DiscoverX

User Manual
cAMP Hunter™
Follitropin Alfa Bioassay Kit

For Chemiluminescent Detection of Follitropin Alfa 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.

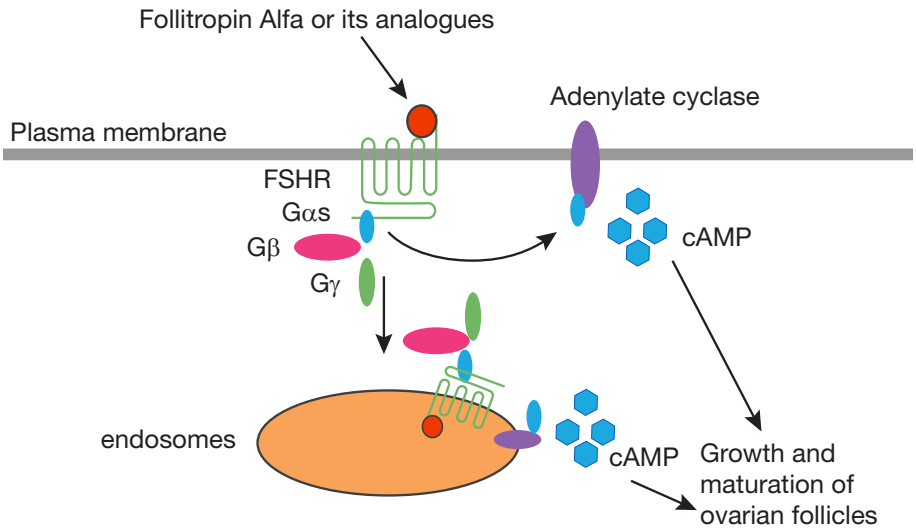
w discoverx.com usa 1.866.448.4864 e SupportUS@discoverx.com eu +44.121.260.6142 e SupportEurope@discoverx.com

Overview

cAMP Hunter Follitropin Alfa Bioassay Kits provide a functional, robust, highly sensitive, and easy-to-use cell-based assay to study potency and neutralizing antibodies for Follitropin Alfa and its analogues. 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.

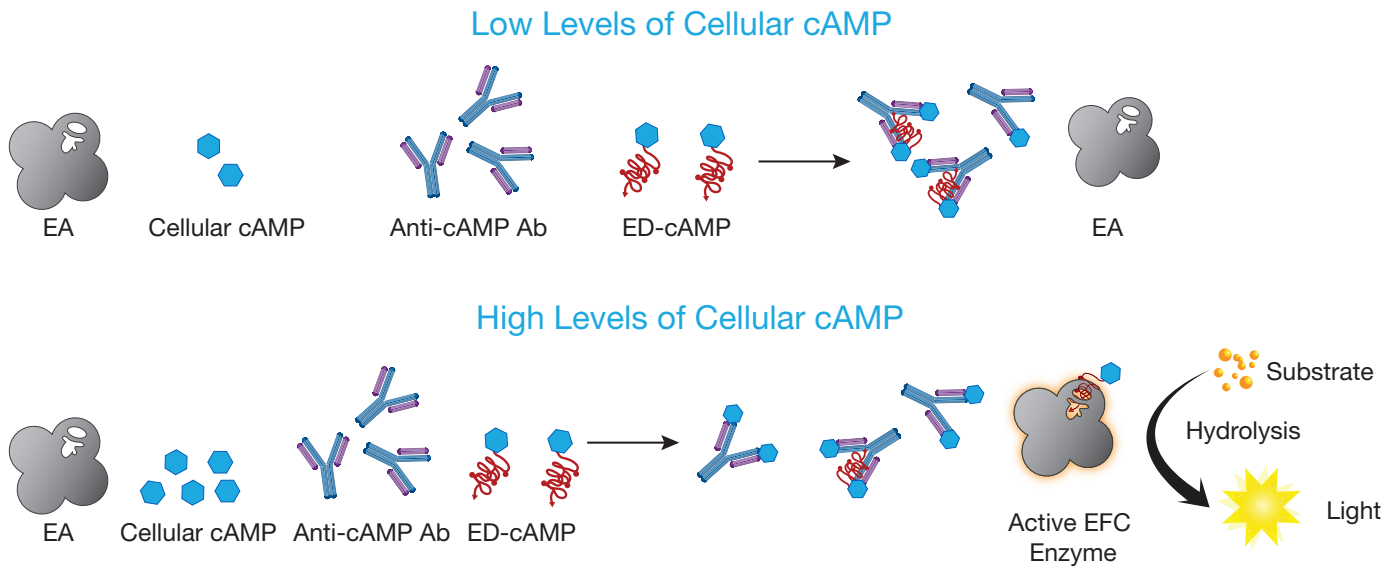
Technology Principle

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 Follitropin Alfa Bioassay Kit, cells overexpressing FSHR utilize the natural coupling status of the GPCR to monitor activation of the $G\alpha_s$-coupled receptor. Following stimulation, the functional activation status of FSHR 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 GPCR activation, the higher the cAMP levels inside the cells, and the larger the signal in this assay.



Materials Provided

List of Components	95-0119Y2-00103	95-0119Y2-00104
cAMP Hunter CHO-K1 FSHR 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	2 X 50 mL	4 X 50 mL
AssayComplete™ Cell Plating Reagent 2	1 X 100 mL	2 X 100 mL
Ultrapure IBMX	1 vial	1 vial
Control Agonist (FSH)	1 vial	1 vial
96-Well Clear-Bottom TC Treated, Sterile Plates w/Lid	2 plates	10 plates

Storage Conditions

cAMP Hunter CHO-K1 FSHR 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.

AssayComplete Cell Plating Reagent 2 (CP2)

Once thawed, CP2 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.

FSH 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.

cAMP Detection Kit & Cell Assay Buffer

Upon arrival, store reagents at -20°C. We recommend that the Detection Reagent kit should be thawed from -20°C to room temperature for at least 24 hours prior to use. After thawing the kit to room temperature, leave it at 2-8°C overnight before use (total ~36 hours of thawing time before using the kit). Ensure that the reagents are at room temperature when using them for the assay to guarantee 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.

Ultrapure IBMX

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 IBMX datasheet. Reconstituted IBMX is stable up to the expiration date listed, when stored at -20°C to -80°C. Avoid multiple freeze-thaw cycles.

96-Well Tissue Culture Treated Plates

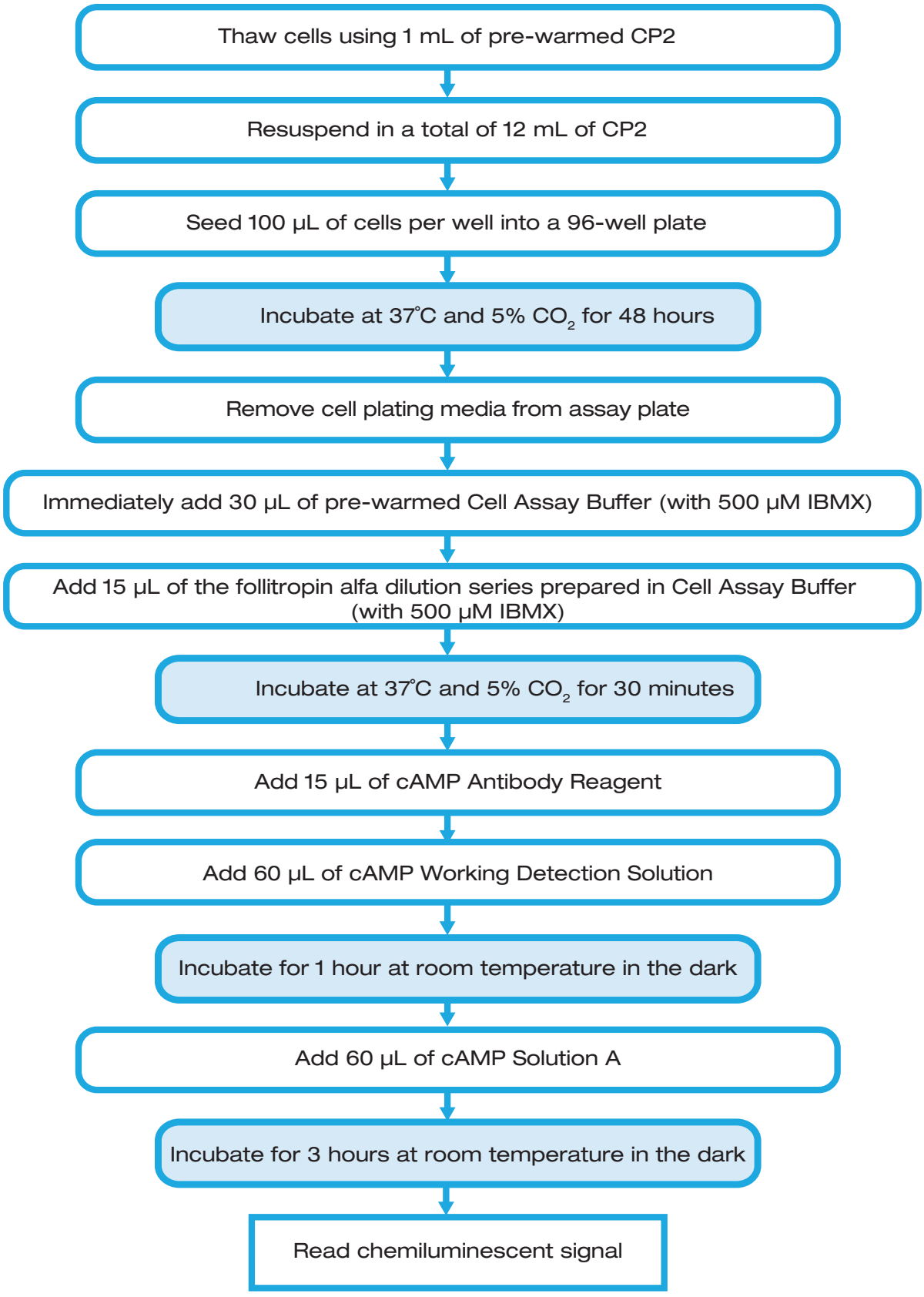
Store at room temperature.

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	ThermoFisher 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 FSHR 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 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.

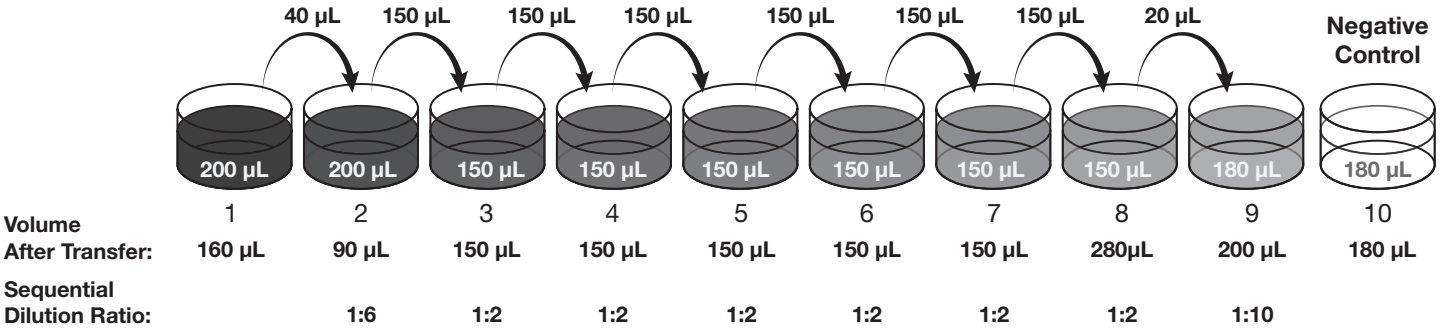
DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% ethanol, and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

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 48 hours at 37ºC 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 5 mL of assay diluent (Cell Assay Buffer + 500 µM IBMX) by adding 5 µL of 500 mM stock IBMX to 5 mL Cell Assay Buffer. Use this assay diluent for diluting Follitropin Alfa.
2. Prepare Follitropin Alfa dilution series at 3X the desired final concentration. Top dose: 0.006 IU/µL or 6 IU/mL.
   a. Add 15 µL of Gonal F from stock 600 IU/mL (300 IU in 0.5 mL) in 485 µL of assay diluent prepared in step 1 above.
   b. Transfer 200 µL of this dilution into well A1 of a master dilution plate to make 3X the desired final concentration.
   c. Add 200 µL of prepared assay diluent (Cell Assay Buffer + 500 µM IBMX) to well A2, 150 µL of assay diluent to wells A3-A8 and add 180 µL of assay diluent to wells A9 & A10 of the master dilution plate.
   d. Using a clean tip, transfer 40 µL from well A1 into well A2 for a 1:6 dilution and mix by pipetting up and down several times.
   e. Replace the pipette tip, and transfer 150 µL from well A2 into A3 for a 1:2 dilution. Repeat, till well A8 is reached.
   f. Using a clean tip, transfer 20 µL from well A8 to A9 for a 1:10 dilution. No sample is transferred to A10 as this will serve as a negative control.



3. Assay Plate Preparation: Completely remove the cell media from the assay plate by careful aspiration.
4. Immediately add 30 µL of pre-warmed Cell Assay Buffer to all empty wells of the plate.
5. Add 15 µL from the Follitropin Alfa curve on the master dilution plate to the appropriate wells of the assay plate.
6. 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.

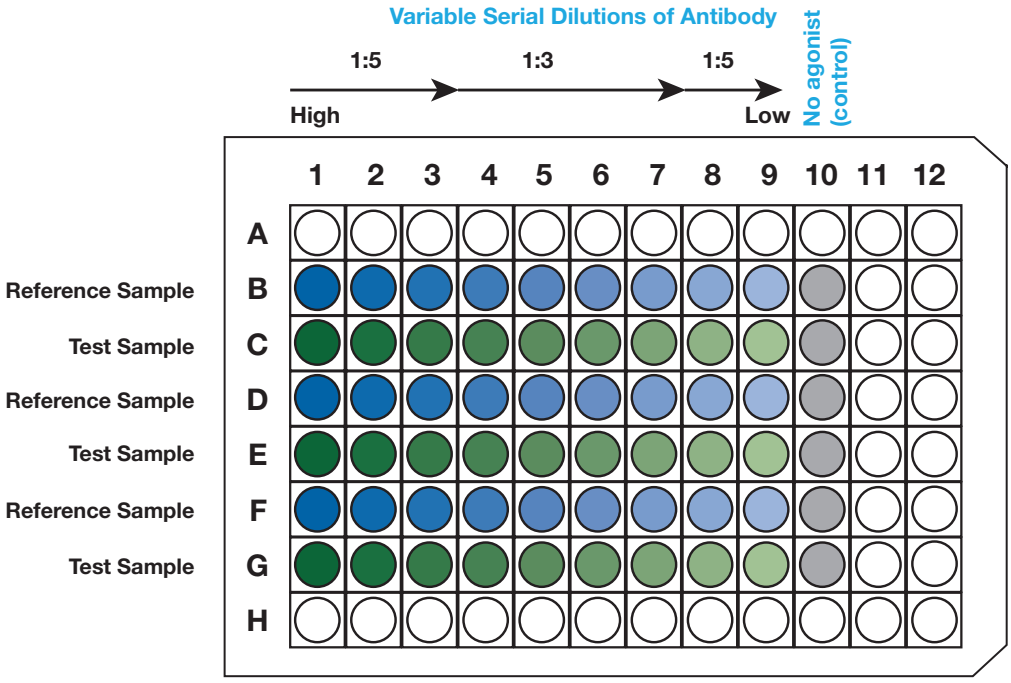
Components	Volume Ratio	Volume per 96-Well Plate (mL)
cAMP Lysis Buffer	19	3.8
Substrate Reagent 1	5	1.0
Substrate Reagent 2	1	0.2
cAMP Solution D	25	5.0
Total Volume		**10**

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 or 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 a 9-point dilution curve with three 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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