DiscoverX

User Manual

cAMP Hunter™

$G\alpha_s$ and $G\alpha_i$ Cell Line

For Chemiluminescent Detection of cAMP and GPCR Activity of Human, Ortholog, and Orphan GPCRs

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

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Overview

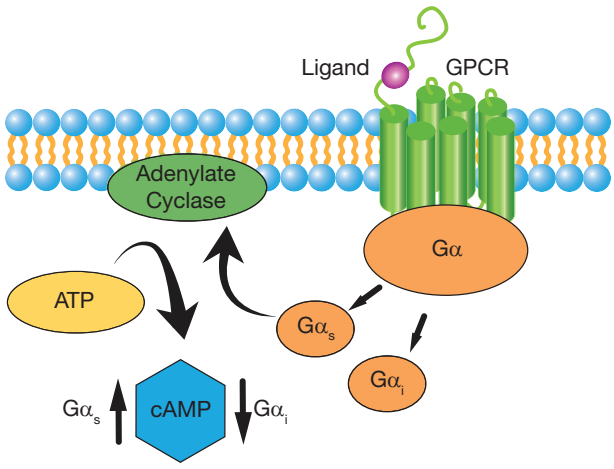
cAMP Hunter Cell Lines are non-force-coupled $G\alpha_s$ and $G\alpha_i$ stable clonal cell lines that expedite drug screening by providing robust responses, with accurate pharmacology. They provide the means for the measurement of an ubiquitous second messenger (cAMP) to determine the mechanism of action of ligands. Each cell line has been characterized for its appropriate GPCR pharmacology, specificity, and stability in culture and can be effectively used for screening both biologics and small molecules. For detection of cAMP, the cAMP Hunter Cell Lines can be paired with DiscoverX's HitHunter cAMP Assays for Small Molecules or Biologics.

Technology Principle

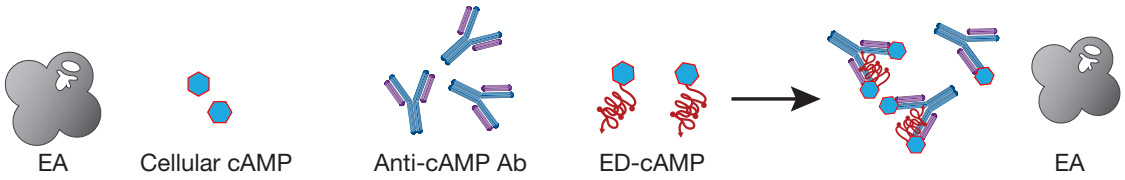
GPCR activation following ligand binding initiates a series of second messenger cascades that result in a cellular response. The signaling involves a membrane bound enzyme called adenylate cyclase. $G\alpha_s$- and $G\alpha_i$-coupled receptors modulate cAMP by either inhibiting or stimulating adenylate cyclase, respectively. With the HitHunter cAMP Assay, cells that overexpress GPCRs utilize the natural coupling status of the GPCR to monitor activation of $G\alpha_s$- and $G\alpha_i$-coupled receptors.

Following ligand stimulation, the functional status of the GPCR is monitored by measuring the cellular cAMP levels using a homogeneous (no wash), gain-of-signal competitive immunoassay based on Enzyme Fragment Complementation (EFC) technology. Thus, the positive signal generated by the assay is directly proportional to GPCR activation.

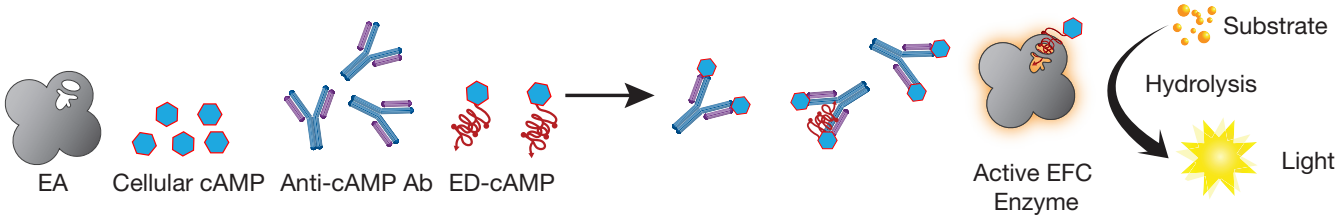
GPCR cAMP Pathway



Low Levels of Cellular cAMP



High Levels of Cellular cAMP



Intracellular cAMP and ED-labeled cAMP (ED-cAMP) compete for anti-cAMP antibody (Ab) binding sites. Antibody-bound ED-cAMP will not be able to complement with EA, but unbound ED-cAMP is free to complement EA to form active enzyme, which subsequently produces a luminescent signal. The amount of signal produced is directly proportional to the amount of cAMP in the cells.

The HitHunter cAMP Assay 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.

The HitHunter cAMP Assay kits provide a robust, highly sensitive, and easy-to-use, cell-based functional assay to study GPCR activity through cAMP production. The kits contain all the reagents needed for the detection of cAMP from cAMP Hunter cells expressing $G\alpha_s$- and $G\alpha_i$-coupled receptors induced with a biologics ligand. The flexible assay system has been designed to work in agonist or antagonist mode for 96- and 384-well plate formats.

The cAMP Hunter Cell Lines can also be used with other cAMP detection technologies (e.g., TR-FRET), but will require further optimization (i.e. cell plating numbers, forskolin concentration, etc.). Please refer to the manufacturer's detection product user manual for protocols for cAMP measurement.

Materials Provided

Number of Components	Configuration
2 vials	Refer to cell line-specific datasheet

Storage Conditions

Cells must arrive in a frozen state on dry ice and should be transferred to the vapor phase of liquid nitrogen storage immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.

Additional Materials Needed

Refer to the cell line-specific datasheet to determine catalog numbers for the media and reagent requirements for the specific cAMP Hunter cell line you are testing. Each cAMP Hunter cell line has been validated for optimal assay performance using the recommended AssayComplete™ Cell Plating Reagent and control ligand indicated on the cell line-specific datasheet along with a HitHunter® cAMP Assay for cAMP detection.

It is highly recommended to not substitute Cell Plating Reagent.

Materials	Ordering Information
HitHunter cAMP Assay for Small Molecules or Biologics	90-0075SM or LM Series*
AssayComplete Thawing Reagents	Refer to cell line-specific datasheet
AssayComplete Cell Plating Reagent	Refer to cell line-specific datasheet
AssayComplete Cell Culture Kits	Refer to cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009
AssayComplete Freezing Reagents	Refer to cell line-specific datasheet
Selection Antibiotics	Refer to cell line-specific datasheet
cAMP Assay Buffer (either HBSS + 10 mM HEPES, or PBS)	
Trypsin-EDTA, 0.25%	
Phosphate-Buffered Saline (PBS)	
Serial Dilution and Assay Plates	discoverx.com/microplates
Disposable Reagent Reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Tissue Culture Disposables (Pipettes 1 mL to 25 mL) and Plastic-Ware (T25 and T75 Flasks, etc.)	
15 mL Polypropylene Tubes and 1.5 mL Microtubes	
Single and Multichannel Micro-Pipettors and Pipette Tips (10 µL to 100 µL)	
Cryogenic Vials for Freezing Cells	
Hemocytometer	
Multimode or Luminescence Plate reader	discoverx.com/instrument-compatibility
Humidified tissue culture incubator (37°C and 5% CO_2)	

* Series refer to the different sizes available for that reagent or kit.

Recommended Materials	Ordering Information
Forskolin	92-0005
Ligands	discoverx.com/ligands

Unpacking Cell Cryovials

Two cryovials are shipped on dry ice and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for safe storage and removal of cryovials from liquid nitrogen storage.

1. Cells must arrive in a frozen state on dry ice.

Contact technical support immediately, if cells are thawed upon arrival.

2. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For longer storage, place vials in the vapor phase of liquid nitrogen storage.

A face shield, gloves, and a lab coat should be worn during these procedures.

3. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least one minute for any liquid nitrogen inside the vial to evaporate.

Cryovials should be stored in the vapor phase of liquid nitrogen. They are not rated for storage in the liquid phase.

4. Proceed with the thawing and propagation protocols in the following section. Refer to the cell line-specific datasheet for appropriate AssayComplete products mentioned in the protocol below.

Care should be taken while handling cells to avoid contamination.

Cell Culture Protocol

The following procedures are for thawing cells in cryovials, seeding and expanding the cells, maintaining the cell cultures once the cells have been propagated, and preparing them for assay detection.

Cell Thawing

The following is a protocol for thawing cells in a T75 flask.

1. Pre-warm AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes.
2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask in a sterile tissue culture hood. Set aside for Step 6 below. DO NOT add selection antibiotics to the thawing reagent.

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid nitrogen storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

3. Remove the cell cryovials from -80°C or liquid nitrogen storage, and immediately place them on dry ice prior to thawing.
4. Place the frozen cell vials briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.
5. Decontaminate the vial by spraying and wiping with 70% ethanol, and transfer it to a tissue culture hood.

6. With a pipette, gently transfer the thawed cells to the pre-filled T75 flask and incubate at 37°C and 5% CO_2.
7. Maintain the cells in culture until they are >70-80% confluent. Then proceed to "Cell Propagation" instructions. Do not split if cells are below this confluency, or growth issues may occur.

Cell Propagation

The following is a protocol for propagating cells once they become >70-80% confluent in a T75 flask.

1. Pre-warm AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.
2. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate media from the T75 flask.
4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire surface of the flask to rinse the cells.
5. Gently aspirate PBS from flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
7. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with trypsin.
8. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm that the cells have detached by viewing them under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells have not detached, return the flask to the incubator for an additional 1 to 2 minutes, and repeat this step until all cells are in suspension.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

10. Add 4 mL of AssayComplete Thawing Reagent to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
12. Split the cells conservatively for the first passage after thaw using AssayComplete Thawing Reagent. Typical (conservative) split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:5
HEK 293	1:3
U2OS	1:2

For example, for CHO-K1 cells, add 4 mL of AssayComplete Thawing Reagent to the flask containing 1 mL of 0.25% Trypsin-EDTA, then transfer 1 mL (1/5th of the total reagent in the flask) into each new tissue culture flask.

13. Fill a new T75 or T225 flask with AssayComplete Thawing Reagent until the total volume equals 12 mL for T75 flasks (or 45 mL for T225 flasks), and add cell suspension (added volume determined at Step 12) to the media in the flask. Transfer flask to a tissue culture incubator and incubate cells for 24 hours at 37°C and 5% CO_2. To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

14. After 24 hours, examine the cells under a microscope. If the cells appear to be healthy (adhering to the surface of the flask with only a small number of cells remaining in suspension) exchange the AssayComplete™ Thawing Reagent with 12 mL for T75 flasks (or 45 mL for T225 flasks) cell culture media supplemented with the recommended concentration of selection antibiotic. Refer to cell line-specific datasheet to determine the correct Cell Culture Kit and the recommended antibiotic and antibiotic concentration for your cell line.

The cell culture media comprises of the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotic.

15. Return the flask to a tissue culture incubator. If the cells do not appear to be healthy or if confluency is <25%, incubate for additional 24 to 48 hours to allow for additional cell recovery before splitting cells.

16. Once the cells become ≥70% confluent in the flask, split the cells every 2 to 3 days, based on the doubling time of the cell line, using cell culture media containing appropriate selection antibiotics. Typical split ratios for common cell backgrounds are indicated in the table below:

Cell Background	Suggested Split Ratio
CHO-K1	1:10
HEK 293	1:5
U2OS	1:3

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 1 x 10^6 per vial).

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.

Care should be taken in handling to avoid contamination.

2. Gently aspirate the media from the flasks
3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.
4. Gently aspirate PBS from the flask.
5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).
6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.

Keep cells on ice during this process and transfer to a cryogenic container.

7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
9. Add 5 mL cell culture media containing appropriate selection antibiotic, to each T75 flask (or 15 mL to each T225 flask).

10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.

11. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional cell culture media containing appropriate selection antibiotic, to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.

12. For the purpose of determining the concentration of cells in the suspension:

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.

    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.

13. Centrifuge the collected cells at 300 X g for 4 minutes.

14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.

15. Based on the total cell number calculated in Step 12 above, re-suspend cells to the desired concentration (e.g. 1 x 10^6 to 2 x 10^6 cells/mL) with ice cold AssayComplete™ Freezing Reagent.

16. Make 1 mL aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.

17. Freeze cells in a –80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

Keep cells on ice during this process to maintain cell viability.

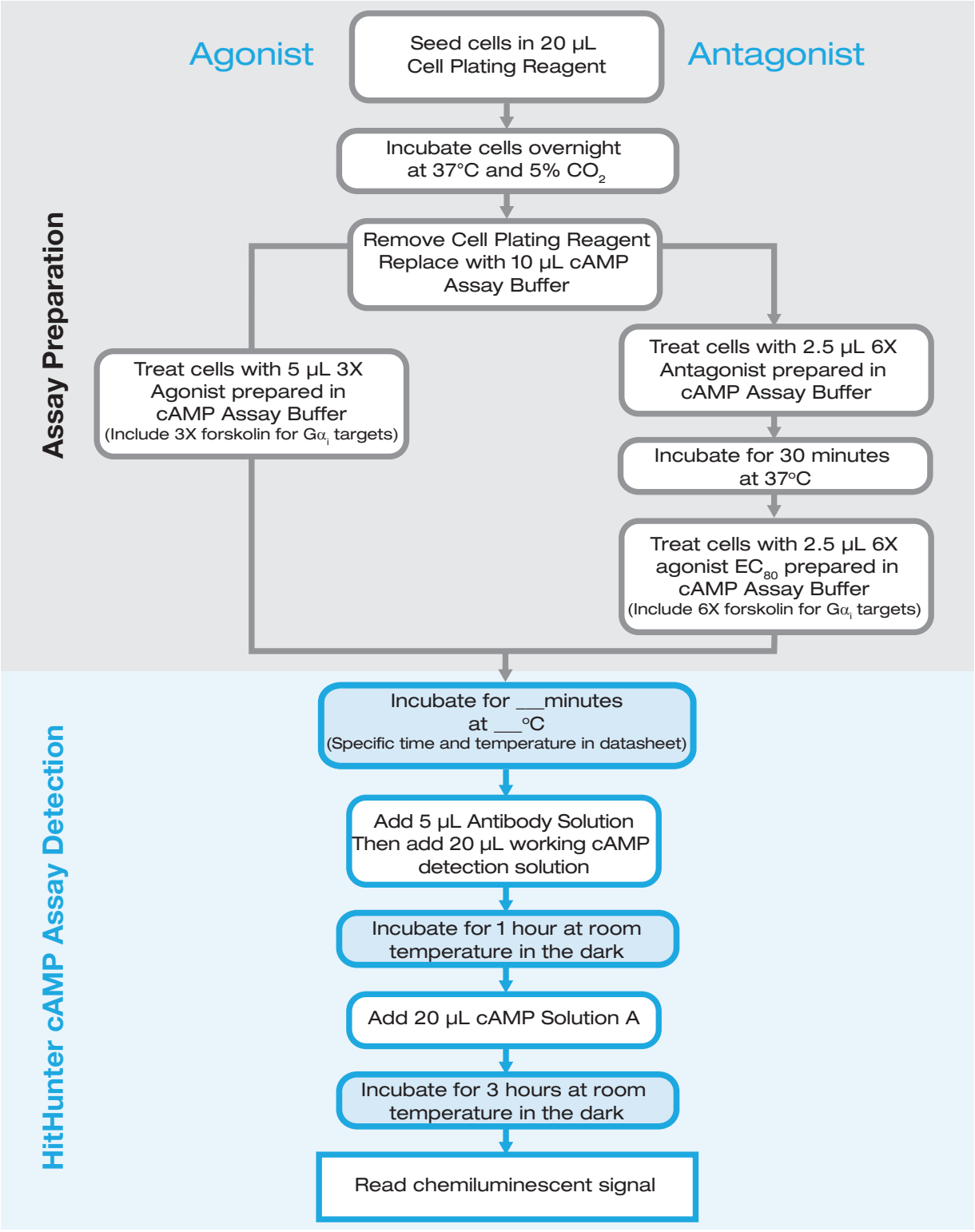
Protocol Schematic

Tip: *Use this sheet to note your assay specific conditions. Post it on your bench to use as a quick reference guide.*

Assay Name:_____ Date: _____

Product Details: _____

Quick-Start Procedure: In a white-walled 384-well tissue culture treated plate, perform the following steps:



Detailed Assay Protocol ($G\alpha_s$ Agonist)

The following is a procedure for testing for a dose dependent agonist response from $G\alpha_s$ receptors in a 384-well assay plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes.

Assay Reagents	96-Well Plate Volume per Well (µL)	384-Well Plate Volume per Well (µL)
AssayComplete™ Cell Plating Reagent*	100*	20*
cAMP Assay Buffer	30	10
Ligand (e.g. Agonist)	15	5
cAMP Antibody Reagent	15	5
Working cAMP Detection Solution	60	20
cAMP Solution A	60	20
Total Assay Volume	**180**	**60**

Note: For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

* The AssayComplete Cell Plating Reagent volume is used when plating cells. The Cell Plating Reagent will then be removed and replaced with the cAMP Assay Buffer at the start of the cAMP assay set-up.

Section I: Cell Preparation and Plating

The following is a protocol for harvesting cells (e.g. cAMP Hunter cell lines) from a confluent T75 or T225 flask, and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that cells have been cultured in AssayComplete Cell Culture Media.

Do not use trypsin for this step; especially with assays interrogating membrane-anchored receptors (e.g. GPCRs), use of trypsin at this step can negatively affect assay results. Additionally, in Step 2 below, it is important to rinse the cells with AssayComplete Cell Detachment Reagent. It is not recommended to use PBS to rinse cells as PBS may inhibit the detachment of cells from the plate.

1. Aspirate the media from the flask.
2. Add 2 mL AssayComplete Cell Detachment Reagent into each T75 flask (or 3 mL to T225 flasks). Swirl to rinse the cells.
3. Gently aspirate AssayComplete Cell Detachment Reagent from flask.
4. Add 1 mL fresh AssayComplete Cell Detachment Reagent to the T75 flasks (or 3 mL to the T225 flasks).
5. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with AssayComplete Cell Detachment Reagent.
6. Incubate at 37°C and 5% CO_2 for 2 to 5 minutes or until the cells have detached.
7. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
8. Add 4 mL AssayComplete Cell Plating Reagent to T75 flasks (or 7 mL to T225 flasks). Note: Refer to datasheet to determine the correct Cell Plating Reagent for this cell line.
9. Using a pipette, gently rinse the cells from the surface of the flask with the added media.
10. Remove the entire amount of cells from the flask and transfer to a 15 mL conical centrifuge tube.

11. For the purpose of determining the concentration of cells in the suspension:

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.

    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.

Keep suspended cells on ice to protect cell viability until ready for transfer to the assay plate.

12. Centrifuge the collected cells at 300 X g for 4 minutes.

13. After centrifugation, discard the supernatant, and re-suspend the cell pellet in AssayComplete™ Cell Plating Reagent. Based on the cell number obtained in Step 11 above, dilute the resuspended cells to the desired concentration (e.g. 500,000 cells/mL or 10,000 cells/20 µL).

14. Transfer 20 µL/well of the cell suspension to a 384-well (or 100 µL/well to a 96-well) assay plate. Leave Rows O and P empty so that they can be used for the cAMP Standard (Section V).

15. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line datasheet for recommended cell incubation time.

16. Proceed to compound preparation and addition.

Section II: Assay Plate Preparation

At this point, cells have been added to the assay plate and incubated overnight to allow cells to attach to the plate and grow. The following steps are for running a cAMP detection assay, starting with removing the Cell Plating Reagent and replacing it with the cAMP Assay Buffer.

1. Completely remove the Cell Plating Reagent from assay wells by aspiration.

2. Immediately add 10 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 10 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve (Section V).

Removing the media completely is crucial for reducing variability of replicates.

Section III: $G\alpha_s$ Agonist Preparation

The following is a procedure for setting up an agonist dose response dilution.

1. Prepare compound (agonist) serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of compound in cAMP Assay Buffer.

2. The concentration of each dilution should be prepared at 3X of the final screening concentration.

    a. For each compound, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.

    b. Add 60 µL of cAMP Assay Buffer to dilution wells No. 2 through No. 12. This exceeds the volume that is enough for four rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

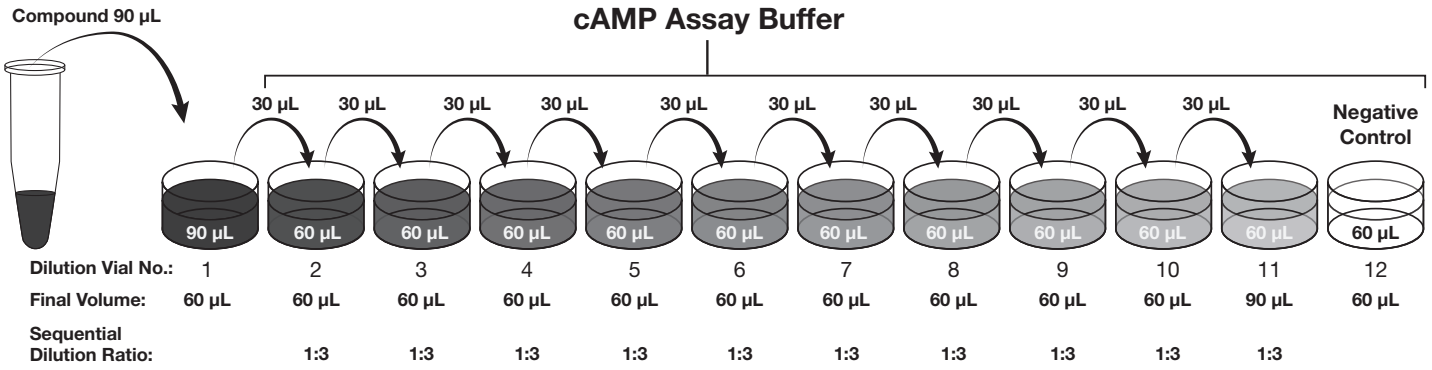
c. Prepare the highest concentration of compound in cAMP Assay Buffer. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the compound. Therefore, prepare a working concentration that is 750X the expected EC_{50} per well to get a 3X working compound concentration. For example, for an expected EC_{50} of 1 nM, prepare the highest working concentration at 750 nM. This is 3X the screening concentration or final highest concentration of 250 nM, and the expected EC_{50} will lie near the center of the dose response curve.

d. Add 90 µL of the highest concentration of compound to well No. 1 (see figure below).

e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

g. Repeat this process until well No. 11 is reached. Do not add compound to well No. 12 since this is the negative control well.

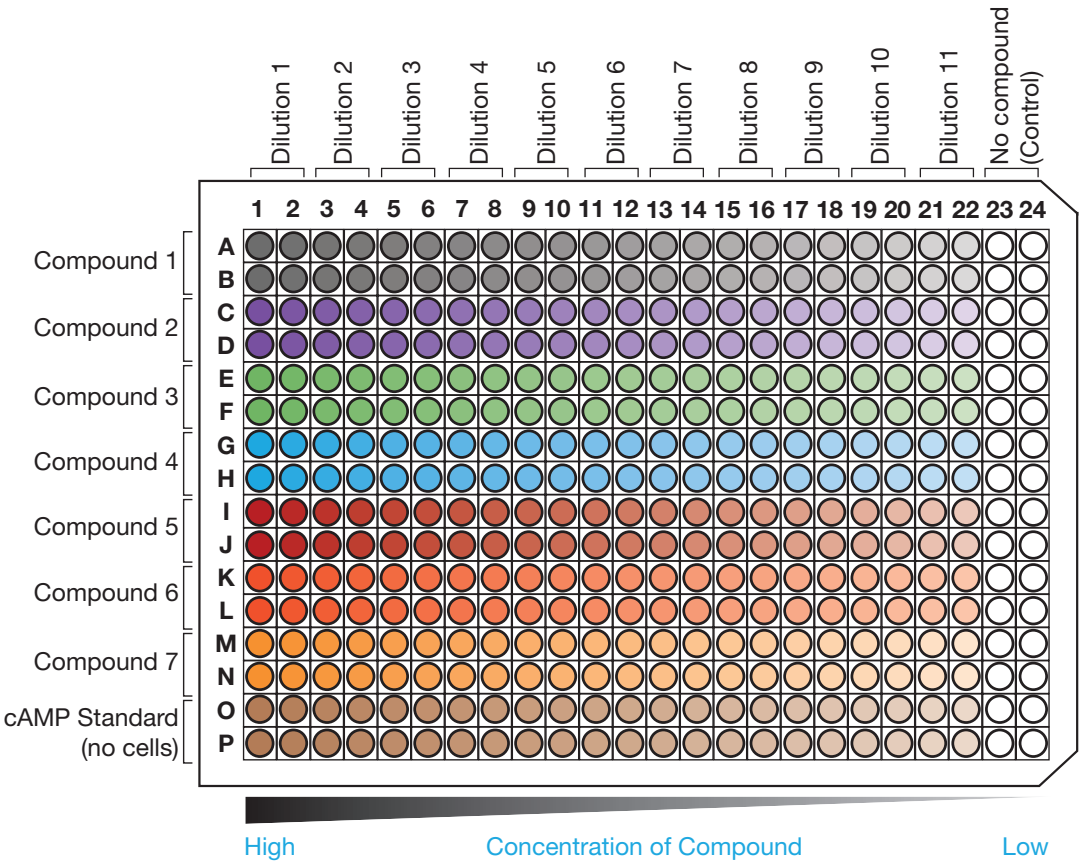


Compound serial dilutions: Create eleven 3-fold serial dilutions of compound in a separate dilution plate.

3. Set up serial dilutions for any additional compounds in a similar manner.

Section IV: Gα$_s$ Agonist Addition

The following is a procedure for adding the agonist dose response dilution to the assay plate.



Compound Assay Plate Map: Create 11-point curves in quadruplicate.

1. Add 5 µL of each 3X agonist serial dilution in quadruplicate to the designated compound Rows (e.g. compound 1 in Rows A and B, Columns 1 and 2; compound 2 in Rows C and D, Columns 1 and 2) of the assay plate as indicated in the Compound Assay Plate Map.

Do not add compound to wells intended for the cAMP Standard curve.

2. Incubate assay plate at the indicated time and temperature for the specific cell line (please refer to the specific cell line datasheet for conditions). For most cell lines, incubate for 30 minutes at 37°C. Any alternative incubation time should be empirically determined.

Section V: cAMP Standard Preparation

When optimizing the assay conditions, always include a cAMP standard curve. The standard curve not only verifies that the kit components are working properly, but also serves as a detection limit guide. If the amount of cAMP being detected exceeds the detection limit of the cAMP detection kit, the EC_{50} will start to shift (depending on the coupling status of Gα$_s$ and Gα$_i$, the shift will be towards the right or left). To avoid this situation, the cell number per well should be optimized. cAMP standard should be prepared fresh before agonist compound addition.

1. Prepare cAMP Standard serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of the standard in cAMP Assay Buffer. This dilution scheme will result in a final high dose of 2.31 x 10^{-6} M cAMP Standard (after all detection reagents and buffers are also added).

   a. Using a separate dilution plate (or polypropylene tubes), label the wells No. 1 to No. 12.

   b. Add 60 µL of cAMP Assay Buffer to wells No. 2 to No. 12.

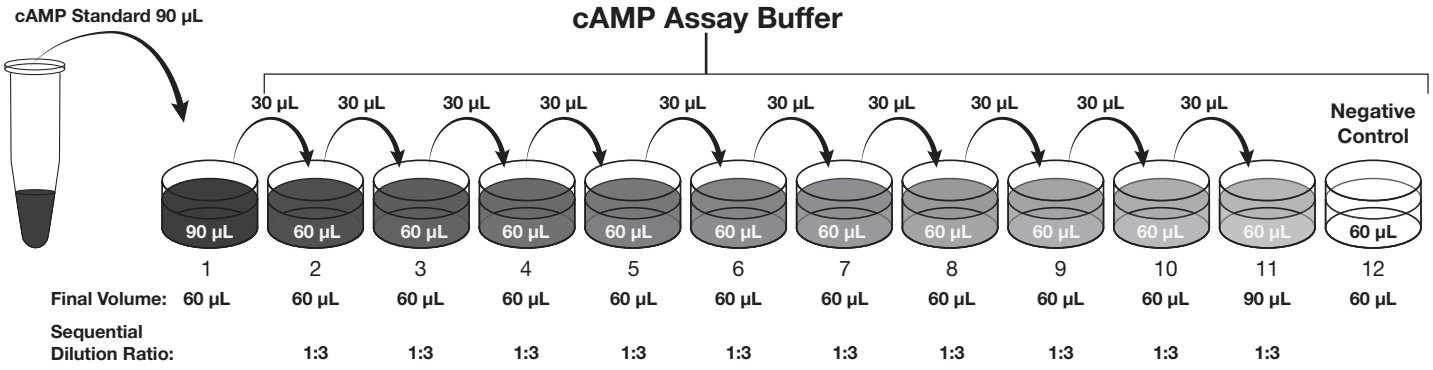
   c. Prepare the highest concentration of cAMP Standard by diluting the cAMP Standard stock [2.5 x 10^{-4} M] in a 1:9 ratio (1-part cAMP standard plus 8-parts cAMP Assay Buffer).

   d. Add 90 µL of the highest concentration cAMP standard (the 1:9 dilution) to well No. 1 (see figure below).

   e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

   f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

   g. Repeat this process until well No. 11 is reached. Do not add cAMP standard to well No. 12 since this is the negative control well.



2. Add 5 µL of the cAMP Standard serial dilution in quadruplicate to the designated cAMP Standard rows (e.g. Rows O and P or wells containing only cAMP Assay Buffer) of the assay plate as indicated in the Compound Assay Plate Map.

Section VI: Antibody and Working Detection Solution Additions

At this point, the agonist stimulation step has been completed. The following Sections VI and VII contain procedures for adding the cAMP assay detection reagents.

Mix the working cAMP detection solution within 8 hours of use.

1. Following agonist incubation, add 5 µL of cAMP Antibody Reagent to all wells.
2. Prepare a stock of working cAMP 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 of Substrate Reagent 2, and 25-parts of cAMP Solution D.
3. Add 20 µL of working cAMP detection solution to all wells of the assay plate (including cAMP Standard wells). Do not mix the wells by pipetting up and down or vortexing the plates.

Working cAMP Detection Solution		
Components	**Volume Ratio**	**Volume per 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**

4. Incubate assay plate for 1 hour at room temperature in the dark for the immunocompetition reaction to occur.

Working cAMP detection solution is light sensitive, thus incubation in the dark is necessary.

Section VII: cAMP Solution A Addition

1. Add 20 µL of cAMP Solution A to all wells of the assay plate (including cAMP Standard wells). Do not mix the wells by pipetting up and down or by vortexing the plates.
2. Incubate assay plate for 3 hours at room temperature in the dark.

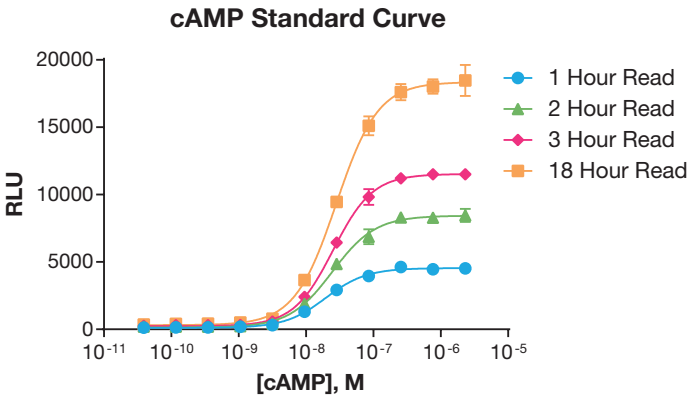
Section VIII: Assay Plate Reading

The following is a procedure for reading the assay plate on a luminometer.

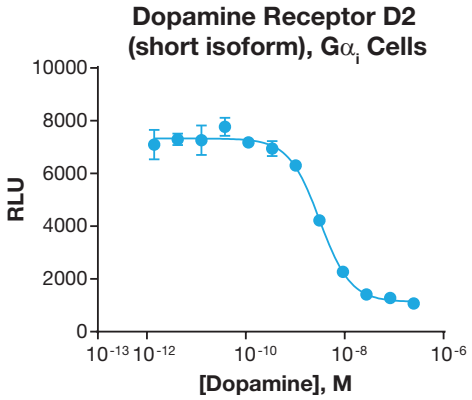
1. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The plate may be incubated overnight and the signal may be measured the next day. In general, the signal continues to increase and reaches a maximum approximately 3 to 6 hours after the last incubation step. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. Luminescence detectors usually collect signal from all wavelengths. Some instrument manufacturers may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.
2. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, BioTek Gen5, Microsoft Excel, etc.).

Typical Results

Typical results shown below of the cAMP standard curve analysis (top) and HitHunter cAMP Assay using the cAMP Hunter Dopamine Receptor D2 (short isoform) Gα$_i$ cells (bottom). Note for the cAMP standard curve graph, the signal continues to increase over time and plates can be read the following day.



	1 Hour Read	2 Hour Read	3 Hour Read	18 Hour Read
EC$_{50}$	19.6 nM	24.7 nM	25.3 nM	18.2 nM
S/B	37.6	42.2	43.1	50.4



EC$_{50}$	1.4 nM
S/B	7.1

Detailed Assay Protocol ($G\alpha_i$ Agonist)

The following is a procedure for testing for a dose dependent agonist response from $G\alpha_i$ receptors in a 384-well assay plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes.

A $G\alpha_i$ agonist assay preparation differs from a $G\alpha_s$ agonist assay preparation in that a $G\alpha_i$ agonist assay requires the use of forskolin in the reaction to stimulate cAMP production.

Assay Reagents	96-Well Plate Volume per Well (µL)	384-Well Plate Volume per Well (µL)
AssayComplete™ Cell Plating Reagent*	100*	20*
cAMP Assay Buffer	30	10
Ligand (e.g. Agonist + Forskolin)	15	5
cAMP Antibody Reagent	15	5
Working cAMP Detection Solution	60	20
cAMP Solution A	60	20
Total Assay Volume	**180**	**60**

Note: For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

*The AssayComplete Cell Plating Reagent volume is used when plating cells. The Cell Plating Reagent will then be removed and replaced with the cAMP Assay Buffer at the start of the cAMP assay set-up.

Section I: Assay Plate Preparation

At this point, cells have been added to the assay plate and incubated overnight to allow cells to attach to the plate and grow. The following steps are for running a cAMP assay, starting with removing the Cell Plating Reagent and replacing it with the cAMP Assay Buffer.

1. Completely remove the Cell Plating Reagent from assay wells by aspiration.
2. Immediately add 10 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 10 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve.

Removing the media completely is crucial for reducing variability of replicates.

Section II: $G\alpha_i$ Agonist Preparation

The following is a procedure for setting up an agonist dose response curve that includes forskolin.

1. Prepare compound (agonist) serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of compound in cAMP Assay Buffer plus forskolin.

Refer to graph on the cell line-specific datasheet to find the recommended final forskolin concentration.

2. The concentration of each dilution plus forskolin should be prepared at 3X of the final screening concentration.

   a. For each compound, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.

   b. Prepare a solution of cAMP Assay Buffer plus forskolin. The concentration of forskolin in the mix should be 3X the final forskolin concentration required for the assay. Add 60 µL of the cAMP Assay Buffer plus forskolin mixture to dilution wells No. 2 through No. 12. This exceeds the volume that is required for four rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

   c. Prepare the highest concentration of compound in cAMP Assay Buffer. The compound preparation must also include forskolin. We recommend preparing a final screening concentration that is 250X the expected EC_{50} of the compound, and also adding forskolin to the mix at 3X the final forskolin concentration required for the assay.

It's recommended that the final concentration of DMSO from the added forskolin be 0.5% or less (i.e. DMSO in 3X forskolin preparation would be 1.5% or less).

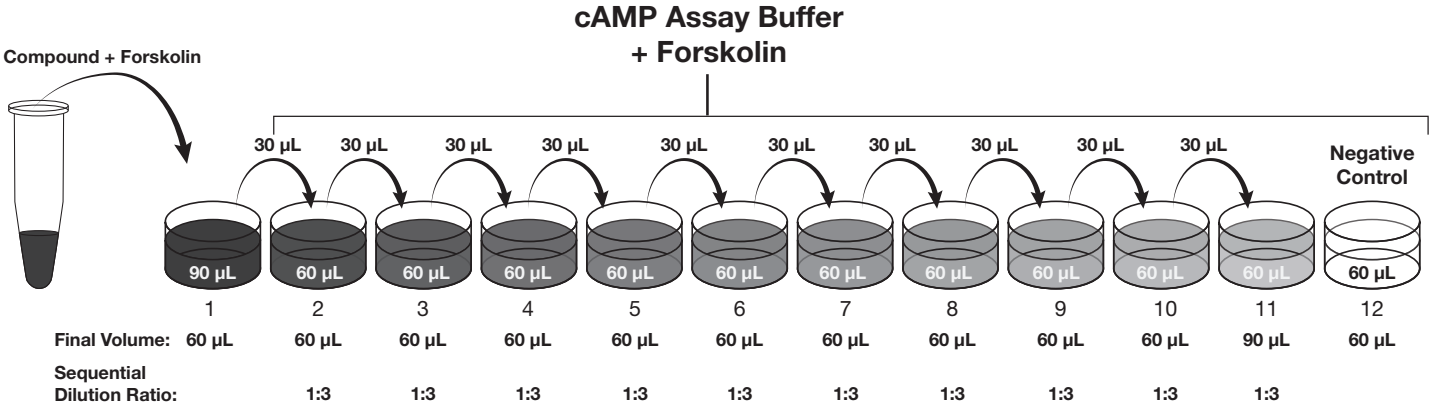
   d. Add 90 µL of the highest concentration of compound plus forskolin mixture to well No. 1 (see figure below).

   e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

   f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

   g. Repeat this process until well No. 11 is reached. Do not add compound to well No. 12 since this is the negative control well.

3. Set up serial dilutions for additional compounds in a similar manner.



Section III: $G\alpha_i$ Agonist Addition

For adding the agonist dose response curve that includes forskolin to the assay plate, follow instructions in the $G\alpha_s$ Agonist Addition (Section IV) under Detailed Assay Protocol ($G\alpha_s$ Agonist).

Section IV: Assay Detection and Plate Reading

Follow the instructions in Antibody and Working Detection Solution Additions (Section VI), cAMP Solution A Addition (Section VII), and Assay Plate Reading (Section VIII) under Detailed Assay Protocol ($G\alpha_s$ Agonist).

Detailed Assay Protocol ($G\alpha_s$ and $G\alpha_i$ Antagonist)

The following is a procedure for testing for a dose dependent antagonist inhibition of either $G\alpha_s$ or $G\alpha_i$ receptors in a 384-well assay plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes. It is important to pay attention to details in the following protocols that are specific to $G\alpha_i$ and $G\alpha_s$ receptor assays.

Antagonist tests are typically run by pre-treating the target cells with antagonist, followed by stimulation of unoccupied receptors with a dose of a receptor agonist. Receptors not occupied by antagonists can be bound by agonists, which will activate the receptors. Receptors that are occupied by antagonist cannot bind agonists and will remain inactive.

Assay Reagents	96-Well Plate Volume per Well (µL)	384-Well Plate Volume per Well (µL)
AssayComplete™ Cell Plating Reagent*	**100***	**20***
cAMP Assay Buffer	30	10
Antagonist	7.5	2.5
Agonist EC_{80} (Plus Forskolin if $G\alpha_i$)	7.5	2.5
cAMP Antibody Reagent	15	5
Working cAMP Detection Solution	60	20
cAMP Solution A	60	20
Total Assay Volume	**180**	**60**

Note: For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of cell number may be required.

* The AssayComplete Cell Plating Reagent volume is used when plating cells. The Cell Plating Reagent will then be removed and replaced with the cAMP Assay Buffer at the start of the cAMP assay set-up.

Section I: Assay Plate Preparation

At this point, cells have been added to the assay plate and incubated overnight to allow cells to attach to the plate and grow. The following steps are for running a cAMP assay, starting with removing the Cell Plating Reagent and replacing it with the cAMP Assay Buffer.

1. Completely remove the Cell Plating Reagent from assay wells by aspiration.

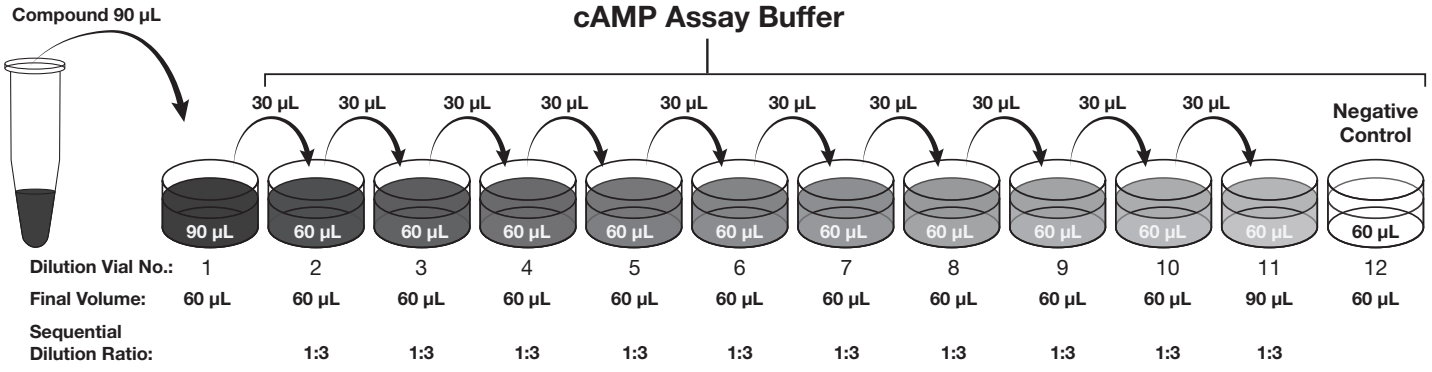
Removing the media completely is crucial for reducing variability of replicates.

2. Immediately add 10 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 10 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve.

Section II: Antagonist or Antibody Preparation

The following is a procedure for setting up an antagonist dose response dilution.

1. Prepare an 11-point series of 3-fold compound (antagonist) serial dilutions in cAMP Assay Buffer in a separate dilution plate. The concentration of each dilution should be prepared at 6X of the final screening concentration.
   a. For each compound, label the wells of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
   b. Add 60 µL of cAMP Assay Buffer to dilution wells No. 2 through No. 12. This exceeds the volume that is required for four rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.
   c. Prepare the highest concentration of compound in cAMP Assay Buffer. We recommend preparing a final screening concentration that is 250X the expected IC_{50} of the compound. Therefore, prepare a working concentration that is 1,500X the expected IC_{50} per well to get a 6X working compound concentration (e.g. for an expected IC_{50} of 1 nM, prepare the highest working concentration at 1,500 nM. This is 6X the screening or final highest concentration of 250 nM, and the expected IC_{50} will lie near the center of the dose response curve).
   d. Add 90 µL of the highest concentration of compound to well No. 1 (see figure below).
   e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.
   f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.
   g. Repeat this process until well No. 11 is reached. Do not add compound to well No. 12 since this is the negative control well.
2. Set up serial dilutions for any additional compounds in a similar manner.



Compound serial dilutions: Create eleven 3-fold serial dilutions of compound in a separate dilution plate.

Section III: Agonist EC_{80} Challenge Preparation

The following is a protocol for preparing an agonist challenge dose that will be added to the cells to stimulate receptors that are not occupied by antagonists. A $G\alpha_i$ agonist preparation differs from a $G\alpha_s$ agonist preparation in that a $G\alpha_i$ assay requires the use of forskolin in the reaction to stimulate cAMP production.

1. Determine the agonist EC_{80} needed for the experiment.
    a. For $G\alpha_s$ receptors follow the $G\alpha_s$ Agonist Preparation and Addition sections in the Detailed Assay Protocol ($G\alpha_s$ Agonist) (Sections III and IV); for $G\alpha_i$ receptors follow $G\alpha_i$ Agonist Preparation and Addition sections in the Detailed Assay Protocol ($G\alpha_i$ Agonist) (Sections II and III) to generate an agonist reference curve.
    b. Plot the agonist response data using a variable slope sigmoidal curve.
    c. Determine EC_{50} and Hill Slope.
    d. Calculate EC_{80} value (refer to the Frequently Asked Questions section for EC_{80} calculation).
2. Prepare an Agonist EC_{80} dilution. Follow the appropriate instructions for $G\alpha_s$ and $G\alpha_i$ receptors below:
    a. For $G\alpha_s$ receptors: Prepare an agonist EC_{80} dilution in cAMP Assay Buffer, in a separate tube, that is 6X the final desired agonist dosage.
    b. For $G\alpha_i$ receptors: Prepare an agonist EC_{80} dilution plus forskolin in cAMP Assay Buffer in a separate tube. Both the agonist and the forskolin in the mixture should be 6X the final desired dosages.
3. Add equal volume aliquots of 6X agonist EC_{80} (or 6X agonist EC_{80}/forskolin for $G\alpha_i$ receptors) to wells No. 1 through No. 12 of a compound dilution plate.

Refer to graph on the cell line-specific datasheet to find the recommended final forskolin concentration.

Section IV: Antagonist and Agonist EC_{80} Additions

The following is a procedure for adding the antagonist dose response curve followed by addition of the agonist EC_{80} dose.

1. Add 2.5 µL of each 6X antagonist serial dilution in quadruplicate to the designated compound (antagonist) rows (e.g. compound 1 in Rows A and B, Columns 1 and 2; compound 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining compounds as indicated on the previously described Compound Assay Plate Map.
2. Incubate assay plate for 30 minutes at 37°C and 5% CO_2.
3. Add 2.5 µL of the 6X agonist EC_{80} dilution (or 6X agonist EC_{80}/forskolin for $G\alpha_i$ receptors) to each assay well.
4. Incubate assay plate at the indicated time and temperature (for agonist incubation) for the specific cell line (refer to the specific cell line datasheet for conditions). For most cell lines, incubate 30 minutes at 37°C and 5% CO_2. For the best results, optimal incubation time should be empirically determined.

Section V: Assay Detection and Plate Reading

Follow the instructions in Antibody and Working Detection Solution Additions (Section VI), cAMP Solution A Addition (Section VII), and Assay Plate Reading (Section VIII) under Detailed Assay Protocol ($G\alpha_s$ Agonist).

Detailed Assay Protocol (Anti-Ligand Antibody)

The following is a procedure for testing for a dose dependent inhibition of an agonist by an anti-ligand antibody in a 384-well assay plate. For assays to be run in a 96-well plate, refer to the table for assay reagent volumes. It is important to pay attention to details in the following protocols that are specific to $G\alpha_i$ and $G\alpha_s$ receptor assays.

Anti-ligand tests are typically run by pre-incubating the agonist with the anti-ligand antibody prior to loading the test sample onto the cell assay. Agonist occupied by the anti-ligand antibody will be unable to bind to and activate the receptor.

Section I: Assay Plate Preparation

At this point, cells have been added to the assay plate and incubated overnight to allow cells to attach to the plate and grow. The following steps are for running a cAMP assay, starting with removing the Cell Plating Reagent and replacing it with the cAMP Assay Buffer.

1. Completely remove the Cell Plating Reagent from assay wells by aspiration.
2. Immediately add 10 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 10 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve.

Removing the media completely is crucial for reducing variability of replicates.

Section II: Anti-Ligand Antibody Preparation

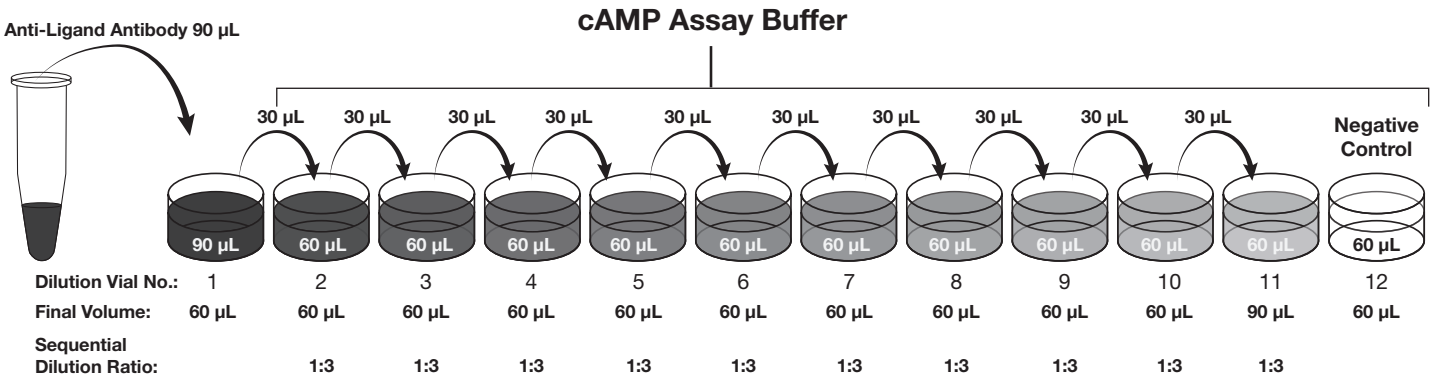
The following is a procedure for setting up an anti-ligand antibody dose response dilution.

1. Prepare an 11-point series of 3-fold compound (anti-ligand antibody) serial dilutions in cAMP Assay Buffer in a separate dilution plate. The concentration of each dilution should be prepared at 6X of the final screening concentration.
    a. For each compound, label the wells in Row A of a dilution plate (or polypropylene tubes) No. 1 through No. 12.
    b. Add 60 µL of cAMP Assay Buffer to dilution wells No. 2 through No. 12. This exceeds the volume that is required for four rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.
    c. Prepare the highest concentration of compound in cAMP Assay Buffer. We recommend preparing a final screening concentration that is 250X the expected IC_{50} of the compound. Therefore, prepare a working concentration that is 1,500X the expected IC_{50} per well to get a 6X working compound concentration (e.g. for an expected IC_{50} of 1 nM, prepare the highest working concentration at 1,500 nM. This is 6X the screening or final highest concentration of 250 nM, and the expected IC_{50} will lie near the center of the dose response curve).
    d. Add 90 µL of the highest concentration of antibody to well No. 1 (see figure below).
    e. Remove 30 µL from well No. 1 and add it to well No. 2. Mix gently.

f. With a clean tip, remove 30 µL from well No. 2 and add it to well No. 3. Mix gently.

g. Repeat this process until well No. 11 is reached. Do not add compound to well No. 12 since this is the negative control well.

2. Set up serial dilutions for additional compounds in a similar manner.



Compound serial dilutions: Create eleven 3-fold serial dilutions of compound in a separate dilution plate.

Section III: Agonist EC$_{80}$ Challenge Preparation

The following is a protocol for preparing an agonist challenge dosage that will be mixed with the anti-ligand antibody dose response curve. A Gα$_i$ agonist preparation differs from a Gα$_s$ agonist preparation in that a Gα$_i$ assay requires the use of forskolin in the reaction to stimulate cAMP production.

1. Determine the agonist EC$_{80}$ needed for the experiment.

   a. For Gα$_s$ receptors follow the Gα$_s$ Agonist Preparation and Addition sections under Detailed Assay Protocol (Gα$_s$ Agonist) (Sections III and IV); for Gα$_i$ receptors follow Gα$_i$ Agonist Preparation and Addition sections in the Detailed Assay Protocol (Gα$_i$ Agonist) (Sections II and III) to generate an agonist reference curve.

Refer to graph on the cell line-specific datasheet to find the recommended final forskolin concentration.

   b. Plot the agonist response data using a variable slope sigmoidal function.

   c. Determine EC$_{50}$ and Hill Slope.

   d. Calculate EC$_{80}$ value (refer to the Frequently Asked Questions section for EC$_{80}$ calculation).

2. Prepare an agonist EC$_{80}$ dilution. Follow the appropriate instructions for Gα$_s$ and Gα$_i$ receptors below:

   a. For Gα$_s$ receptors: Prepare an agonist EC$_{80}$ dilution in cAMP Assay Buffer, in a separate tube, that is 6X the final desired agonist dosage.

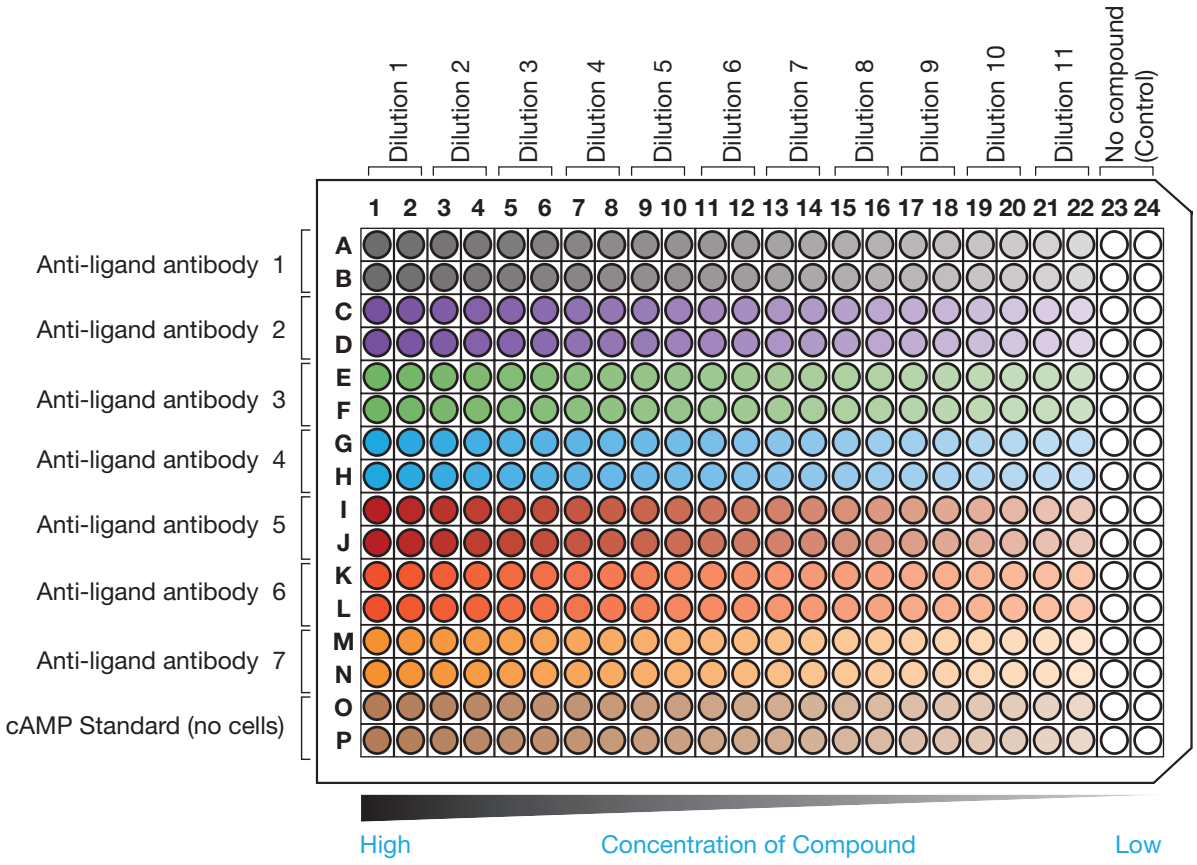
   b. For Gα$_i$ receptors: Prepare an agonist EC$_{80}$ dilution plus forskolin in cAMP Assay Buffer in a separate tube. Both the agonist and the forskolin in the mixture should be 6X the final desired dosages.

3. In Row B of the compound dilution plate, aliquot 30 µL of the 6X agonist EC$_{80}$ (or 6X agonist EC$_{80}$/forskolin for Gα$_i$ receptors) dilution into wells No. 1 through No. 12.

Section IV: Antibody/Agonist Pre-Incubation and Addition

The following is a procedure for the mixing and pre-incubation of the anti-ligand antibody with the agonist EC_{80} dose.

1. Using a multi-channel pipette, transfer 30 µL of 6X antibody dilution that is in wells No. 1 through No. 12 of Row A to the 30 µL of agonist EC_{80} (or 6X agonist EC_{80}/forskolin for Gα$_i$ receptors) that is in wells No. 1 through No. 12 of Row B on the dilution plate. Gently mix by pipetting up and down. The result is a mix of 3X anti-ligand antibody plus 3X agonist EC_{80}.
2. Pre-incubate the anti-ligand and agonist mix for at least 15 minutes. The optimal pre-incubation time and temperature should be determined empirically.
3. Add 5 µL of each 3X antibody/agonist mix in quadruplicate to the designated antibody rows (e.g. antibody/agonist mix 1 in Rows A and B, Columns 1 and 2; antibody/agonist mix 2 in Rows C and D, Columns 1 and 2). Repeat for the remaining antibody/agonist mix as indicated in the Compound Assay Plate Map.
4. Incubate assay plate at the indicated time and temperature (for agonist incubation) for the specific cell line (refer to the specific cell line datasheet for conditions). For most cell lines, incubate 30 minutes at 37°C and 5% CO_2. For the best results, the optimal incubation time should be determined empirically.



Section V: Assay Detection and Plate Reading

Follow the instructions in Antibody and Working Detection Solution Additions (Section VI), cAMP Solution A Addition (Section VII), and Assay Plate Reading (Section VIII) under Detailed Assay Protocol (Gα$_s$ Agonist).

Supplemental Information

Allosteric Modulators

For positive allosteric modulators (PAMs), refer to the antagonist protocol above, but use an agonist challenge concentration of EC_{20} instead of EC_{80}.

For negative allosteric modulators (NAMs), follow the antagonist protocol.

Experiments Using Crude Biologic Samples

The HitHunter™ assays can be run in the presence of high levels of serum or plasma without significantly impacting assay performance. Therefore, samples can be prepared in neat serum or plasma, and added directly to cells without further dilution. For the best results, the optimized minimum required dilution of crude samples should be empirically determined. Also, after sample treatment, it may ultimately be necessary to remove the biologic test sample from the cells, and replace it with 45 µL fresh cAMP Assay Buffer prior to the additions of the cAMP Antibody Reagent, the working cAMP detection solution, and the cAMP Solution A. The necessity of adding such a step must be determined empirically. High levels of protein in the wells may interfere with the EFC reaction or the optics of the plate reader.

High-Throughput Screening Protocol (Optional)

1. For high-throughput screening, one reagent addition step can be eliminated by adding the cAMP Antibody Reagent, premixed with cAMP Assay Buffer, to the assay wells prior to addition of compound.
2. Prepare a solution of cAMP Assay Buffer and cAMP Antibody Reagent by mixing 1-part of Antibody Reagent with 2-parts of cAMP Assay Buffer.
3. Completely remove the cell media from assay wells by aspiration.
4. Immediately add 15 µL of cAMP Assay Buffer/cAMP Antibody Reagent mixture to all wells of the assay plate.
5. Prepare 4X agonist dilution (or 4X agonist plus 4X forskolin mixture for $G\alpha_i$ receptors).
6. Add 5 µL 4X agonist dilution to the assay wells.
7. Incubate assay plate at the indicated time and temperature for the specific cell line (please refer to the specific cell line datasheet for conditions). For most cell lines, incubate for 30 minutes at 37°C. For the best results, the optional incubation time should be empirically determined.
8. Follow Antibody and Working Detection Solution Additions (Section VI), cAMP Solution A Addition (Section VII), and Assay Plate Reading (Section VIII) in the Detailed Assay Protocol ($G\alpha_s$ Agonist).

Frequently Asked Questions

Do you perform any quantitative expression analysis of your recombinant cell lines?

- No. Expression level is crucial to induce the correct response to control ligands. We select clones according to ligand response rather than expression level.

At what passage number do you freeze down your inventory lots?

- Passage 2-3.

I understand that you generate your cell lines via retroviral infection as it is a very efficient system. Do the final cell lines produce any viable retroviral particles?

- No. We have confirmed the lack of retroviral particles using a Marker Rescue Assay.

What is the general liquid dispensing speed that you would recommend when using robotic dispensing instruments?

- This will depend on the particular instrument and has to be determined empirically, but in general, we would recommend a speed of 15-20 µL/second.

Can your assays be run in 96-, 384-, and 1536-well formats?

- Yes.

How do I run the assay with cells in suspension?

- cAMP Hunter cell lines are typically adherent cells but assays can be run in suspension mode. Assay performance may or may not be similar to data on cell line-specific datasheet. Additional assay optimization may be required.
- Harvest and resuspend suspension cells in cAMP Assay Buffer (either 1X HBSS + 10 mM HEPES or PBS) at the optimal cell density. Typical suspension cell density is approximately 10,000 cells per well in a standard 384-well plate. The optimal cell number should be determined empirically.

What if there is no or low signal?

- If plated on clear-bottom assay plates, visually inspect the cells before and after compound incubation to ensure that they are healthy. Make sure proper cell plating procedure is followed to ensure assay performance.
- Make sure detection reagents are stored and prepared properly.
- For any experiments involving $G\alpha_i$- coupled receptors, forskolin must be mixed with the agonist to generate a detectable signal.
- Make sure the proper assay mode is used ($G\alpha_s$ or $G\alpha_i$ mode; agonist or antagonist mode).
- Make sure the plate reader is setup properly to read luminescence.
- High levels of protein in the wells may interfere with enzyme fragment complementation. If high levels are present, a media exchange step could be performed just prior to the detection reagent addition.

What if the response is lower than expected (lower than expected S:B)?

- Make sure that the ligand is prepared properly, take extra care to observe ligand solubility.
- Make sure DMSO and other solvents are not too high and not more than 1% final concentration.
- When testing ligands at high concentrations, make sure the vehicle is compatible.
- Make sure ligand is incubated for the designated time and at designated temperature.
- Make sure plates are protected from light during incubation.

What if the EC_{50} does not match reported values?

- 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.
- Make sure ligands are incubated at the proper temperature.
- The source of a ligand, particularly for biologics, can have a large effect on the observed pharmacology.
- Changing tips during serial dilutions can help to avoid carryover.
- Receptor expression level may cause receptor reserve issues in ligand testing. Select a cell line that has medium to low expression of receptors.

What if the variability between replicates is high?

- Make sure dispensing equipment is properly calibrated and proper pipetting technique is used.

What if my compound is in media containing high concentrations of serum, can I use it as is or will the serum interfere with the assay?

- Our assays are highly tolerant to high serum content (as high as 80% serum). To generate optimal results, it may be necessary to aspirate the high serum media and replace it with cAMP Assay Buffer prior to adding the cAMP Antibody Reagent, the working cAMP detection solution, and the cAMP Solution A.

How do you determine EC_{80} from the agonist reference curve?

- Determine the EC_{50} value and the Hill Slope from the agonist reference curve. Use an online EC_{80} calculator like QuickCalc by GraphPad (graphpad.com/quickcalcs/Ecanything1. cfm), or
- Use the formula below where F is the percent response and H is the Hill Slope from the agonist reference curve:

$$EC_F = \left(\frac{F}{100 - F}\right)^{\frac{1}{H}} \cdot EC_{50}$$

- An example of EC_{80} calculation:

$$EC_F = \left(\frac{F}{100 - F}\right)^{\frac{1}{H}} \cdot EC_{50}$$

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