User Manual

HitHunter® cAMP Assay for Biologics

For the Chemiluminescent Detection of cAMP and GPCR Activity

For Assay Kits:

90-0075LM2: 2-Plate Kit

90-0075LM10: 10-Plate Kit

90-0075LM25: 25-Plate Kit

90-0075LM100: 100-Plate Kit

Document Number 70-318 Revision 5

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Overview

The HitHunter cAMP Assay for Biologics kit provides a robust, highly sensitive, and easy-to-use assay cell-based functional assay for monitoring G-protein coupled receptor (GPCR) activation based on 3'-5'-cyclic adenosine monophosphate (cAMP) production in cells. This kit is ideal for testing biologics samples (e.g., crude biologic samples in serum or plasma, purified or non-purified antibodies [anti-ligand or anti-receptor], and hybridoma supernatants). The kits contain all the reagents needed for detecting cAMP from cAMP Hunter™ cells expressing $G\alpha_s$- and $G\alpha_i$-coupled receptors induced with a ligand or agonist biologic. The flexible assay system has been designed to work in agonist or antagonist mode for 96- and 384-well plate formats.

Cell lines from other sources are also compatible with the HitHunter cAMP Assay.

For screening and profiling small molecules, please refer to the HitHunter cAMP Assay for Small Molecules (Cat. No. 90-0075SM Series) designed specifically for such applications.

The Detailed Assay Protocol sections in this user manual contain procedures for testing agonists on $G\alpha_s$ receptor assays, $G\alpha_i$ Agonist, $G\alpha_s$ and $G\alpha_i$ Antagonist, Anti-Ligand Antibody, as well as suggestions for testing PAMs and NAMs. An alternate protocol suitable for high-throughput screening is also included.

Assay Principle

GPCR activation following ligand binding initiates a series of second messenger cascades that result in a cellular response. This signaling involves the membrane-bound enzyme adenylate cyclase. $G\alpha_s$- and $G\alpha_i$- coupled receptors modulate cAMP by either stimulating or inhibiting adenylate cyclase, respectively. With the HitHunter cAMP Assay, cells that overexpress GPCRs utilize the natural coupling status of the GPCR to monitor the 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 (Figure 1).

EFC technology is based on the use of a split β-galactosidase (β-gal) enzyme. The two enzyme fragments are designated the Enzyme Donor (ED) and the Enzyme Acceptor (EA). Independently, these fragments do not have any β-gal enzymatic activity; however, in solution, the two components rapidly complement to form an active β-gal enzyme.

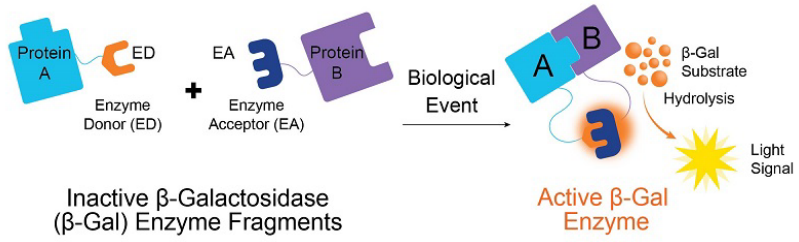


Figure 1. Enzyme Fragment Complementation (EFC) technology

ED-labeled cAMP (ED-cAMP) provided in the kit, and cAMP produced by the activation of the cells compete for anti-cAMP antibody (Ab) binding sites (Figure 2). Antibody-bound ED-cAMP will not be able to complement with EA, but unbound ED-cAMP is free to complement EA to form the active enzyme to act upon the assay substrate to produce a luminescent signal. The amount of light signal produced is directly proportional to the amount of cAMP in the cells.

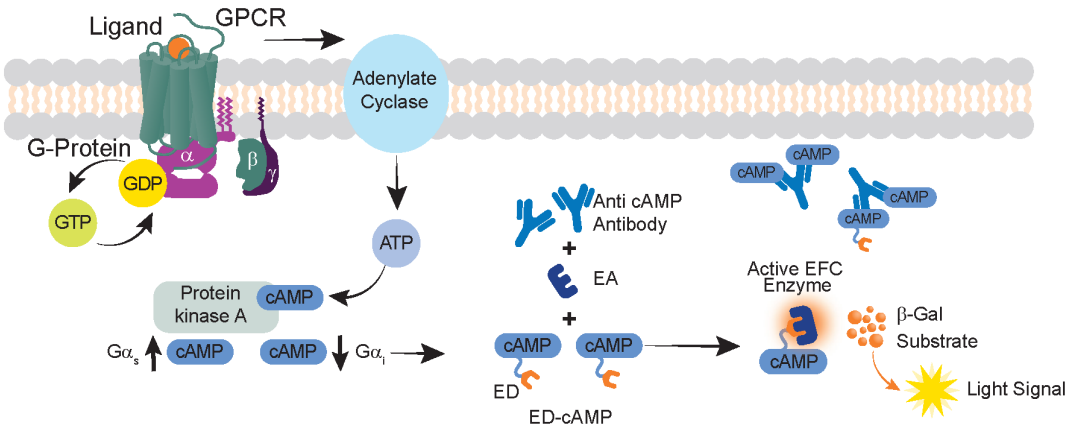


Figure 2. Assay Principle

Assay Workflow

HitHunter cAMP Assays are functional cell-based immunoassays with a chemiluminescent readout. The kits contain all the reagents needed for detecting cAMP from whole cells expressing $G\alpha_i$- and $G\alpha_s$-coupled receptors induced with a biologic or small molecule ligand (Biologics [LM] and Small Molecule [SM] kits, [respectively]). After plating and stimulation of cells, the user simply adds the HitHunter cAMP Assay reagents to the cells following the homogeneous, simple protocol provided (Figure 3). The flexible assay system works in agonist or antagonist mode for 96- and 384-well plate formats.

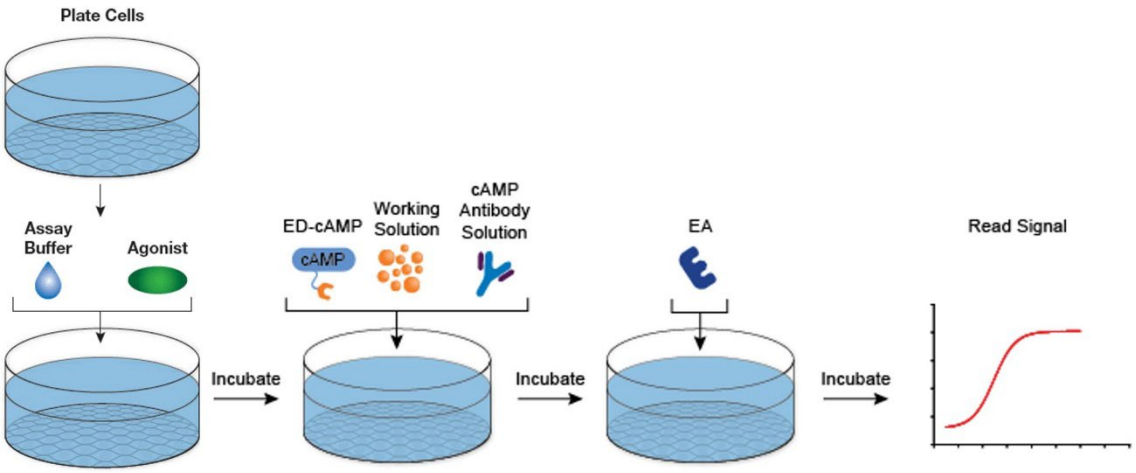


Figure 3. Assay Workflow - Agonist

Materials Provided

Catalog Number	90-0075LM2	90-0075LM10	90-0075LM25	90-0075LM100
Number of Plates*	2	10	25	100
96-Well, Number of Data Points	200	1,000	2,500	10,000
384-Well, Number of Data Points	800	4,000	10,000	40,000
Kit Components	**Total Volume (mL)**			
cAMP Standard (250 µM)	0.2	1	2.5	10
cAMP Antibody Reagent	5	25	62.5	250
cAMP Lysis Buffer	7.6	38	95	380
Substrate Reagent 1	2	10	25	100
Substrate Reagent 2	0.4	2	5	20
cAMP Solution D	10	50	125	500
cAMP Solution A	16	80	200	800

*Plates not provided with the kit.

Table 1. Materials Provided

Storage Conditions

Store reagents at -20°C upon arrival. 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. The detection kit is stable until the expiration date indicated on the kit box outer label if left frozen at -20°C. After thawing, store reagents for up to 1 month at 2-8°C. For long-term storage, aliquots of all the components may be re-frozen in opaque containers at -20°C. The reagents can be thawed and frozen for a total of 3 times without loss in performance.

Additional Materials Required

Materials	Ordering Information
Serial Dilution and Assay Plates	discoverx.com/product-category/cell-culture-kits-reagents/
Ligands	discoverx.com/product-category/control-ligands-inhibitors/
Cell Culture Kits, Reagents, and Consumables	discoverx.com/product-category/cell-culture-kits-reagents/
cAMP Assay Buffer [1X HBSS + 10 mM HEPES; HBSS should contain Mg^{++} and Ca^{++}]	
Tissue Culture Disposables (Pipettes 1 mL to 25 mL) and Plastic-Ware (T25 and T75 Flasks, etc.)	
Cryogenic Vials for Freezing Cells	
Humidified Tissue Culture Incubator (37°C and 5% CO_2)	
Single and multi-channel micropipettes and pipette tips (10 µL-1,000 µL)	
Multimode or Luminescence plate reader	Refer to the Instrument Compatibility Chart at discoverx.com/tools-resources/instrument-compatibility/
Sterile disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar

Table 2. Additional Materials Required

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 96-well tissue culture treated plate, perform the following steps.

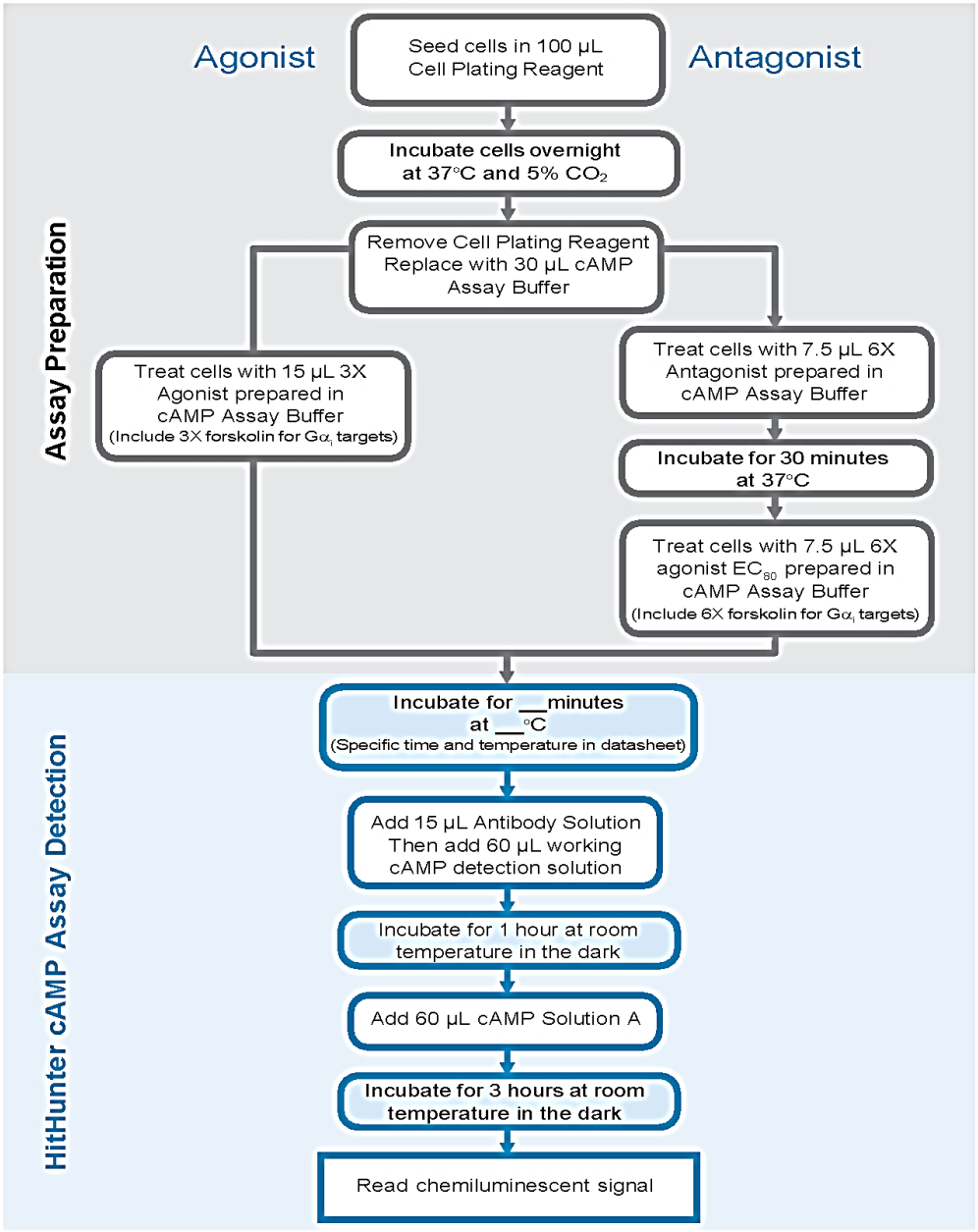


Figure 4. Quick-start Procedure

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 96-well assay plate. For assays to be run in a 384-well plate, refer to the table below 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**

Table 3. Assay reagent volumes per well for 96-well and 384-well plates ($G\alpha_s$ Agonist)

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 the cell number per well 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 (see Additional Materials section for formula) at the start of the cAMP assay set-up.

Experiments Using Crude Biologic Samples

The HitHunter cAMP assays can be run in the presence of high serum or plasma levels 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 of fresh cAMP Assay Buffer prior to the addition 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.

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 DiscoverX 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 the flask.
4. Add 1 mL of 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 it 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 the 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 them to a 15 mL conical centrifuge tube.

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

11. To determine 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 and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.

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 re-suspended cells to the desired concentration (e.g., 200,000 cells/mL or 20,000 cells/100 µL).

14. Transfer 100 µL/well of the cell suspension to a 96-well (or 20 µL/well to a 384-well) assay plate. Leave Rows G and H empty to 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 assay plate preparation.

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.

Complete removal of the media is crucial for reducing the variability of replicates.

   a. Alternate method if vacuum aspiration is not available: With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin for 30 seconds in a plate centrifuge set at 1000 rpm.
   Note: The centrifuge will not reach the speed of 1000 rpm within 30 seconds.

2. Immediately add 30 µL of cAMP Assay Buffer to all assay wells in the assay plate. This step includes adding 30 µL cAMP Assay Buffer to the cell-free wells saved for the cAMP Standard curve (Section V).

Section III: Gαs Agonist Preparation

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

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

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

    a. For each biologic sample, label the wells of a dilution plate (or polypropylene tubes) sequentially number 1 through number 12.

    b. Add 60 µL of cAMP Assay Buffer to dilution wells numbers 2 through number 12. This volume exceeds the requirement for two rows of wells for each concentration per sample (e.g., as shown in Figure 6). The dilution volume may be adjusted according to the number of wells desired.

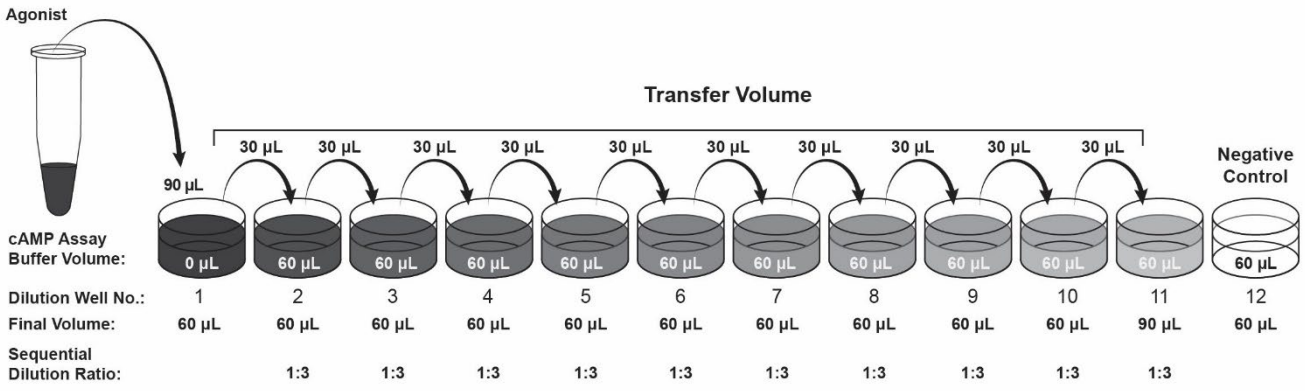
    c. Prepare the highest concentration of the agonist in the cAMP Assay Buffer. We recommend preparing a final screening concentration that is not higher than 10 µg/mL. Therefore, prepare a working concentration of 3X the desired agonist concentration. For example, for a desired agonist concentration of 9 µg/mL per well, prepare the highest working concentration at 27 µg/mL. This is 3X the desired screening concentration of the final highest screening concentration of 9 µg/mL per well and the expected EC_{50} will lie near the center of the dose response curve.

    d. Add 90 µL of the highest concentration of biologic sample to well number 1 (see figure below).

    e. Remove 30 µL from well number 1 and add it to well number 2. Mix gently.

    f. With a clean pipette tip, remove 30 µL from well number 2 and add it to well number 3. Mix gently.

    g. Repeat this process sequentially until well number 11 is reached. Do not add biologic sample to well number 12 since this is the negative control well.



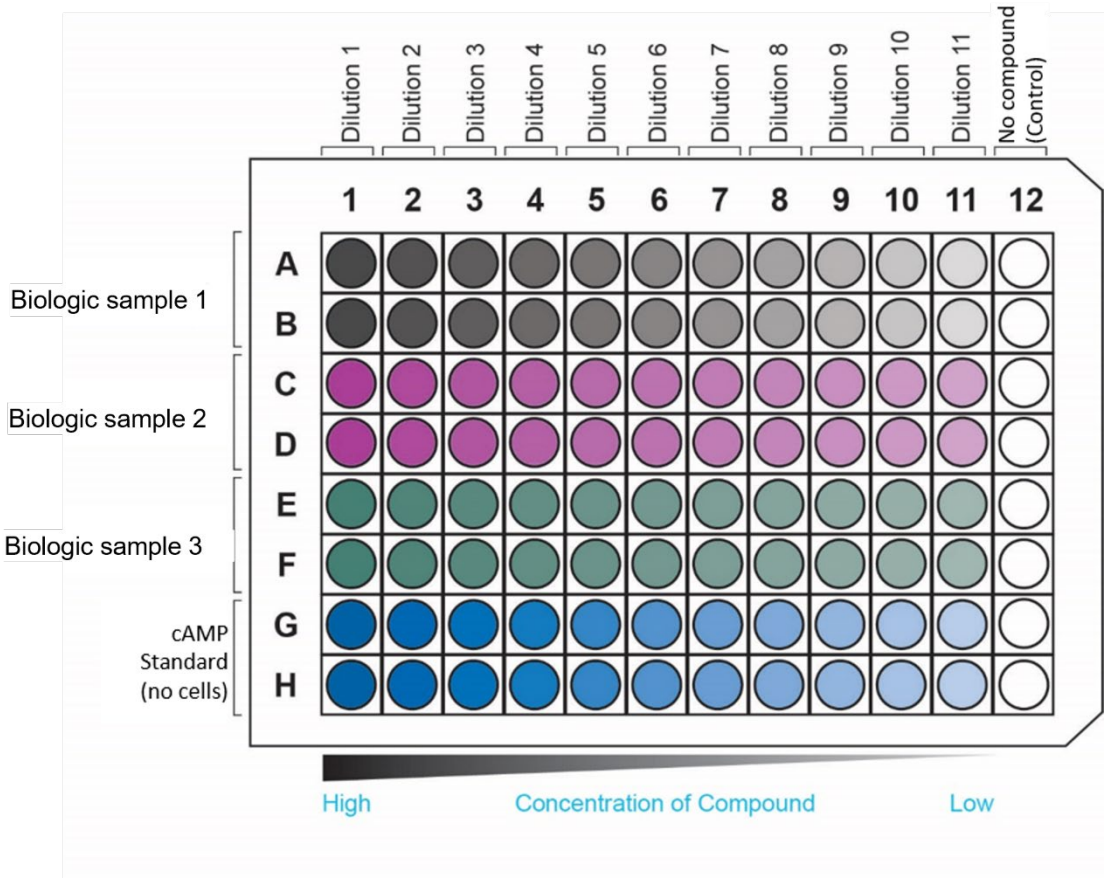
Create 11 three-fold serial dilutions of Agonist in a separate dilution plate.

Figure 5. Biologic sample serial dilutions - Agonist

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

Section IV: Gαs Agonist Addition

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



Create 11-point curves in duplicate.

Figure 6. Biologic sample Assay Plate Map – Agonist dose response dilution addition

1. Add 15 µL of each 3X agonist serial dilution in duplicate to the designated biologic sample rows (e.g., Biologic sample 1 in Rows A and B, Biologic sample 2 in Rows C and D) of the assay plate as indicated in the Biologic sample Assay Plate Map.

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

2. Incubate the 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 $G\alpha_s$ and $G\alpha_i$, the shift will be towards the right or left). To avoid this situation, the cell number per well should be optimized. The cAMP standard should be prepared fresh before agonist biologic sample addition.

1. Prepare the cAMP Standard serial dilutions in a separate dilution plate in an 11-point series of 3-fold dilutions of the standard in the 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 sequentially from number 1 to number 12.

    b. Add 60 µL of cAMP Assay Buffer to wells numbers 2 through number 12.

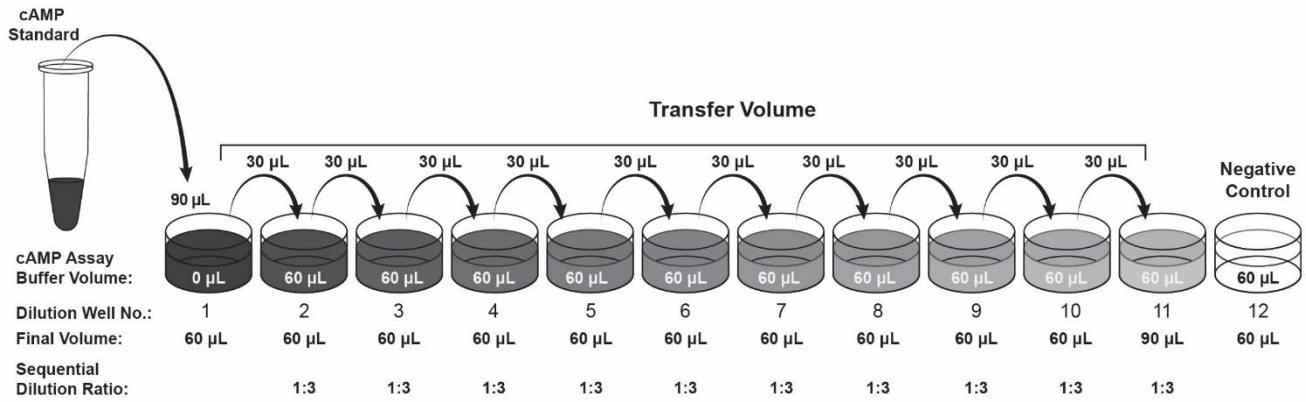
    c. Prepare the highest concentration of cAMP Standard by diluting the cAMP Standard stock [2.5 x 10^{-4} M] in a 1:9 ratio (1-part cAMP standard plus 8-parts cAMP Assay Buffer).

    d. Add 90 µL of the highest concentration cAMP standard (the 1:9 dilution) to well number 1 (see figure below).

    e. Remove 30 µL from well number 1 and add it to well number 2. Mix gently.

    f. With a clean tip, remove 30 µL from well number 2 and add it to well number 3. Mix gently.

    g. Repeat this process until well number 11 is reached. Do not add cAMP standard to well number 12 since this is the negative control well.



Create 11 three-fold serial dilutions of cAMP Standard in a separate dilution plate.

Figure 7. Biologic sample serial dilutions – cAMP Standard

2. Add 15 µL of the cAMP Standard serial dilution in duplicate to the designated cAMP Standard rows (e.g., Rows G and H or wells containing only cAMP Assay Buffer) of the assay plate as indicated in the Biologic sample 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 15 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.

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**

Table 4. Working cAMP Detection Solution

3. Add 60 µL of the 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.

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

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

Section VII: cAMP Solution A Addition

1. Add 60 µL of the 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 the 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 an 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 of approximately 3 to 6 hours after the last incubation step. Lab conditions, such as temperature, affect the actual signal characteristics over time, and the user should establish an optimal read time. Luminescence detectors usually collect signals 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 are shown below. Please refer to the APPLICATIONS tab on this web page, discoverx.com/target-class/gpcr-camp-product-solutions/ for additional examples.

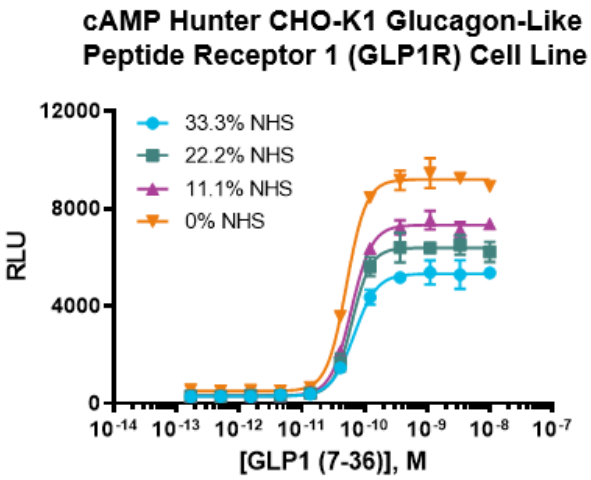


Figure 8. Example of serum tolerance of cAMP Assays (GLP1R)

Experiment using a cAMP Hunter™ glucagon-like peptide receptor 1 (GLP1R) cell line to evaluate human serum tolerance levels. The results show similar EC_{50}s for all samples tested, ranging from only 52 pM for the control sample (no serum) to 68 pM for the sample containing 33.3% human serum. This indicates HitHunter cAMP assays will tolerate high serum levels while maintaining robust assay performance.

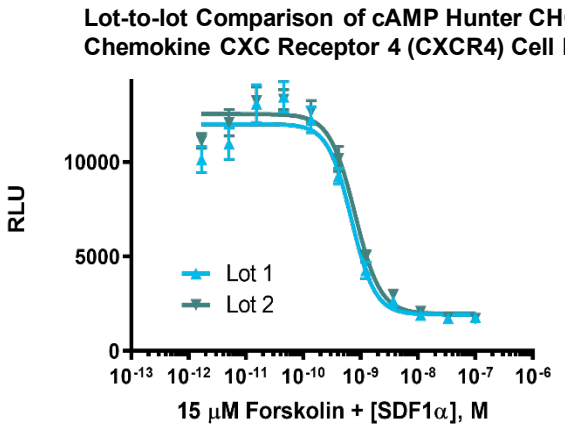


Figure 9. Example of lot-to-lot consistency in G_i-coupled CXCR4 cell line

Evaluation of lot-to-lot consistency using a biologic ligand, SDF1α, and the cAMP Hunter Gαi-coupled CXCR4 receptor (chemokine C-X-C motif receptor 4) cell line. Results show high sensitivity detection and excellent reproducibility with overlapping S:B ratios of ~6 and EC_{50}'s ranging from only 689 pM to 796 pM.

Detailed Assay Protocol (Gα$_i$ Agonist)

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

A Gα$_i$ agonist assay preparation differs from a Gα$_s$ agonist assay preparation in that a Gα$_i$ 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**

Table 5. Assay reagent volumes per well for 96-well and 384-well plates (Gα$_i$ Agonist)

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 per well 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, the cells have been added to the assay plate and incubated overnight to allow the 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.

Complete removal of the media is crucial for reducing the variability of replicates.

   a. Alternate method if vacuum aspiration is not available: With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin for 30 seconds in a plate centrifuge set at 1000 rpm.
   Note: The centrifuge will not reach the speed of 1000 rpm within 30 seconds.

2. Immediately add 30 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 30 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve.

Section II: Gαi Agonist Preparation

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

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

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

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

   a. For each biologic sample, label the wells of a dilution plate (or polypropylene tubes) number 1 through number 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 numbers 2 through number 12. This exceeds the volume that is required for two rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

   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).

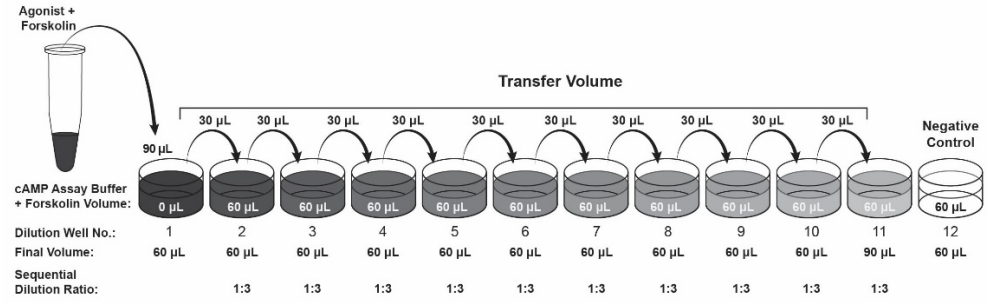
   c. Prepare the highest concentration of the Gαi agonist in the cAMP Assay Buffer. We recommend preparing a final screening concentration that is not higher than 10 µg/mL. The biologic sample preparation must also include forskolin. Therefore, prepare a working concentration of 3X the desired Gαi agonist concentration and add forskolin to the mix at 3X the final forskolin concentration required for the assay.

   d. Add 90 µL of the highest concentration of agonist plus forskolin to well number 1 (Figure 10).

   e. Remove 30 µL from well number 1 and add it to well number 2. Mix gently.

   f. With a clean tip, remove 30 µL from well number 2 and add it to well number 3. Mix gently.

   g. Repeat this process until well number 11 is reached. Do not add biologic sample to well number 12 since this is the negative control well.



Create 11 three-fold serial dilutions of Agonist + Forskolin in a separate dilution plate.

Figure 10. Biologic sample serial dilutions – Agonist + Forskolin

3. Set up serial dilutions for additional biologic samples 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 96-well assay plate. For assays to be run in a 384-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 an 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 an 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**

Table 6. Assay reagent volumes per well for 96-well and 384-well plates ($G\alpha_s$ and $G\alpha_i$ Antagonist)

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/well 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 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.

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

a. Alternate method if vacuum aspiration is not available: With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin for 30 seconds in a plate centrifuge set at 1000 rpm.
Note: The centrifuge will not reach the speed of 1000 rpm within 30 seconds.

2. Immediately add 30 µL of cAMP Assay Buffer to all assay wells in the assay plate. This step includes adding 30 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 series.

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

2. The concentration of each dilution should be prepared at 6X of the final screening concentration.

    a. For each biologic sample, label the wells of a dilution plate (or polypropylene tubes) sequentially number 1 through number 12.

    b. Add 60 µL of cAMP Assay Buffer to dilution wells numbers 2 through number 12. This exceeds the volume that is required for two rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

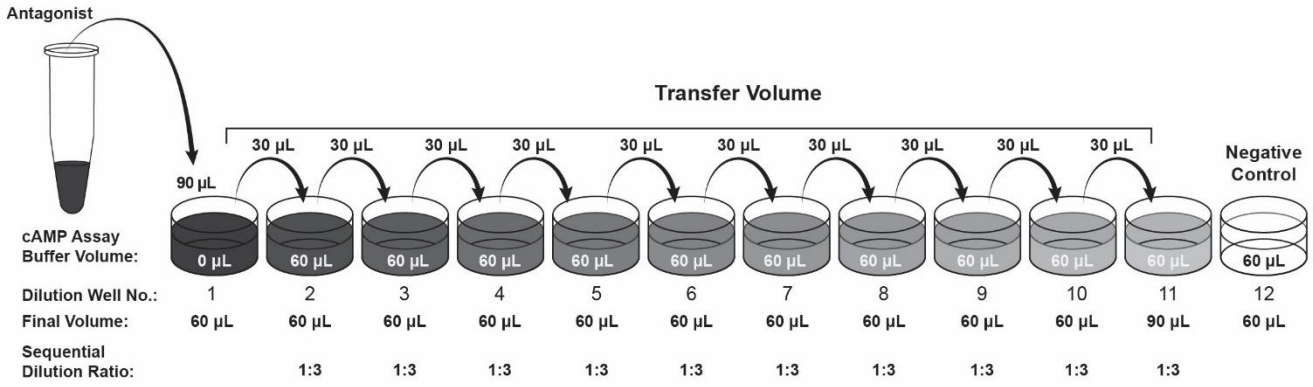
    c. Prepare the highest concentration of the biologic sample in the cAMP Assay Buffer. For antagonists or antibodies, the highest concentration will need to be determined empirically. After determining the highest concentration for the biologic sample, prepare a working concentration of 6X this top concentration.

    d. Add 90 µL of the highest concentration of the biologic sample to well number 1 (Figure 11).

    e. Remove 30 µL from well number 1 and add it to well number 2. Mix gently.

    f. With a clean pipette tip, remove 30 µL from well number 2 and add it to well number 3. Mix gently.

    g. Repeat this process until well number 11 is reached. Do not add biologic sample to well number 12 since this is the negative control well.



Create 11 three-fold serial dilutions of Antagonist in a separate dilution plate.

Figure 11. Biologic sample serial dilutions - Antagonist

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

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 number 1 through number 12 of a biologic sample 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 the addition of the agonist EC_{80} dose.

1. Add 7.5 µL of each 6X antagonist serial dilution in duplicate to the designated biologic sample (antagonist) rows (e.g., biologic sample 1 in Rows A and B; biologic sample 2 in Rows C and D). Repeat for the remaining biologic samples as indicated on the previously described Biologic sample Assay Plate Map.

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

3. Add 7.5 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 the 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 96- well assay plate. For assays to be run in a 384-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 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.

Complete removal of the media is crucial for reducing the variability of replicates.

   a. Alternate method if vacuum aspiration is not available: With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin for 30 seconds in a plate centrifuge set at 1000 rpm.
   Note: The centrifuge will not reach the speed of 1000 rpm within 30 seconds.

2. Immediately add 30 µL of cAMP Assay Buffer to all assay wells in the assay plate. This includes adding 30 µL cAMP Assay Buffer to the cell-free wells that were saved for the cAMP Standard curve.

Section II: Anti-Ligand Antibody Preparation

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

1. Prepare an 11-point series of 3-fold biologic sample (anti-ligand antibody) serial dilutions in cAMP Assay Buffer in a separate master dilution plate.

2. The concentration of each dilution should be prepared at 6X of the final screening concentration.

   a. For each antibody, label the wells in Row A of a master dilution plate (or polypropylene tubes) number 1 through number 12.

   b. Add 60 µL of cAMP Assay Buffer to dilution wells numbers 2 through number 12. This exceeds the required volume for two rows of wells for each concentration. The dilution volume may be adjusted according to the number of wells desired.

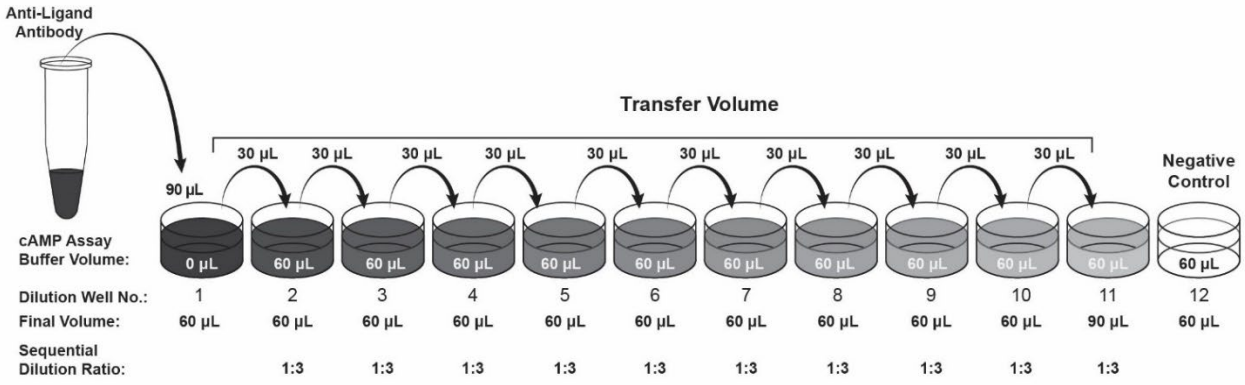
   c. Prepare the highest concentration of the antibody in the cAMP Assay Buffer. For antibodies, the highest concentration will need to be determined empirically as this will depend on each antibody's affinity for its target. After determining the top antibody concentration, prepare a working concentration of 6X the desired biologic sample concentration.

   d. Add 90 µL of the highest concentration of antibody 1 to well number 1 (see figure below) of Row A.

   e. Remove 30 µL from well number 1 and add it to well number 2. Mix gently.

   f. With a clean tip, remove 30 µL from well number 2 and add it to well number 3. Mix gently.

   g. Repeat this process until well number 11 is reached. Do not add antibody to well number 12 since this is the negative control well.



Create 11 three-fold serial dilutions of Anti-Ligand Antibody in a separate dilution plate.

Figure 12. Anti-Ligand Antibody serial dilutions

3. Set up serial dilutions for additional antibodies in a similar manner in additional rows of the master 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 $G\alpha_i$ 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 under 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.

   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 $G\alpha_s$ 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. In Row A of a fresh dilution plate (e.g., the ligand-neutralizing dilution plate), aliquot 30 µL of the 6X agonist EC_{80} (or 6X agonist EC_{80}/forskolin for $G\alpha_i$ receptors) dilution into wells number 1 through number 12.

4. Prepare additional rows of the 6X agonist EC_{80} (or 6X agonist EC_{80}/forskolin for $G\alpha_i$ receptors) for each antibody dilution series to be tested.

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.

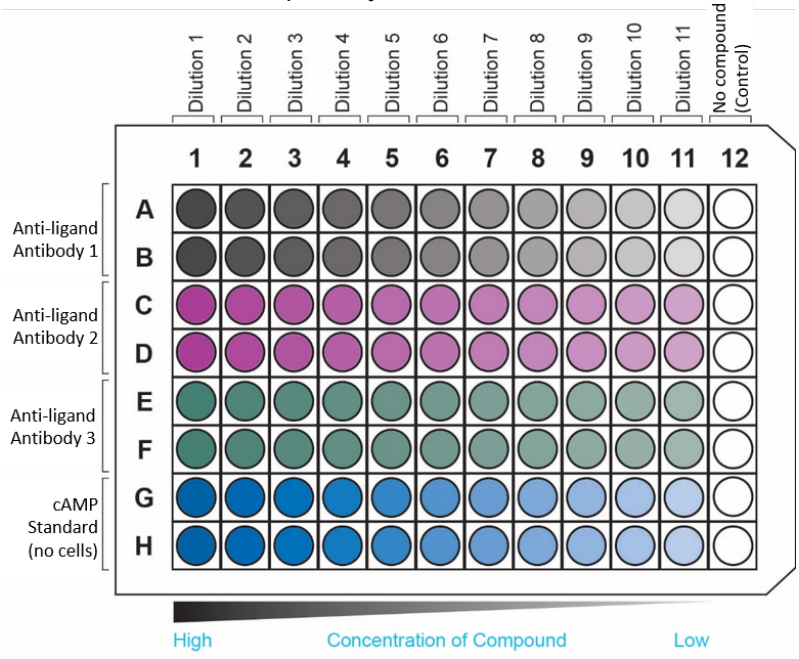
1. Using a multi-channel pipette, transfer 30 µL of 6X antibody dilution series from Row A of the master dilution plate to Row A of the ligand neutralizing plate containing the 30 µL of agonist EC_{80} (or 6X agonist EC_{80}/forskolin for $G\alpha_i$ receptors). Gently mix by pipetting up and down. The result is a mix of 3X anti-ligand antibody plus 3X agonist EC_{80}.

2. Transfer 30 µL of any additional antibody dilution series from their location in the master dilution plate to the corresponding row in the ligand neutralizing plate and mix by pipetting up and down several times.

3. Pre-incubate the anti-ligand and agonist mix for at least 15 minutes. The optimal pre-incubation time and temperature should be determined empirically.

4. Add 15 µL of each 3X antibody/agonist mix in duplicate to the designated rows in the assay plate as indicated in the Anti-Ligand Antibody Assay Plate Map (Figure 13). E.g., Rows A of the ligand neutralizing plate (Anti-ligand Antibody 1) to Rows A and B of the assay plate; Row B of the ligand neutralizing plate (Anti-ligand Antibody 2) to Rows C and D of the assay plate; Row C of the ligand neutralizing plate (Anti-ligand Antibody 3) to Rows E and F of the assay plate.

5. Incubate the 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.



Create 11-point curves in duplicate.

Figure 13. Anti-ligand Antibody Assay Plate Map

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).

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.

High-Throughput Screening Protocol (Optional)

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 the addition of the biologic sample.

1. Prepare a solution of cAMP Assay Buffer and cAMP Antibody Reagent by mixing 1-part of the Antibody Reagent with 2-parts of cAMP Assay Buffer.
2. Completely remove the cell media from assay wells by aspiration.
    a. Alternate method if vacuum aspiration is not available: With a flicking motion, remove liquid from the assay wells in the plate. Then, place the assay plate upside down on a lab-grade low lint tissue/paper towel and spin for 30 seconds in a plate centrifuge set at 1000 rpm.
    Note: The centrifuge will not reach the speed of 1000 rpm within 30 seconds.
3. Immediately add 45 µL of cAMP Assay Buffer/cAMP Antibody Reagent mixture to all wells of the assay plate.
4. Prepare 4X agonist dilution (or 4X agonist plus 4X forskolin mixture for $G\alpha_i$ receptors).
5. Add 15 µL 4X agonist dilution to the assay wells.
6. Incubate the 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.
7. 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

Is IBMX necessary in the DiscoverX cAMP kits?

IBMX is not necessary, and we do not use it for our internal testing. However, the kit is compatible with the use of IBMX. If IBMX is to be used, cell number per well and IBMX concentration needs to be optimized so that the amount of cAMP generated stays within the optimal detection range of the assay kit.

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 re-suspend suspension cells in cAMP Assay Buffer [1X HBSS + 10 mM HEPES; HBSS should contain Mg^{++} and Ca^{++}] at the optimal cell density. Typical suspension cell density is approximately 20,000 cells per well in a standard 96-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 biologic sample 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 biologic sample 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/), or
- Use the formula below where F is the percent response and H is the Hill Slope from the agonist reference curve:

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

- An example of EC_{80} calculation:

$$EC_F = \left(\frac{80}{100 - 80}\right)^{\frac{1}{H}} \times EC_{50}$$

For additional information or Technical Support see contact information below.

Document Revision History

Revision Number	Date Released	Revision Details
5	November 2023	Document Revision (Addition of 90-0075LM100 kit, Eurofins DiscoverX format, minor spelling corrections) This document replaces the following User Manual: 1. HitHunter® cAMP Assay for Biologics User Manual (70-318 Rev. 4)

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