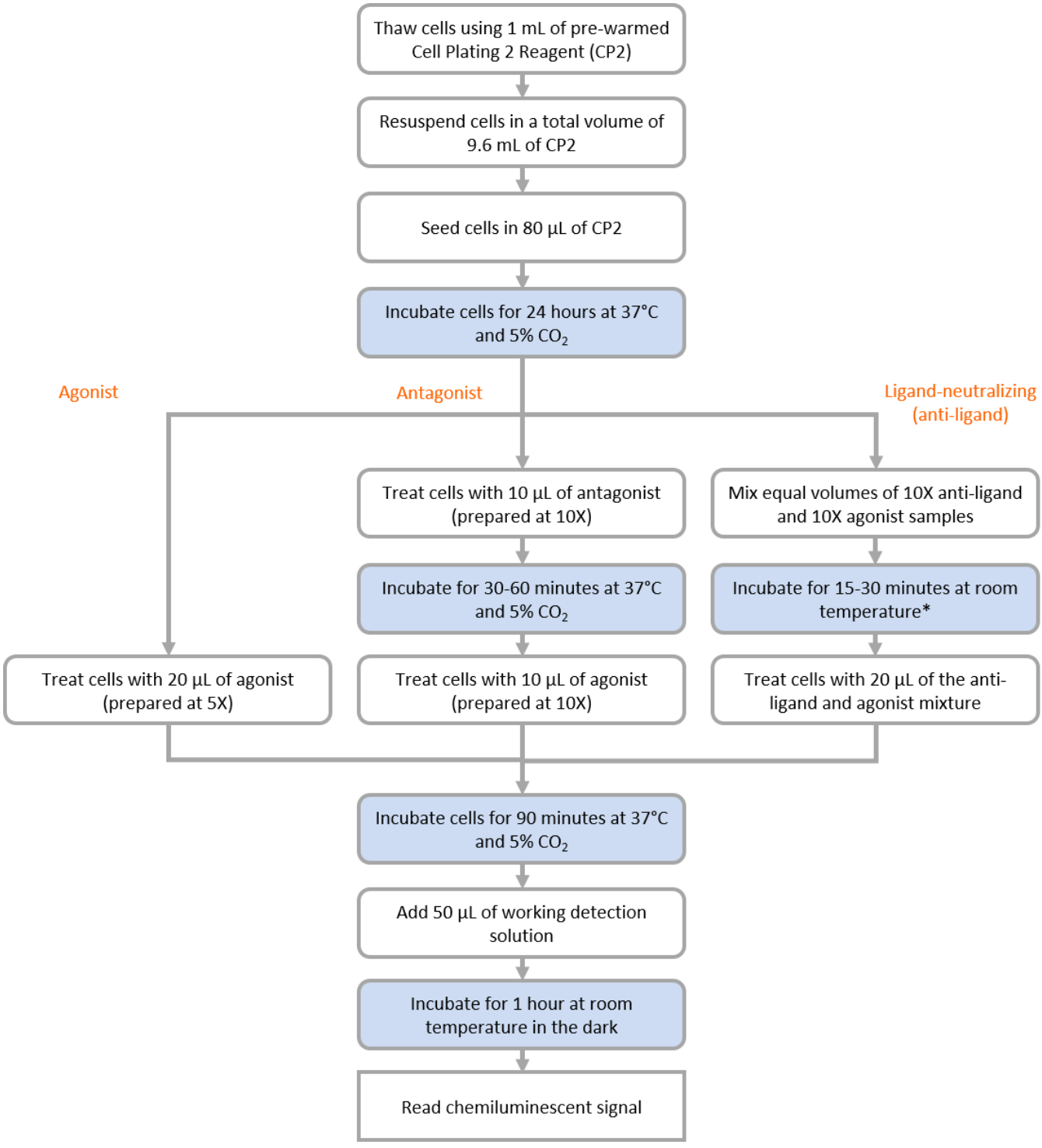
Additional Materials Required

The following equipment and additional materials are required to perform this assay:

Materials	**Ordering Information**
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettors and pipette tips (10 µL-1000 µL)	
Polypropylene tubes (50 mL and 15 mL)	
Microcentrifuge tubes (1.5 mL)	

Protocol Schematic

Quick-Start Procedure: In a 96-well tissue culture treated 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 the cryopreserved PathHunter CHO-K1 MRGPRX2 Bioassay Cells from cryovials.

1. Prior to thawing the cells, ensure that all the required materials are set up in the tissue culture hood. These include:
    a. One sterile 25 mL reagent reservoir
    b. One sterile 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 AssayComplete™ Cell Plating 2 Reagent (CP2), 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 9.6 mL of CP2 into the 15 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 CP2 from the 15 mL conical tube, to the cryovial to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet up and down several times to uniformly suspend the cells.
5. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP2. Remove all the suspension from the cryovial tube to ensure maximum recovery of all the cells.
6. Replace the cap on the conical tube and mix by gentle inversion several times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension. Pour it immediately into the 25 mL 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, to minimize potential edge effects.
8. Incubate the assay plate at 37°C and 5% CO_2 for 24 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 determined empirically.

A 1:3 serial dilution for the control agonist, Cortistatin-14, has been used in this protocol, as shown in Figure 2. 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 CP2 to Wells A2 to A12 of the master dilution plate.
2. Prepare the control agonist (Cortistatin-14) dose-response curve:
   Cortistatin-14 will serve as a positive control in this assay. The agonist is prepared at 5X the desired final concentration as it will be diluted 5-fold by adding 20 µL to 80 µL of the medium present in the assay plate.
   - 2.1. Add 290 µL of the supplied Reconstitution Buffer to the vial containing 0.5 mg of the lyophilized Cortistatin-14 powder to make a 1 mM stock solution. Mix by slowly pipetting up and down several times.
   - 2.2. Make a 500 µM intermediate stock solution of Cortistatin-14 by adding 25 µL of the 1 mM stock solution prepared in Step 2.1, into 25 µL of CP2, in a sterile microcentrifuge tube.
   - 2.3. Add 180 µL of CP2 to Well A1 of the master dilution plate. Transfer 20 µL of the 500 µM Cortistatin-14 stock prepared in Step 2.2 to Well A1 and mix well by pipetting up and down several times. This represents a 5X stock of the top screening dose of 50 µM.
   - 2.4. Using a clean pipette tip, transfer 40 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times.
   - 2.5. Replace the pipette tip and transfer 40 µL from Well A2 to Well A3, and mix well. Repeat this process until Well A11 is reached, resulting in an 11-point, 1:3 dilution series. No sample is transferred to any Well A12, as this will serve as a negative control.
3. Prepare any agonist test samples in a similar manner.
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 (or prepared test samples) from the master dilution plate 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 90 minutes.

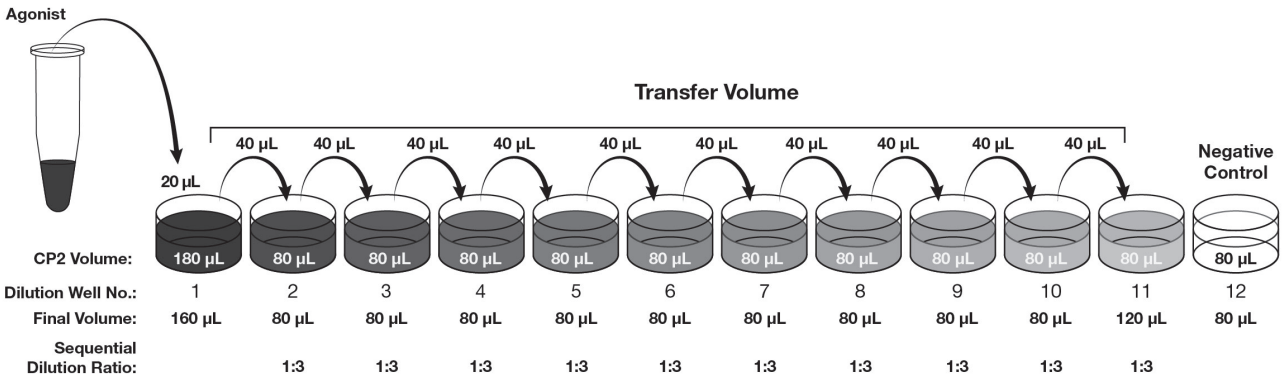


Figure 2. Agonist Serial Dilutions: Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

7. Prepare the antagonist dilution series. If antagonist samples are not being tested, then skip Steps 7-15 and proceed to Day 2: Ligand-Neutralizing Sample Preparation or Day 2: Detection. The antagonist is prepared at 10X the desired final concentration, in 11-point dilution series, similar to that described for Cortistatin-14 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.
8. Remove the assay plate from the incubator and place it in the tissue culture hood.
9. 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.
10. 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.
11. Prepare the agonist challenge for the antagonist dilution series. The agonist challenge is prepared at 10X the desired final concentration (e.g. at EC_{80} of the agonist).
12. Remove the assay plate from the incubator and place it in the tissue culture hood.
13. Transfer the 10X agonist challenge into a 25 mL reagent reservoir.
14. Transfer 10 µL of the 10X agonist challenge prepared in Step 11, to the appropriate wells (i.e. wells with antagonist dilution series) of the assay plate using a multichannel pipette.
15. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 90 minutes.

Day 2: Ligand-Neutralizing Sample Preparation

This 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 for your sample 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 11 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 slowly 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 90 minutes.

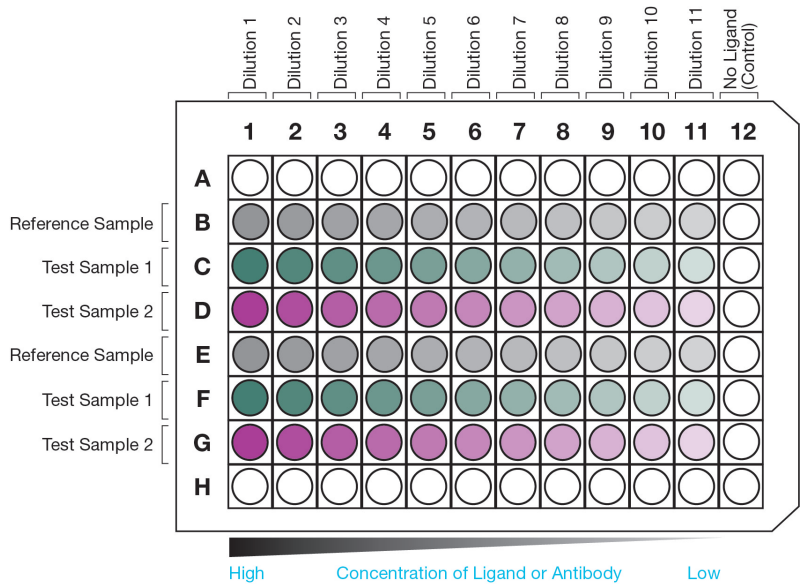


Figure 3. Representative Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with two replicates per dose point, for two test samples and one reference sample tested using the same dilution scheme.

Day 2: Detection

1. Prepare a working detection solution by mixing the three detection reagent components in a 15 mL conical tube. Refer to the table below for the volume ratio.

Working Detection Solution		
Component	**Ratio**	**Volume per Plate (mL)**
Cell Assay Buffer	19	5.7
Substrate Reagent 1	5	1.5
Substrate Reagent 2	1	0.3
Total Volume		**7.5**

2. Replace the cap of the tube and mix by gentle inversion. Transfer 50 µL (50% of the assay volume) of the working detection solution to the appropriate wells of the assay plate.
3. Incubate the assay plate for 1 hour at room temperature in the dark.


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

4. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

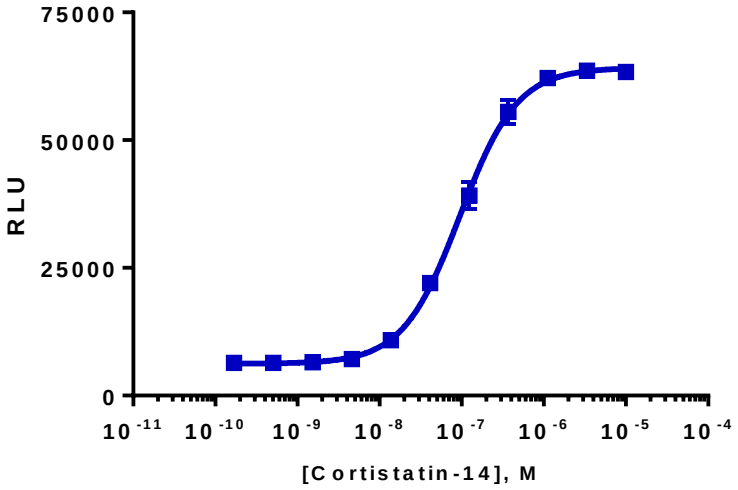
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel etc.

Typical Results

The following graph is an example of a typical dose response curve for the MRGPRX2 Bioassay generated using the protocol outlined in this manual. The data shows potent, dose-dependent recruitment of β-arrestin, when treated with Cortistatin-14.

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

A



B

EC_{50} (nM)	95
S/B	10.8

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 Cortistatin-14-mediated activation of MRGPRX2, 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 datasheet for recommended ligand and assay conditions.
	Incorrect preparation of ligand (agonist or antagonist)	Refer to the vendor-specific datasheet to ensure proper handling, dilution, and storage of ligand.
	Sub-optimal time course for induction	Optimize time course of induction with agonist and antagonist.
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	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) Sub-optimal agonist challenge concentration	Some ligands are difficult to handle. Confirm the final concentration of ligands. Perform agonist curve to reassess EC_{80} with the ligand provided in the kit. Perform antibody titrations with EC_{80} and EC_{90} agonist challenge concentrations to re-optimize the assay window.
EC_{50} is right-shifted	Improper ligand handling or storage	Check ligand handling requirements
	Difference in agonist binding affinity	Refer to the Certificate of Analysis for the ligand provided in the kit to confirm that the ligand used is comparable.
	Problems with plate type and compound stability	Hydrophobic compounds should be tested for solubility and may require evaluation in different dilution buffers.
		Non-binding surface plates may be necessary for hydrophobic compounds.

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

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