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User Manual

PathHunter® CHO-K1 CNR1 Bioassay Kit

For Detection of β-Arrestin Recruitment by Ligand-Activated CNR1

Document Number: 70-404 Revision 0

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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.

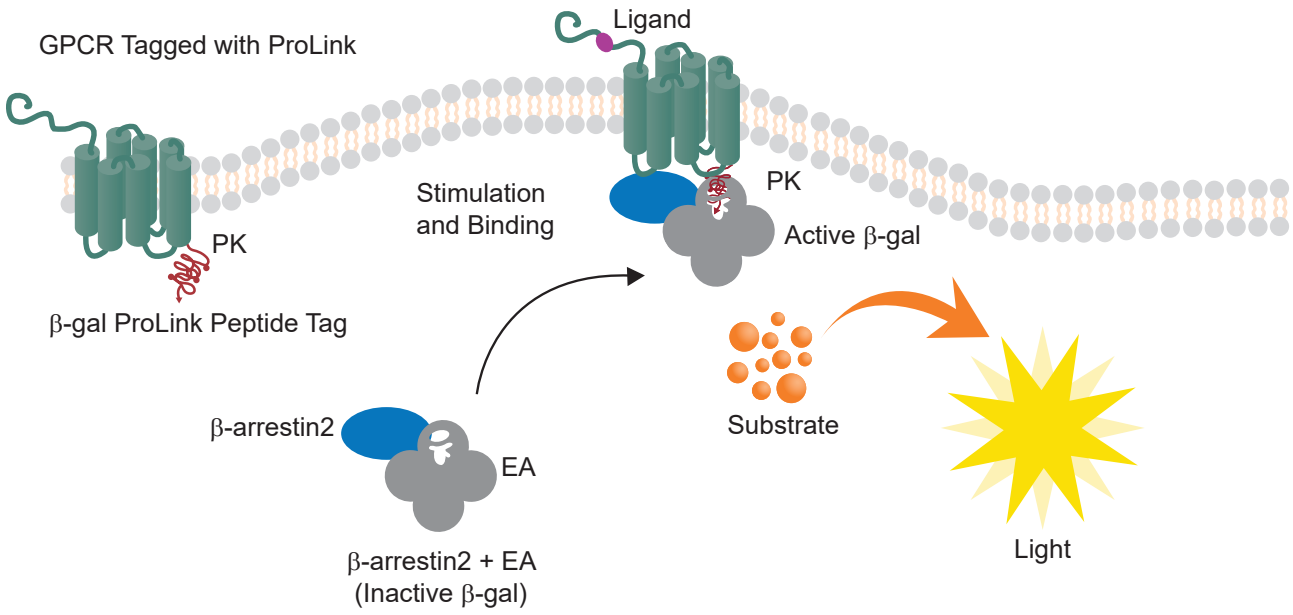
Overview

The PathHunter CNR1 Bioassay kit provides a functional, robust, highly sensitive, and easy-to-use-cell based assay to study drug potency and 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 cryopreserved cells have been manufactured for single-use, which are provided in a convenient ready-to-assay format that saves time. 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 CNR1 Bioassay monitors GPCR activity by detecting β-Arrestin2 recruitment by the ligand-activated CNR1. In this system, the GPCR is fused, in frame, to the small 42 amino acid fragment of β-gal, PK and co-expressed in cells stably expressing a fusion protein of β-Arrestin2 with the larger, N-terminal deletion mutant of β-gal, EA. Activation of the GPCR stimulates the recruitment of β-Arrestin2 by the ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β-gal enzyme. This action leads to an increase in enzyme 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.



Materials Provided

List of Components	93-0959Y2-00119	93-0959Y2-00120
PathHunter CHO-K1 CNR1 Bioassay Cells (0.6 x 10^6 cells in 0.1 mL per vial)	2	10
PathHunter Bioassay Detection Kit (No. of datapoints) Detection Reagent 1 (Bottle) Detection Reagent 2 (Bottle)	200 1 x 3 mL 1 x 12 mL	1000 1 x 15 mL 1 x 60 mL
AssayComplete™ Cell Plating 2 Reagent* (100 mL per bottle)	1	2
CP 55,940 (5 mg per vial)	1	1
96-well White, Clear Flat-Bottom, TC Treated, Sterile Plates with Lid	2	10

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

Storage Conditions

PathHunter CHO-K1 CNR1 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.

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 long-term 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.

For the ten-plate kit: If all the plates will not be used 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 reagent volumes are provided in the kit to make these aliquots.

If the reagents will be used for a single plate, then the remaining Detection Reagents can be frozen. The detection reagents can be thawed and frozen for a total of three times without loss in performance.

AssayComplete™ Cell Plating 2 Reagent (CP2)

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.

CP 55,940 Control Agonist

Store at -20°C, until ready to use (up to the expiration 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.

96-Well Tissue Culture Treated Plates

Store at room temperature.

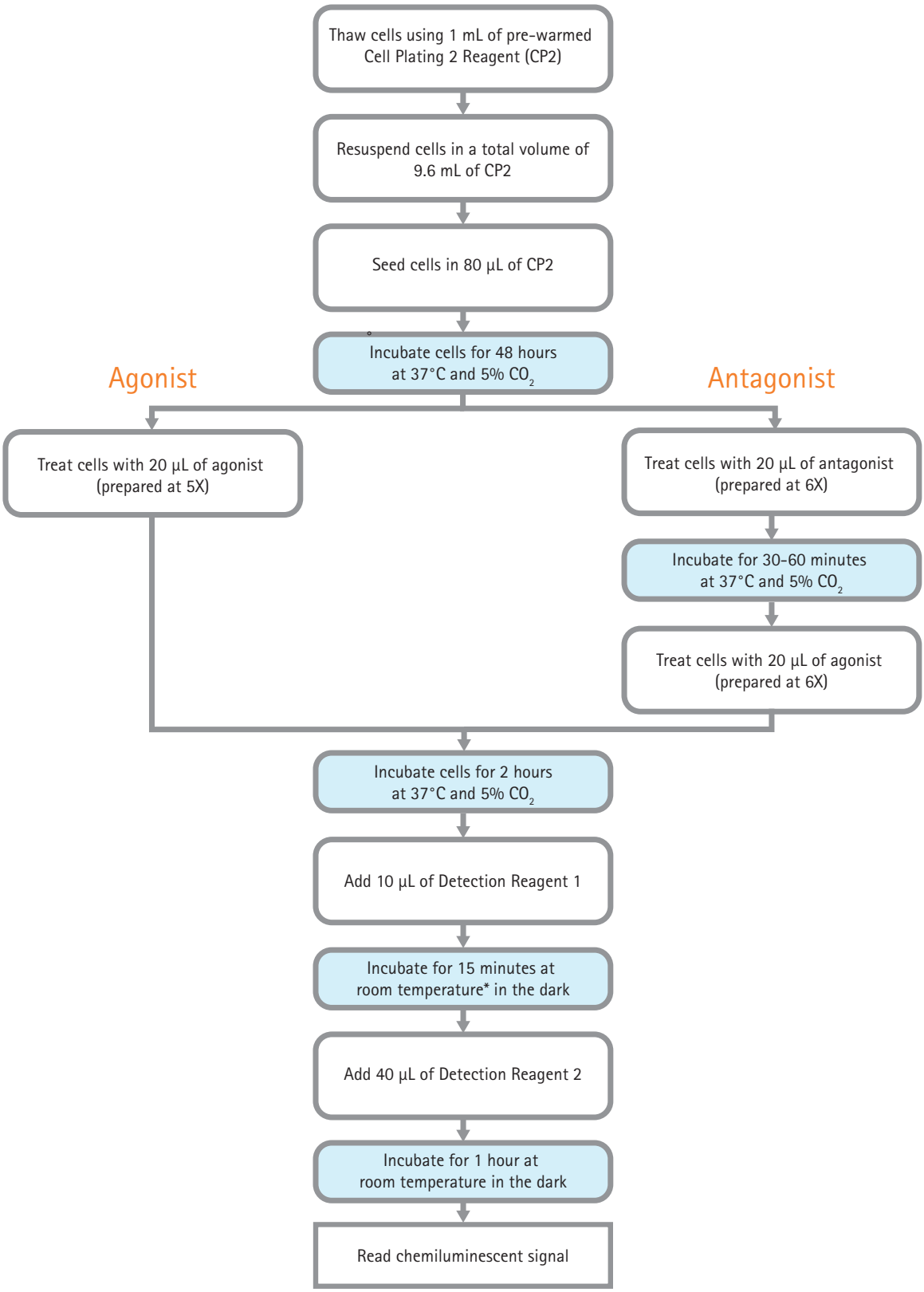
Additional Materials Required

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

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
DMSO (for compound reconstitution and intermediate dilutions)	
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettes and pipette tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	

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

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter CHO-K1 CNR1 Bioassay cells from cryovials.

1. Before thawing the cells, ensure that all the necessary materials required are set up in the tissue culture hood. These include:
    a. One 25 mL reagent reservoir
    b. One 15 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 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)
2. Dispense 9.6 mL of CP2 into the 15 mL conical tube.
3. Remove the cryovial from liquid nitrogen and immediately place in dry ice.

DO NOT use a heated water bath to thaw the vial. Hold the cryovial 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. Gently pipet up and down several times to uniformly resuspend 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 to ensure maximum recovery of all the cells.
6. Gently invert the conical tube several times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension, and immediately pour the suspension 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 48 hours before proceeding with the assay.

Day 3: 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, CP 55,940, has been used in this protocol. The volumes listed below are designed for running samples from one dose-response curve in triplicate on the assay plate (Refer to the Representative Plate Map).

1. Add 200 µL of Cell Plating 2 Reagent (CP2) to Wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX, Cat. No. 92-0011 or similar).

2. Prepare the control agonist (CP 55,940) dose-response curve:
   CP 55,940 will serve as a positive control in this assay. The agonist is prepared at 5X the desired concentration, as it will be diluted by adding 20 µL to 80 µL of the medium present in the assay plate.

   2.1 Add 1.3 mL of the supplied reconstitution reagent (DMSO) to the vial containing 5 mg of lyophilized CP 55,940 powder, to make a 10 mM stock solution. Gently pipet up and down several times.

   2.2 Make a 100 µM intermediate stock solution of CP 55,940 by adding 10 µL of the 10 mM solution prepared in Step 2.1, into 990 µL of DMSO in a sterile microcentrifuge tube. Mix well.

   2.3 Add 190 µL of CP2 to Well A1 of the master dilution plate. Add 10 µL of the intermediate stock solution to well A1. Mix thoroughly by pipetting up and down several times. This results in a 5 µM solution (5X the final 1 µM highest dose).

   2.4 Using a clean pipette tip, transfer 100 µL from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times. Replace the pipette tip and transfer 100 µL from well A2 into Well A3, and mix well. Repeat this process until Well A11, resulting in an eleven-point, 1:3 dilution series. No sample is transferred Well A12 of the dilution plate, as this will serve as a negative control.

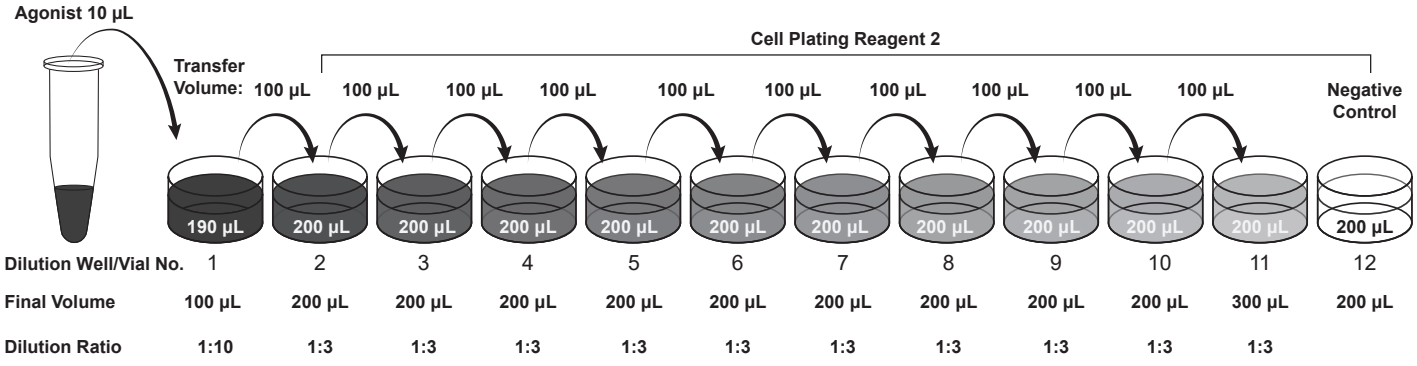
The agonist dilutions should be added to the cells immediately. Leaving the agonist dilutions on the benchtop for prolonged periods of time may cause them to stick to the plastic of the plate, thus introducing variability in the assay.

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 from the master dilution plate to the appropriate wells of the assay plate using a multichannel pipette.

6. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 2 hours.



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

7. Prepare the antagonist dilution series:

   7.1 The antagonist is prepared at 6X the desired final concentration, in 11-point dilution series, similar to that described for CP 55,940 in Steps 1 and 2. However, an appropriate top dose and dilution scheme (e.g. a 1:3 dilution scheme) must be determined empirically. Note: No antagonist is added to any wells in column 12, as these will be the negative control wells.

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

   7.3 Transfer 20 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 antagonist dilution series:
The EC_{80} of the supplied agonist, CP 55,940, was determined to be approximately 22 nM. If CP 55,940 from a different vendor is used, the EC_{80} should be determined empirically prior to testing samples. Prepare the agonist challenge at 6X the desired concentration.

The following steps provide an adequate amount of the agonist challenge for two assay plates:

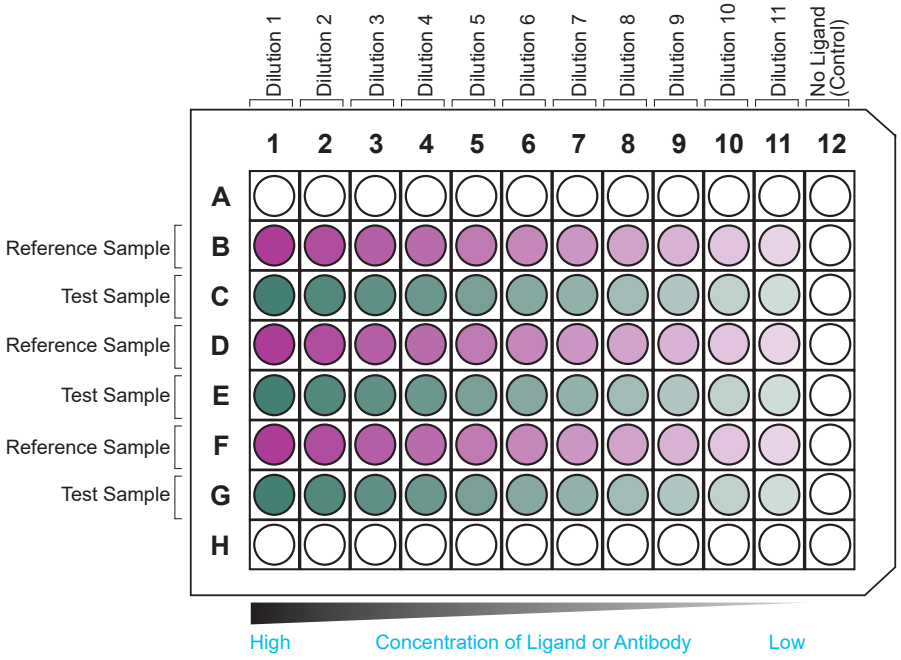
8.1 Prepare a 132 nM stock solution of the CP 55,940 in CP2 containing 5% DMSO: Transfer 5.3 µL of the 100 µM intermediate solution prepared in Step 2.2, into 3,994.7 µL of CP2 containing 5% DMSO. The final DMSO concentration in the 6X agonist challenge stock will be ~5%.

8.2 Transfer the 6X agonist challenge into a 25 mL reagent reservoir. Transfer 20 µL of the 6X 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.

9. Transfer 20 µL of the 6X 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.

The agonist challenge should be added to the cells immediately after preparation. Do not allow the diluted agonist to sit on the benchtop prior to use.

10. Incubate the assay plate at 37°C and 5% CO_2 in a humidified incubator for 2 hours.



Assay Plate Map: This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test and reference sample tested with their respective dilution schemes.

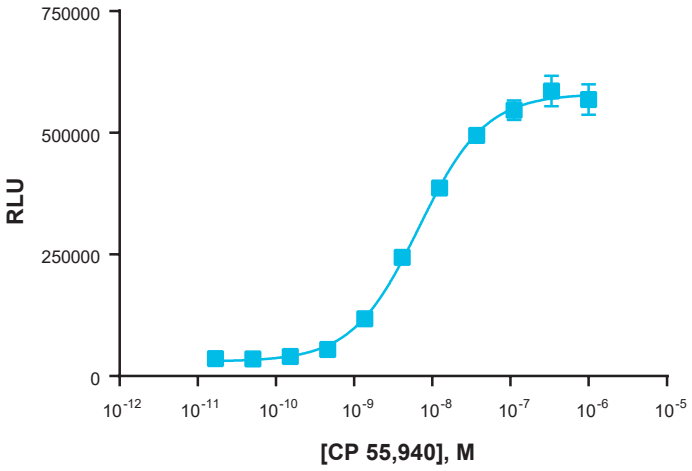
Day 3: Detection

1. 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. 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 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 imager.

Note: For crude biologic samples, gently removing the liquid from all wells of the assay plate and replacing with 100 µL of Cell Plating Reagent, before the addition of the detection reagents, can result in a higher signal. Additional Cell Plating Reagent will be required for this method.

Typical Results

The following graph is an example of a typical dose-response curve for the CNR1 Bioassay generated using the protocol outlined in this user manual. The data shows potent, dose-dependent recruitment of β-arrestin, when treated with CP 55,940.



EC_{50} (nM)	16.6
S/B	6.7

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or improper ligand incubation time	See datasheet for recommended ligand and assay conditions.
	Incorrect preparation of ligand (agonist or antagonist)	Refer to 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	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 assay at correct incubation temperature as indicated on the assay datasheet.
	Incorrect preparation of ligand (agonist or antagonist) Suboptimal 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 technical questions about this product, please contact technical support using the information listed at the bottom of this page.

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