DiscoverX

User Manual
cAMP Hunter™
Liraglutide Bioassay Kit

For Chemiluminescent Detection of Liraglutide Activity

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

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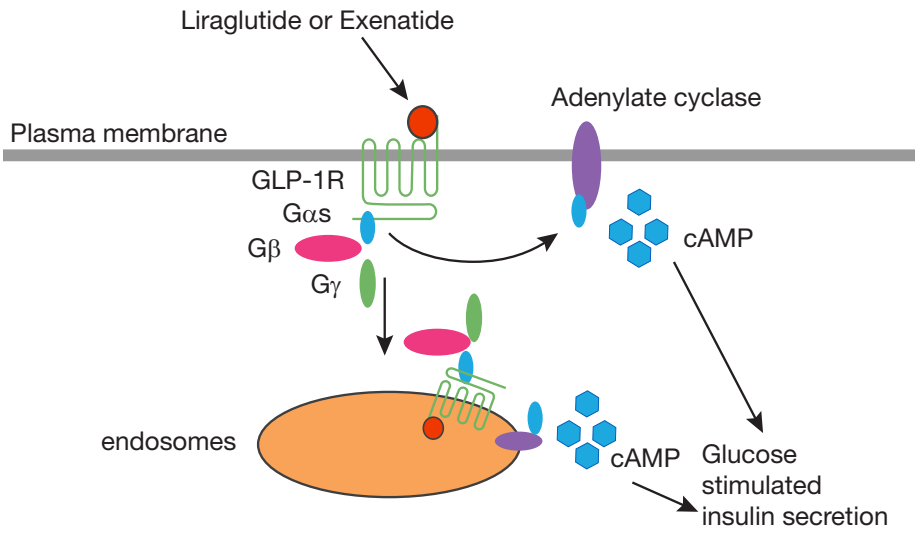
Overview

cAMP Hunter Liraglutide Bioassay Kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study Liraglutide potency and neutralizing antibodies. The bioassay kits contain all the reagents needed for a complete assay including cells, detection reagents, cell plating reagent, positive control agonist, and assay plates. The pre-qualified, frozen cells have been manufactured for single-use and are provided in a ready-to-assay format that saves time and adds convenience.

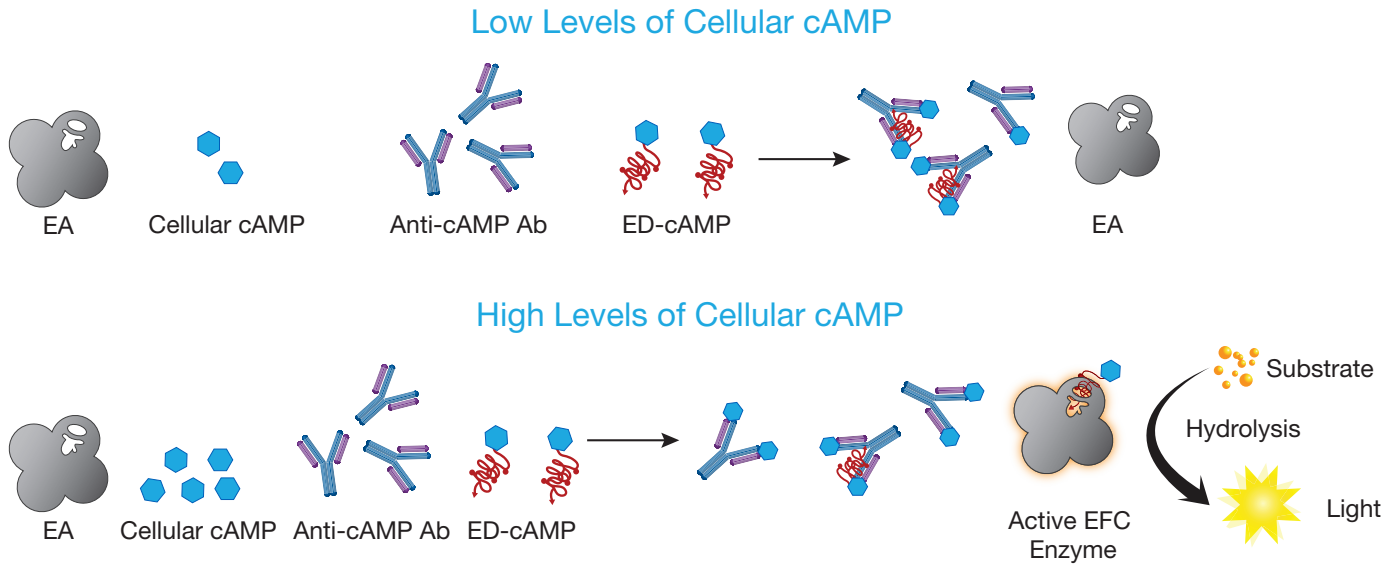
Technology Principle

GPCR activation mobilizes a series of pathways that result in a cellular response. One of those pathways is the activation of the cyclic AMP (cAMP) response, involving a membrane bound enzyme called adenylate cyclase. $G\alpha_i$- and $G\alpha_s$- coupled GPCR receptors modulate cAMP by either inhibiting or stimulating adenylate cyclase, respectively. With the cAMP Hunter Liraglutide Bioassay Kit, cells overexpressing GLP1R utilize the natural coupling status of the GPCR to monitor activation of the $G\alpha_s$-coupled receptor. Following stimulation, the functional status of GLP1R is monitored by measuring the cellular cAMP levels using a homogeneous, gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology.



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 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 will not be able to complement with EA, but unbound ED-cAMP is free to complement EA to form an active enzyme, which subsequently produces a luminescent signal. The signal from the assay is directly proportional to the amount of cellular cAMP in the well, i.e., the greater the amount of GLP1R activation, the higher the cAMP levels inside the cells, and the larger the signal in this assay.



Materials Provided

List of Components	95-0062Y2-00099	95-0062Y2-00100
cAMP Hunter CHO-K1 GLP1R Bioassay Cells	2 vials	10 vials
cAMP Detection Kit for Bioassays		
cAMP Standard (250 µM) (mL)	0.2	1
cAMP Antibody Reagent (mL)	5	25
cAMP Lysis Buffer (mL)	7.6	38
Substrate Reagent 1 (mL)	2	10
Substrate Reagent 2 (mL)	0.4	2
cAMP Solution D (mL)	10	50
cAMP Solution A (mL)	16	80
Cell Assay Buffer	1 X 50 mL	2 X 50 mL
AssayComplete™ Cell Plating Reagent 2	1 x 100 mL	2 X 100 mL
Protein Dilution Buffer– B2	1 X 50 mL	2 X 50 mL
Control Agonist (Exendin-4)	1 vial	1 vial
96-Well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

cAMP Hunter CHO-K1 GLP1R Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, thaw the vials as soon as possible upon receipt. If continued storage of the frozen vials is necessary, store as follows:

- Short term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for more 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 a 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.

cAMP Detection Kit & Cell Assay Buffer

Upon arrival, 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 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 Reagent 2 (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 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.

Protein Dilution Buffer–B2 (PDB-B2)

Once thawed, the Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed on the kit 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, 10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on your stock sample concentrations and should be adjusted accordingly.

Recombinant Exendin-4 Control Agonist

Store at -20°C until ready to use (up to the expiration date listed on the kit certificate of analysis). Centrifuge the vial prior to opening to maximize recovery and reconstitute as noted in the ligand datasheet. 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

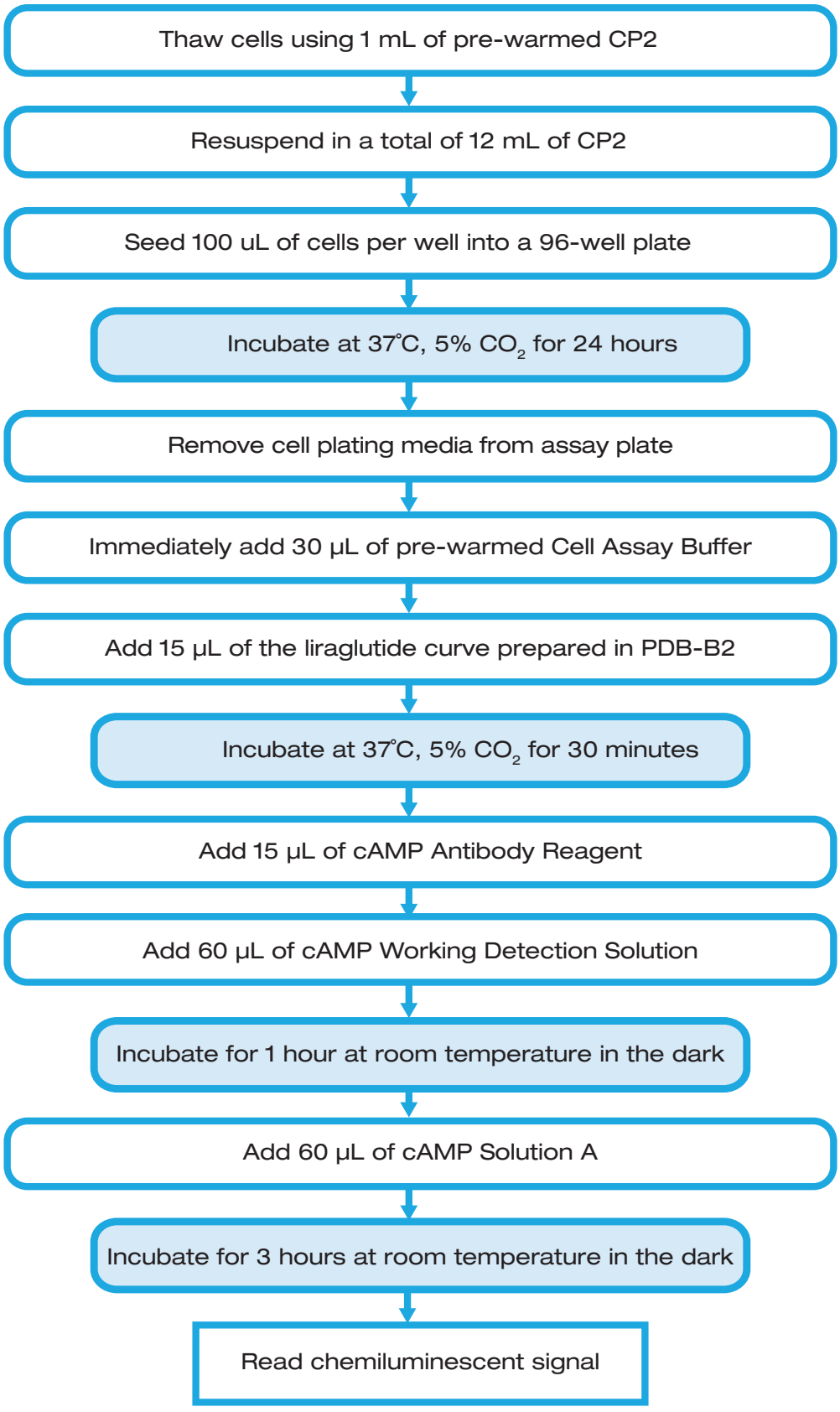
Store at room temperature.

Additional Materials Required

Material	Ordering Information
V-Bottom 96-well ligand dilution plates	92-0011
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Single and multichannel micro-pipettors and pipette tips	
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar

Protocol Schematic

In a 96-well tissue culture treated plate provided in the kit, perform the following steps:



Detailed Protocol

Day 1: cAMP Hunter Bioassay Cell Preparation

The following protocol is for thawing and plating frozen cAMP Hunter CHO-K1 GLP1R 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 micropipettor (P1000) set to dispense 1 mL.
    d. A multichannel pipette and tips set to dispense 100 µL.
    e. A bottle of Cell Plating Reagent 2 (CP2, pre-warmed in a 37˚C water bath for 15 minutes).
    f. A white-walled, clear bottom 96-well assay plate.
2. Dispense 12 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 cryovial quickly with 70% EtOH, and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

4. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP2 from the 15 mL conical tube to the cryovial, thawing the cell pellet. The medium should be added slowly along the side of the wall of cryovial tube. Mix the cells gently by pipetting up and down several times to break up any clumps. Transfer the cell suspension to the conical tube containing the remaining 11 mL of CP2. Remove any medium/suspension left in the tube to ensure complete recovery of all the cells from the vial.
5. Mix the tube by inversion to ensure the cells are properly mixed in the medium without creating any froth in the suspension and immediately pour the suspension into the 25 mL reservoir.
6. Add 100 µL of cells to each well of the 96-well assay plate using the multichannel pipette.
7. Let plate sit for 15 minutes at room temperature to allow cells to settle, reducing potential edge effects.
8. Incubate for 24 hours at 37°C and 5% CO_2 in a humidified tissue culture incubator.

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. Therefore, standard curves of control can typically be prepared in neat serum or plasma and added directly to cells without further dilution. For the best results, the optimized minimum required dilution (MRD) of crude samples should be empirically determined.

1. Prepare Liraglutide curve. Liraglutide is prepared at 3X the desired final concentration. Top dose: 10 ng/mL.

    a. Prepare 1:1000 dilution of Liraglutide stock concentration of 6 mg/mL to 6 µg/mL of working stock concentration. This can be done in a two-step process of adding and mixing 20 µL of 6 mg/mL Liraglutide in 980 µL of Protein Dilution Buffer-B2 (PDB-B2) for a 1:50 dilution, and then taking 20 µL of that dilution and adding it to 380 µL of PDB-B2.

    b. Add 20 µL of the 6 µg/mL dilution in 980 µL of PDB-B2 for another 1:50 dilution and then finally add 200 µL from this final dilution to 600 µL of PDB-B2. This will make a final concentration of 30 ng/mL of Liraglutide to make 3X the desired final concentration of 10 ng/mL.

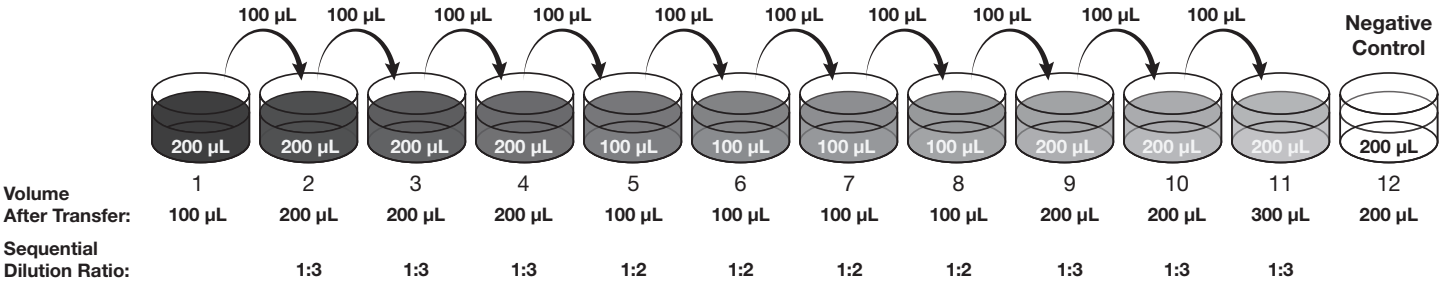
    c. Transfer 200 µL of the 30 ng/mL dilution to well A1 on the master dilution plate.

    d. Add 200 µL of PDB-B2 to wells A2-A4 and A9-A12 in the master dilution plate and add 100 µL of PDB-B2 to wells A5-A8.

    e. Using a clean tip, transfer 100 µL from well A1 into well A2 for a 1:3 dilution and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 into A3 for a 1:3 dilution. Repeat this process for A3 to A4.

    f. Using a clean tip, transfer 100uL from well A4 to A5 for a 1:2 dilution. Repeat, till well A8 is reached.

    g. Using a clean tip, transfer 100uL from well A8 to A9 for a 1:3 dilution and repeat for wells A10 and A11. No sample is transferred to A12 as this will serve as a negative control.



2. Assay Plate Preparation: Completely remove the cell media from the assay plate by careful aspiration.

3. Immediately add 30 µL of Cell Assay Buffer to all empty wells of the plate.

4. Add 15 µL from the Liraglutide curve on the master dilution plate to the appropriate wells of the assay plate.

5. Incubate assay plate at 37°C and 5% CO_2 incubator for 30 minutes.

Day 2: cAMP Detection

1. Following agonist incubation, add 15 µL of cAMP Antibody Reagent to all wells.
2. Prepare a stock of cAMP Working Detection Solution in a separate 15 ml polypropylene tube, by mixing 19-parts of cAMP Lysis Buffer, 5-parts of Substrate Reagent 1, 1-part Substrate Reagent 2, and 25-parts of cAMP Solution D. Store in the dark before use.

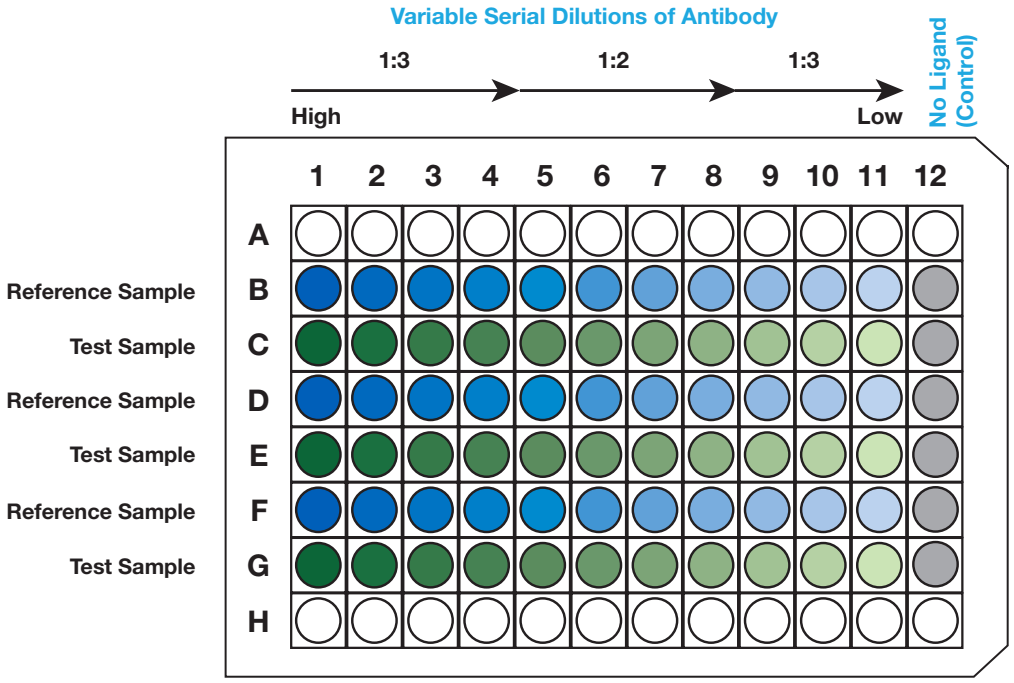
Components	Volume Ratio	Volume per 96-well Plate (mL)
cAMP Lysis Buffer	19	3.8
Substrate Reagent 1	5	1.0
Substrate Reagent 2	1	0.2
cAMP Solution D	25	5.0
Total Volume		10

cAMP Working Detection Solution is light sensitive, thus storage and incubation in the dark is necessary.

3. Add 60 µL of cAMP Working Detection Solution to all wells of the assay plate. Do not pipette up and down in the wells to mix or vortex plates.
4. Incubate assay plate for 1 hour at room temperature in the dark.
5. Enzyme Acceptor addition: Add 60 µL of cAMP Solution A to all wells of the assay plate. Do not pipette up and down in the wells to mix or vortex plates.
6. Incubate assay plate for 3 hours at room temperature in the dark.
7. Read sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers of 5-10 seconds for imager.

cAMP Working Detection Solution is light sensitive, thus storage and incubation in the dark is necessary.

Representative Plate Map for Sample Curve



This plate map shows an 11-point dose curve with 3 data points at each concentration for one reference and one test sample per plate, with a variable serial dilution scheme.

Troubleshooting Guide

Problem	Cause	Solution
No response	Improper thawing procedure	Refer to thawing instructions in this user manual. Thawing process can have a significant effect on cell viability.
	Improper ligand used or improper ligand incubation time	See certificate of analysis for recommended ligand and assay conditions.
	Improper preparation of ligand (agonist or antagonist)	Refer to specific datasheet to ensure proper handling, dilution and storage of ligand.
	Improper time course for induction	Optimize time course of induction with agonist and antagonist.
Decreased response	Cells are not adherent and exhibit incorrect morphology	Confirm adherence of cells using microscope.
Low or no signal	Improper preparation of detection reagents	Detection reagents should ideally be prepared just prior to use and are sensitive to light.
	Problem with microplate reader	Microplate reader should be in luminescence mode. Read at 0.1-1 seconds/well.
Experimental S:B does not match datasheet value	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	Make sure 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 serial dilutions can help to avoid 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 ligands.
High variability between replicates	Instrument calibration	Ensure dispensing equipment is properly calibrated, and proper pipetting technique is used.

For additional information or technical support, please contact Technical Support listed below.

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