User Manual

cAMP Hunter™ CHO-K1 GPBAR1 Bioassay Kit

For the Measurement of Ligand-Mediated cAMP Accumulation

For Bioassay Kits:

95-0049Y2-00139: 2-Plate Kit

95-0049Y2-00140: 10-Plate Kit

Document Number 70-417 Revision 0

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

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Overview

The cAMP Hunter CHO-K1 GPBAR1 Bioassay Kit provides a robust and highly sensitive functional, cell-based assay to monitor 3'-5'-cyclic adenosine monophosphate (cAMP) production in cells, as a result of ligand-mediated GPCR activation. The bioassay kit contains all the materials needed to run the 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 long-term assay reproducibility, and faster implementation from characterization to QC lot release. This bioassay has been optimized for a 96-well format. The protocol can be optimized further for running the assay in a high-throughput 384-well format.

Assay Principle

Ligand-mediated GPCR stimulation leads to the activation of G-proteins, which in turn triggers downstream signaling pathways by recruiting, activating or inhibiting cellular enzymes. One such enzyme is adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) to cAMP. Adenylate cyclase is either stimulated or inhibited by the G-protein subunits, $G\alpha_s$ and $G\alpha_i$, respectively. The cAMP Hunter GPBAR1 Bioassay monitors activation of the naturally $G\alpha_s$-coupled receptor, GPBAR1, by measuring the cellular cAMP levels using a homogeneous, gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology, which is explained in the cAMP Detection Kit Principle section.

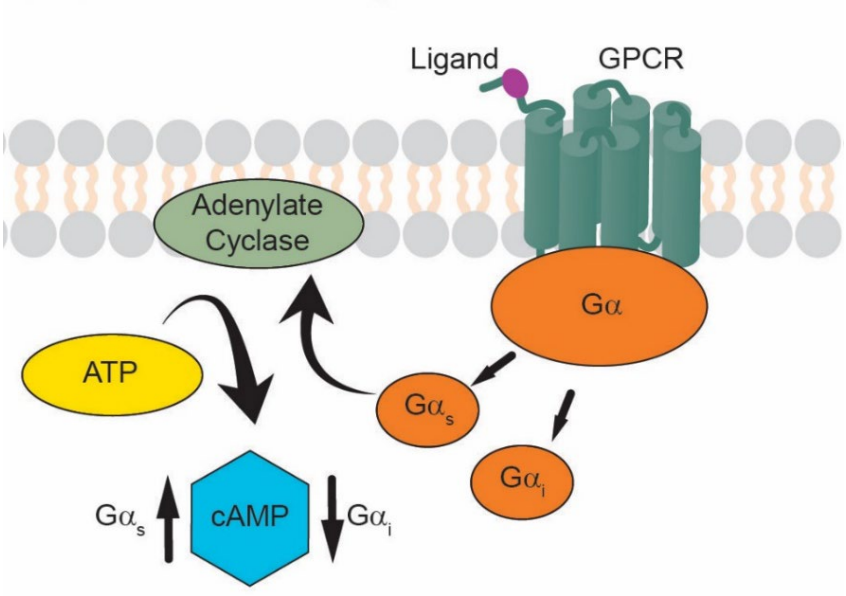


Figure 1. Assay Principle: GPCR cAMP Pathway: Ligand-mediated activation of GPCR either stimulates or inhibits adenylate cyclase to modulate cellular cAMP levels. In the case of GPBAR1, its activation by a ligand stimulates adenylate cyclase, which in turn enables the production of cAMP.

cAMP Detection Kit Principle

The EFC technology uses a β-galactosidase (β-gal) enzyme split into two fragments, the Enzyme Donor (ED) and Enzyme Acceptor (EA). Independently these fragments have no β-gal enzymatic activity; however, in solution they rapidly complement to form an active β-gal enzyme.

In this assay, cAMP from cell lysates and ED-labeled cAMP (ED-cAMP) compete for anti-cAMP antibody (Ab). Antibody-bound ED-cAMP does not complement with EA, but unbound ED-cAMP is free to complement with EA to form an active enzyme, which subsequently produces a chemiluminescent signal. The signal from the assay is directly proportional to the amount of cellular cAMP in the well, i.e., the higher the GPBAR1 activation, the greater the cAMP levels inside the cells become, and the larger the signal in the assay.

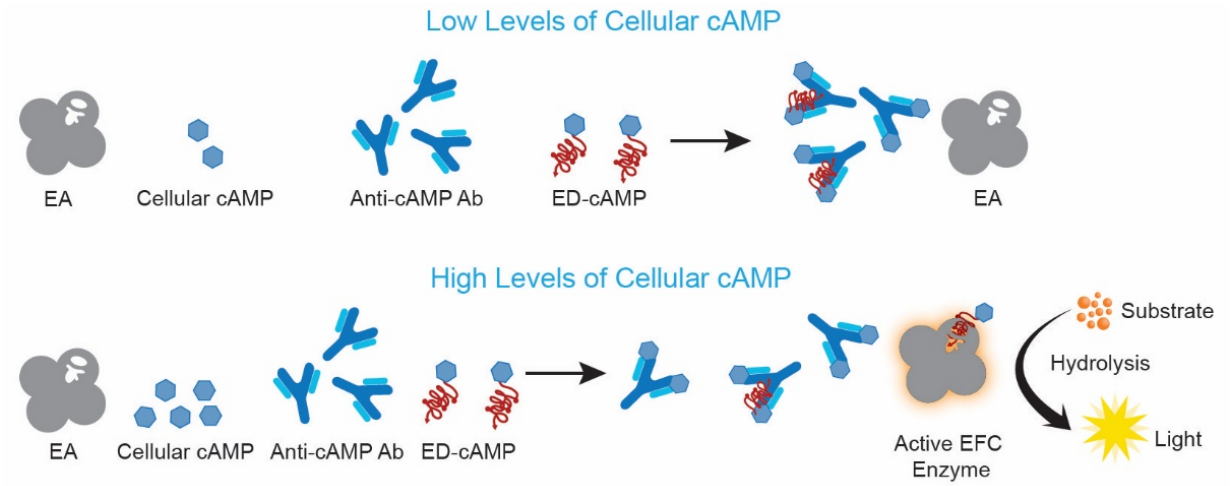


Figure 2. cAMP Detection Kit Principle: When cellular cAMP levels are low, ED-labeled cAMP successfully binds with the anti-cAMP-antibody, as there aren't enough cellular cAMP molecules to compete against. In contrast, when cellular cAMP levels are high, they bind to the anti-cAMP antibody, leaving ED-labeled cAMP relatively free. Upon the addition of the detection reagent containing EA, the ED and EA fragments undergo complementation, which successfully forms an active enzyme that hydrolyzes the substrate and generates a chemiluminescent signal.

Materials Provided

List of Components	95-0049Y2-00139	95-0049Y2-00140
cAMP Hunter CHO-K1 GPBAR1 Bioassay Cells (1.2 x 10^6 cells in 0.1 mL per vial)	2	10
AssayComplete™ Cell Plating 2 Reagent (100 mL per bottle)	1	2
AssayComplete Cell Assay Buffer (50 mL per bottle)	1	2
TGR5 Receptor Agonist (5 mg per vial)	1	1
cAMP Detection Kit for Bioassays		
cAMP Standard (250 µM) (Vial)	1 x 0.2 mL	1 x 1 mL
cAMP Antibody Reagent (Bottle)	1 x 5 mL	1 x 25 mL
cAMP Lysis Buffer (Bottle)	1 x 7.6 mL	1 x 38 mL
Substrate Reagent 1 (Vial)	1 x 2 mL	1 x 10 mL
Substrate Reagent 2 (Vial)	1 x 0.4 mL	1 x 2 mL
cAMP Solution D (Bottle)	1 x 10 mL	1 x 50 mL
cAMP Solution A (Bottle)	1 x 16 mL	1 x 80 mL
96-Well White, Flat-Bottom, TC-Treated, Sterile Plates with Lid	2	10

Storage Conditions

cAMP Hunter CHO-K1 GPBAR1 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.

cAMP Detection Kit for Bioassays

Upon receipt, store reagents at -20°C. It is important to thaw the kit from -20°C to room temperature at least 24 hours prior to using the kit. After thawing the kit to room temperature, leave it at 2-8°C overnight before use. Ensure that the reagents are at room temperature for best performance.

After thawing, store reagents for up to 4 weeks at 2-8°C. 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 in opaque containers until needed. Avoid multiple freeze-thaw cycles.

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 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.

AssayComplete Cell Assay Buffer

Upon receipt, store reagents at -20°C. It is important to thaw the Cell Assay Buffer from -20°C to room temperature at least 24 hours prior to using in the assay. After thawing to room temperature, leave the bottle at 2-8°C overnight before use. Ensure that the reagents are equilibrated to room temperature before using in the assay for best performance.

TGR5 Receptor Agonist

Upon receipt, store at -20°C until ready to use (up to the expiration date listed on the kit's Certificate of Analysis). Centrifuge the vial prior to opening to maximize recovery, and reconstitute as recommended in the ligand datasheet. The 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

Upon receipt, store at room temperature.

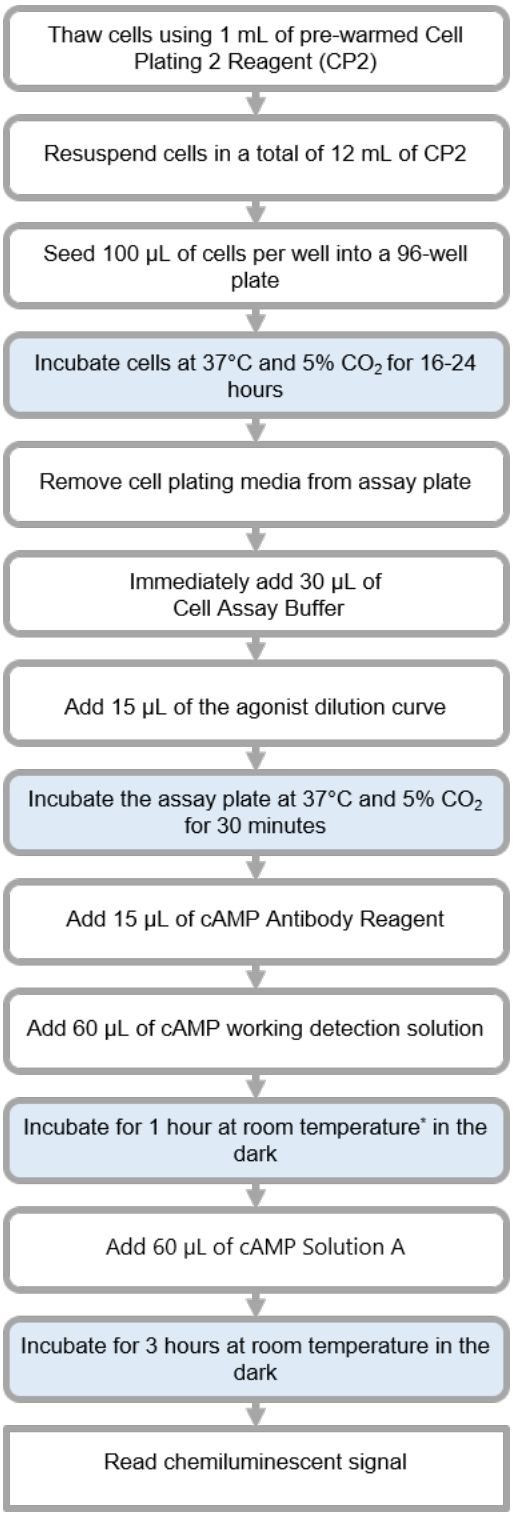
Additional Materials Required

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)	

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 GPBAR1 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 15 mL conical tube
    c. A micropipette (P1000) set to dispense 1 mL
    d. A multichannel pipette and tips set to dispense 100 µL
    e. A bottle of AssayComplete™ Cell Plating 2 Reagent (CP2), pre-warmed in a 37°C water bath for 15 minutes and equilibrated to room temperature
    f. A 96-Well White, 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 12 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 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 11 mL of CP2. Remove any remaining liquid 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 CP2, without creating any froth in the suspension. Immediately transfer the suspension into the sterile 25 mL reagent reservoir.
7. Transfer 100 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 16-24 hours before proceeding with the assay.

Day 2: Sample Preparation

The following protocol is designed for testing purified biologics. The cAMP Hunter 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.

1. Add 200 µL of the AssayComplete Cell Assay Buffer to Wells A2 to A12 of the master dilution plate.
2. Prepare the TGR5 Receptor Agonist curve:
   The TGR5 Receptor Agonist curve is prepared at 3X the desired final concentration, with a top dose of 10 µM.
   - 2.1. Add 692 µL of DMSO to the vial containing 5 mg of the lyophilized TGR5 Receptor Agonist powder, to make a 20 mM stock solution. Make suitable aliquots (e.g. 30 µL) and store at -20°C until needed.
   - 2.2. Make a 900 µM intermediate stock solution from the 20 mM stock solution prepared in Step 2.1, by adding 10 µL of the 20 mM stock into 213 µL of DMSO in a sterile microcentrifuge tube. Mix well by pipetting up and down several times.
   - 2.3. Make a 1:30 dilution of the control agonist, to generate a 30 µM solution (3X the final 10 µM dose). Add 290 µL of the Cell Assay Buffer to Well A1 of the master dilution plate. Add 10 µL of the agonist intermediate stock solution prepared in Step 2.2 to Well A1. Mix well by slowly pipetting up and down several times.
   - 2.4. Using a clean pipette tip, transfer 100 µL of solution 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 to A3. Repeat this process until Well A11 is reached. No sample is transferred to Well A12 as this is the negative control.

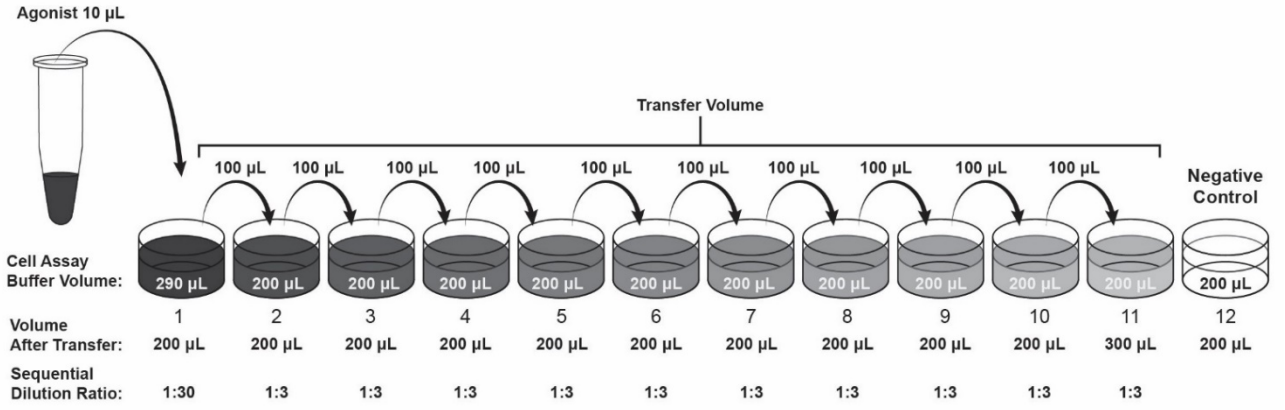


Figure 3. Agonist Serial Dilutions Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

3. Remove the cell media from the assay plate completely, by careful aspiration.
4. Immediately add 30 µL of the Cell Assay Buffer to all the empty wells of the plate.
5. Add 15 µL of sample from the agonist dilution curve prepared in Step 2, to the appropriate wells of the assay plate. Refer to Figure 4. Representative Assay Plate Map.
6. Incubate the assay plate in a humidified incubator at 37°C and 5% CO_2 for 30 minutes.

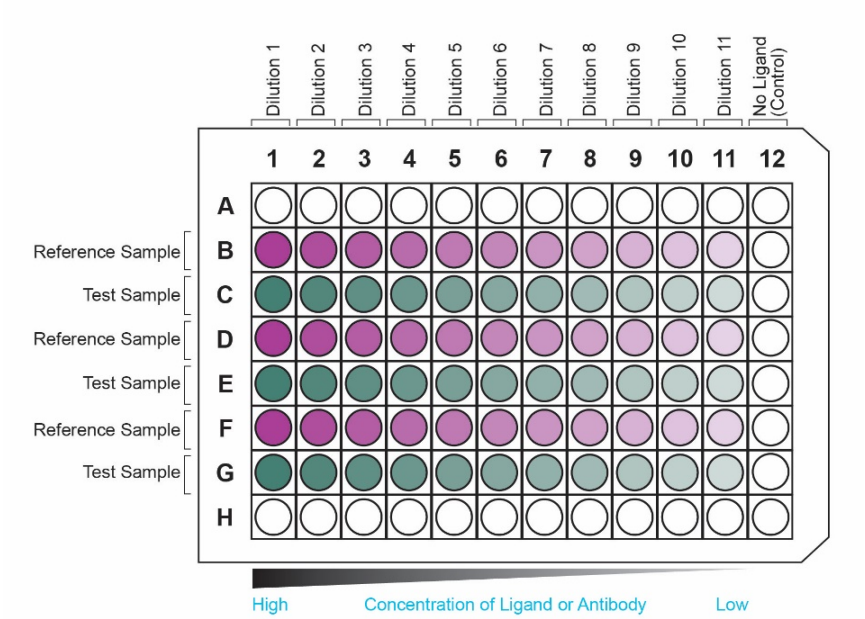


Figure 4. Representative Assay Plate Map This plate map shows two interdigitated 11-point dose curves, with three replicates per dose point, for a test and reference sample tested using the same dilution scheme.

Day 2: cAMP Detection

1. Following agonist incubation, add 15 µL of the cAMP Antibody Reagent to all wells of the assay plate.
2. Prepare a stock of the cAMP working detection solution in a separate 15 mL polypropylene tube. Refer to the table below for the volume ratio.

Components	Volume ratio	Volume per 96-well Plate (mL)
cAMP Lysis Buffer	19	3.8
Substrate Reagent 1	5	1.0
Substrate Reagent 2	1	0.2
cAMP Solution D	25	5.0
Total Volume		10

3. Add 60 µL of the cAMP working detection solution to all wells of the assay plate. Do not pipette up and down or vortex the plate to mix.
4. Incubate the assay plate for 1 hour at room temperature in the dark.

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

5. Add 60 µL of cAMP Solution A to all wells of the assay plate. Do not pipette up and down or vortex the plate to mix.

6. Incubate the assay plate for 3 hours at room temperature in the dark.
7. 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.

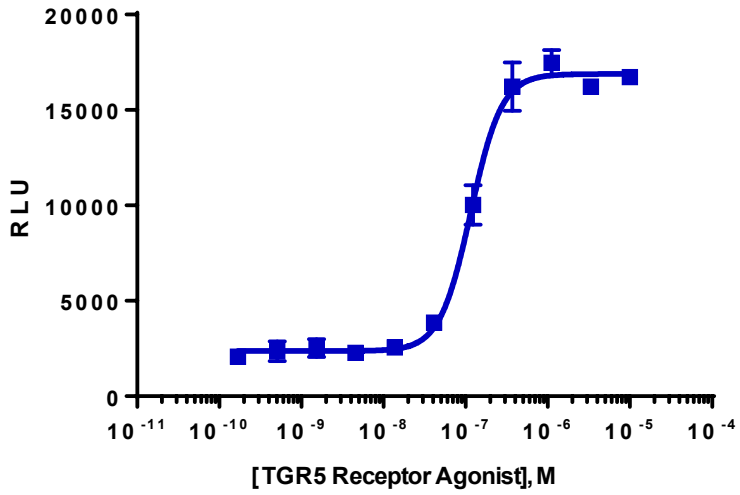
Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, and Microsoft Excel etc.

Typical Results

The following graph is an example of a typical dose-response curve for the GPBAR1 Bioassay generated using the protocol outlined in this manual. The data shows potent, dose-dependent cAMP production when treated with TGR5 Receptor Agonist.

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

A



B

EC_{50} (nM)	115.1
S/B	8.0

Figure 5. Typical Results: Representative **A,** dose-response curve and **B,** the EC_{50} and assay window for TGR5 Receptor Agonist-mediated GPBAR1 activation, 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.
		Include a cAMP standard curve in the assay to ensure that the ligand dose curve is in the dynamic range of the cAMP detection kit. Moving out of the dynamic range may shift the EC_{50} of the ligands.
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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