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User Manual

PathHunter® CHO-K1 BDKRB2 Bioassay Kit

For Chemiluminescent Detection of β-Arrestin Recruitment

Document Number: 70-405 Revision 0

Table of Contents

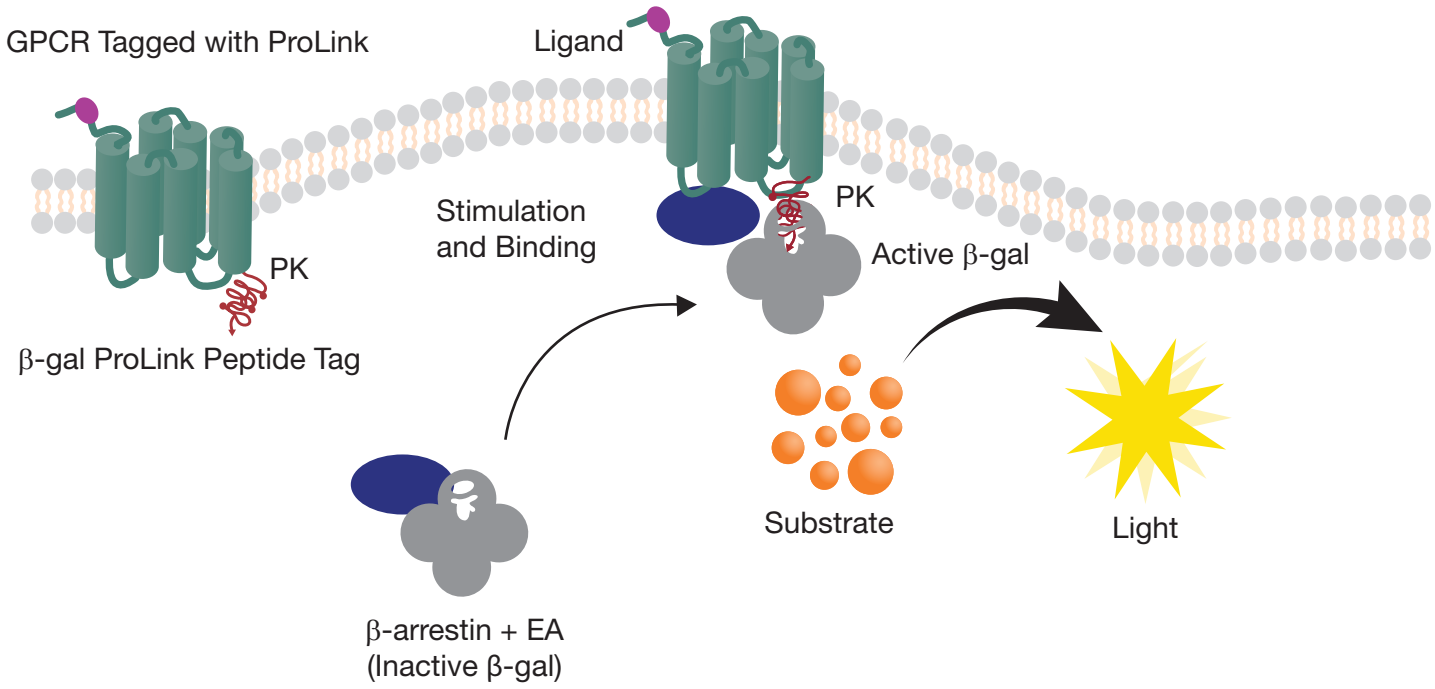
Please read this entire user manual before proceeding with the assay.
For additional information or Technical Support, see contact information below.

Overview

The PathHunter BDKRB2 Bioassay kit is a ready-to-use kit that contains all reagents necessary to perform a functional assay with live cells, without any continuous cell culture. This kit has been designed to rapidly detect the potency of your biologic or the presence of neutralizing antibodies against the biologic. Each Bioassay kit includes single use vials of frozen cells expressing the BDKRB2 receptor, chemiluminescent detection reagents, control agonist, optimized cell plating reagent / dilution buffer and plates. Simply thaw and plate the pre-validated cells and treat with test reagents within 48 hours. Assays have been designed for 96-well formats.

Technology Principle

The PathHunter BDKRB2 Bioassay monitors GPCR activity by quantifying the interaction of β-Arrestin 2 with ligand-activated BDKRB2 using β-galactosidase (β-gal) enzyme fragment complementation (EFC). In this system, BDKRB2 is fused in frame with the small 42 amino acid fragment of β-gal called ProLink™ and co-expressed in cells stably expressing a fusion protein of β-Arrestin 2 with the larger, N-terminal deletion mutant of β-gal (called enzyme acceptor or EA). Activation of BDKRB2 stimulates binding of β-Arrestin 2 to 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 chemiluminescent PathHunter Detection Reagent. Because 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	93-0189Y2-00113	93-0189Y2-00114
PathHunter CHO-K1 BDKRB2 Bioassay Cells (No. of Vials)	2	10
PathHunter Detection Kit (No. of datapoints)	200	1000
Cell Assay Buffer (mL)	11.4	57
Substrate Reagent 1 (mL)	3	15
Substrate Reagent 2 (mL)	0.6	3
AssayComplete™ Cell Plating 2 Reagent*	1 X 100 mL	2 X 100 mL
Bradykinin	1 vial	1 vial
96-well White, Clear-Bottom, TC Treated, Sterile Plates with Lid	2 plates	10 plates

* Cell Plating 2 Reagent is also used for diluting control agonist in the bioassay.

Storage Conditions

PathHunter CHO-K1 BDKRB2 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 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 (less than 24 hours): 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. 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 Detection

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 List of Components), 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.

AssayComplete Cell Plating 2 Reagent (CP2)

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 List of Components), 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.

Bradykinin Control Ligand

Store at -20°C until ready to use (up to the expiration date listed in the kit's List of Components). Centrifuge the vial prior to opening to maximize recovery.

96-Well Tissue Culture Treated Plates

Store at room temperature.

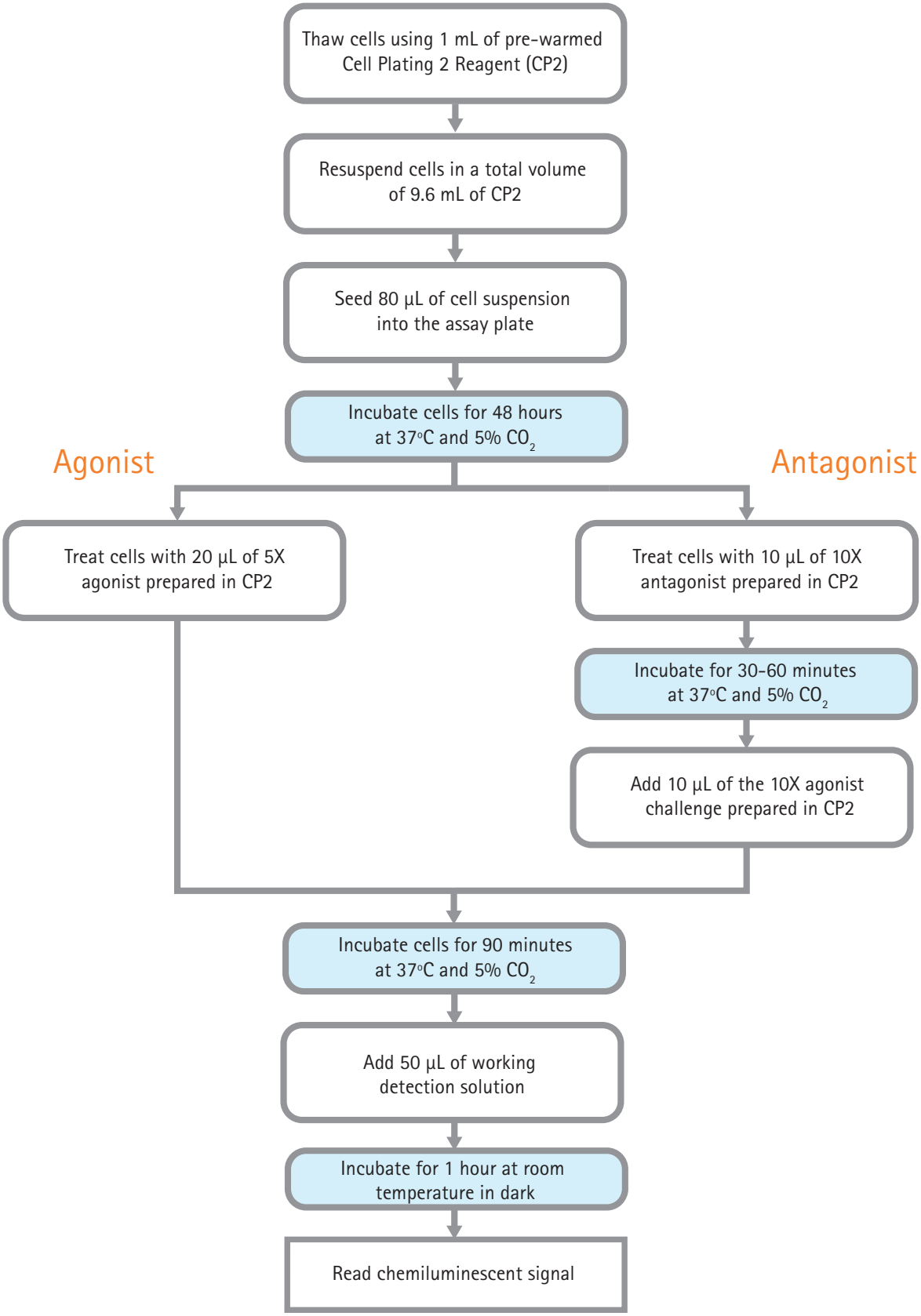
Additional Materials Required

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

Materials	Ordering Information
V-Bottom 96-well ligand dilution plates	92-0011
Multimode or Luminescence 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 micro-pipettors 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.



Detailed Protocol

Day 1: Bioassay Cell Preparation

The following protocol is for thawing and plating frozen PathHunter BDKRB2 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 pipette set to dispense 1 mL
    d. A multichannel pipette and tips set to dispense 80 µL
    e. A bottle of Cell Plating 2 Reagent (CP2), pre-warmed in a 37˚C water bath for 15 minutes
    f. A 96-well white-walled, clear flat-bottom tissue culture-treated sterile assay plate (provided with the kit)
    g. A ligand dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX 92-0011 or similar); labeled as 'master' dilutions
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 heated water bath to thaw the vial. Wipe down the outside surface of the cryovial quickly with 70% ethanol, and immediately bring it into the tissue culture hood. Hold the cryovials at the cap, DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet.

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 side of the wall of the cryovial tube. Mix the cells gently by pipetting 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 medium/suspension in the tube to ensure maximum recovery of all the cells from the vial.
6. Gently invert the 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 reagent reservoir.
7. Add 80 µL of cells to each well of the 96 well assay plate using the multichannel pipette.
8. Replace 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.
9. Gently place the assay plate in a tissue culture incubator set to 37˚C and 5% CO_2 for 48 hours before proceeding with the assay.

Day 2: Sample Preparation and Testing

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 adversely impacting assay performance. In some assays, standard curves of control ligand or antibody 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.

1. Prepare the reference agonist (Bradykinin) dose response curve, which will serve as a positive control in this assay. Agonist is prepared at 5X the desired final concentration as it will be diluted by adding to the 80 µL of medium present in the assay plate.

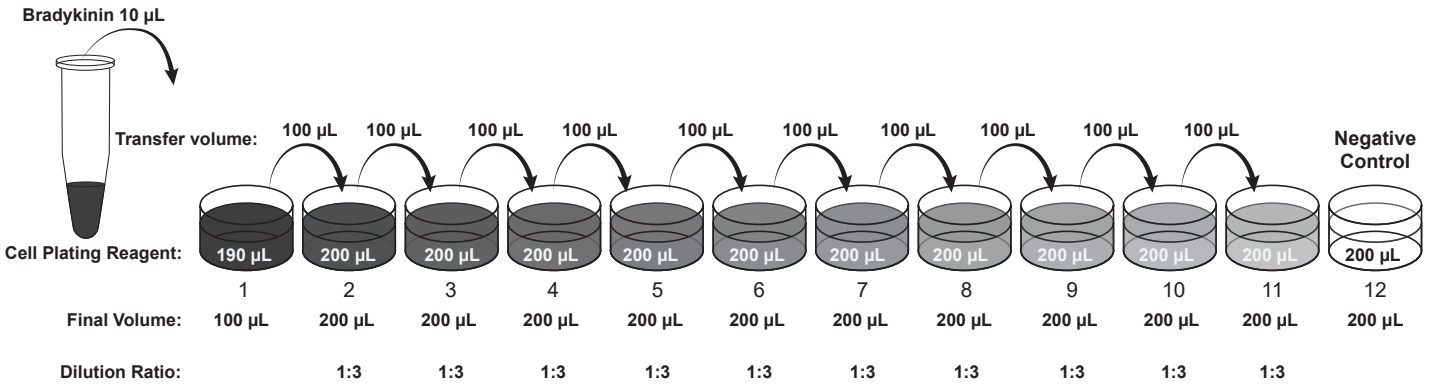
    a. Add 472 µL of the supplied reconstitution buffer to the vial containing 5 mg of bradykinin (lyophilized powder) to make a 10 mM stock solution. Make suitable aliquots (e.g. 30 µL) and store at -20°C until needed.

    b. In sterile 1.5 mL microcentrifuge tube, prepare a 1:100-fold intermediate dilution (100 µM bradykinin): to 990 µL of CP2, add 10 µL of 10 mM bradykinin stock.

    c. Add 200 µL of CP2 to wells A2 to A12 of the master dilution plate.

    d. Prepare a 1:20 dilution of the 100 µM bradykinin stock solution in CP2 to generate the top 5X concentration of 5 µM. Add 190 µL of CP2 to well A1 in master dilution plate. Add 10 µL of 100 µM bradykinin stock. Mix gently by pipetting up and down several times.

    e. Prepare a 1:3 dilution series in the master dilution plate. Using a clean tip, transfer 100 µL from well A1 into well A2. Mix thoroughly 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. Prepare agonist test samples in a similar manner.

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

4. Add 20 µL from each well of the ligand curve on the master dilution plate to the appropriate wells of the assay plate.

5. Place the assay plate in the 37°C and 5% CO_2 incubator and incubate for 90 minutes.

For antagonist assay format:

1. Prepare the antagonist dilutions in CP2, as 10X stocks on a master dilution plate, using appropriate dilution series (e.g. 1:3).

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

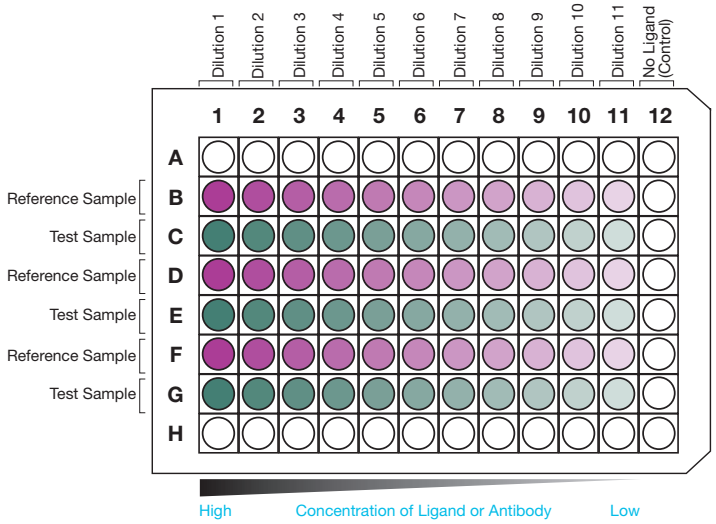
3. Add 10 µL from each well of the antagonist curve on the master dilution plate to the appropriate wells of the assay plate.
4. Place the assay plate in the 37°C, 5% CO_2 incubator and incubate for 30-60 minutes.
5. Prepare the agonist challenge at 10X the desired final concentration (e.g. typically the EC_{80}).
6. Add 10 µL of the 10X agonist challenge to the appropriate wells in the assay plate.
7. Optional: add 20 µL from the agonist reference curve on the master dilution plate (wells A1-A12) to the appropriate wells in the assay plate.
8. Place the assay plate in the 37°C and 5% CO_2 incubator and incubate for 90 minutes.

Day 2: Detection

1. Prepare working detection solution by mixing the three detection reagent components in a 15 mL conical tube as shown in the table below. Mix by gently inverting the tube and store the solutions in dark at room temperature prior to use. The Working Detection Solution is stable for at least 24 hours at room temperature with no adverse impact on assay performance.

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

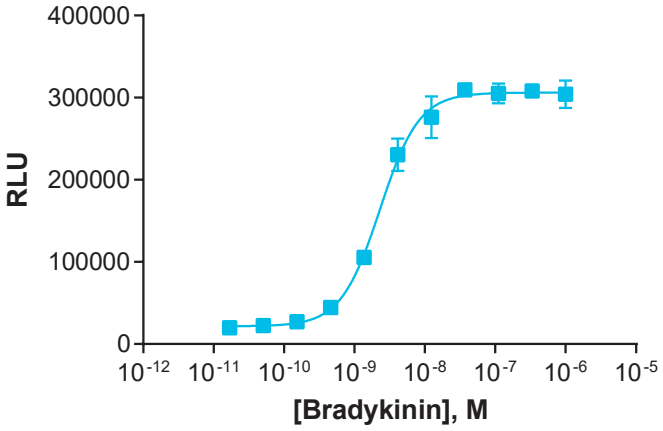
2. Add 50 µL (this is 50% of the assay volume) of working detection solution to each well of the assay plate.
3. Incubate the plate at room temperature for 60 minutes in the dark.
4. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for an imager.



Assay Plate Map: This plate map shows a typical plate layout for relative potency, with two interdigitated 11-point dose curves, with 3 replicates per dose point, for a test and reference sample tested with their appropriate dilution schemes.

Typical Results

The following graph is an example of a typical dose-response curve for the BDKRB2 Bioassay, generated using the protocol outlined in this user manual.



EC_{50} (nM)	2.3
S/B	15.3

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No Response	Incorrect thawing procedure	Refer to thawing instructions in Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or incorrect 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 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.
	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	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 information or support, please contact technical support as listed below.

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