cAMP Hunter™ NPY2R Bioassay Kit

For chemiluminescent detection of cAMP and NPY2R activity

Simple Solutions for Complex Biology

DiscoveRx

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Please read entire booklet before proceeding with the assay.
For additional information or technical support, contact SupportUS@discoverx.com or visit www.discoverx.com.

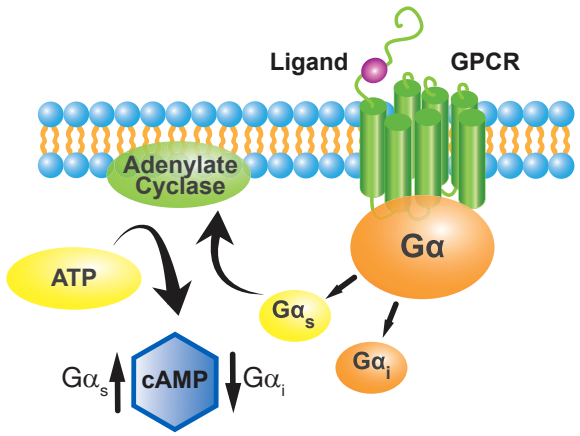
Overview

The cAMP Hunter NPY2R Bioassay kits provide a robust, highly sensitive and easy-to-use assay for monitoring peptide YY activation based on 3'-5'-cyclic adenosine monophosphate (cAMP) production in cells.

Technology Principle: cAMP Hunter NPY2R Bioassay

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. $G\alpha_i$- and $G\alpha_s$-coupled receptors modulate cAMP by either inhibiting or stimulating adenylate cyclase, respectively. With the cAMP Hunter Bioassay kit, cells overexpressing NPY2R utilize the natural coupling status of the GPCR to monitor activation of the $G\alpha_i$-coupled receptor. Following ligand stimulation, the functional status of NPY2R 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.

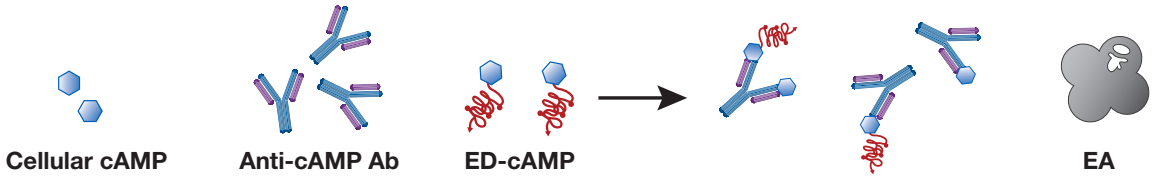
GPCR cAMP Pathway



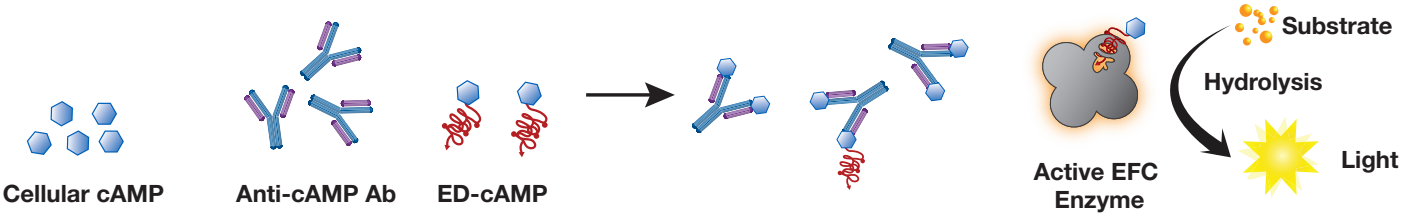
The cAMP Hunter Bioassay EFC technology uses a β-galactosidase (β-gal) enzyme split into two fragments, the Enzyme Donor (ED) and Enzyme Acceptor (EA). Independently these fragments have no β-gal activity; however, in solution they rapidly complement to form an active β-gal enzyme.

In this assay, cAMP from cell lysates and ED-labeled cAMP (ED-cAMP) compete for anti-cAMP antibody 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 an active enzyme, which subsequently produces a luminescent signal. The amount of signal produced is directly proportional to the amount of cAMP in the cell supernatant.

Low Levels of Cellular cAMP



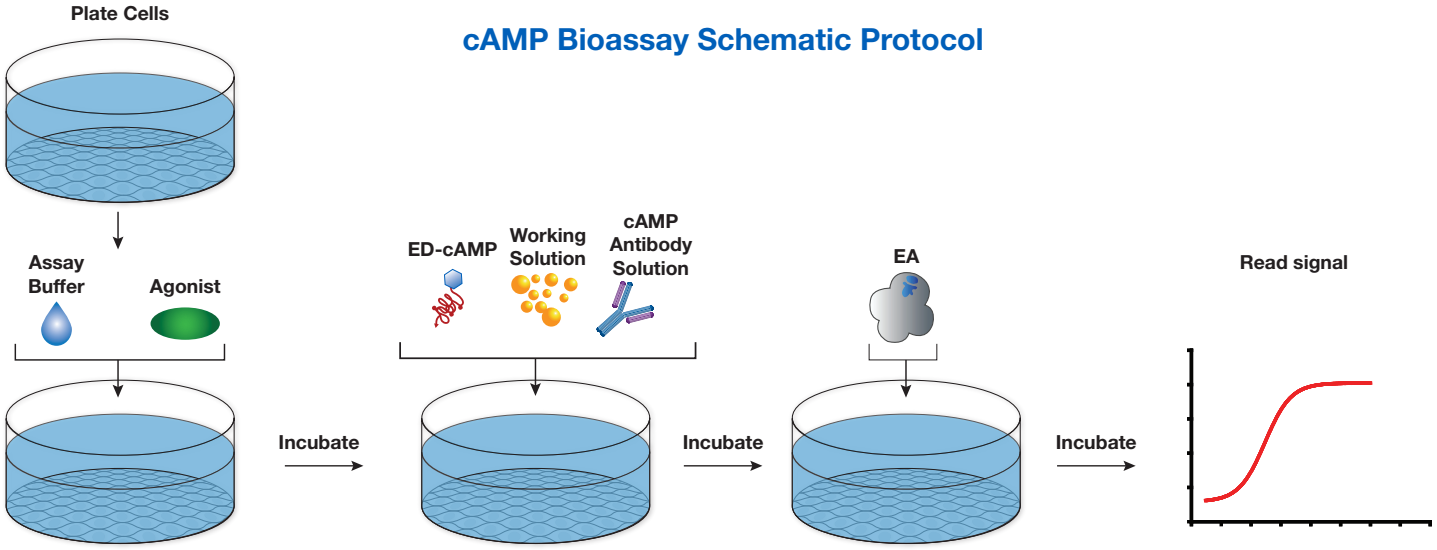
High Levels of Cellular cAMP



Intended Use

cAMP Hunter NPY2R Bioassay kits provide a robust, highly sensitive and easy-to-use, cell-based functional assay to study peptide YY potency and neutralizing antibodies through cAMP production. The Bioassay kits contain all the reagents needed for a complete assay including live cells, cAMP detection reagents, cell plating reagents, positive control agonist and assay plates. The pre-qualified, frozen Bioassay cells have been manufactured for short term use and are provided in a ready-to-assay format that saves time and adds convenience. Assays have been designed for 96-well and 384-well plate formats.

cAMP Bioassay Schematic Protocol



Materials Provided and Storage Conditions

List of Components	95-0077Y2-00055	95-0077Y2-00056
cAMP CHO-K1 NPY2R Bioassay Cells (No. of vials)	2 vials	10 vials
cAMP Hunter™ Detection Reagents (No. of Datapoints)	200	1,000
cAMP Standard (250 mM) (mL)	0.2	1
cAMP Antibody Reagent (mL)	5	25
cAMP Lysis Buffer (mL)	7.6	38
Substrate Reagent 1 (mL)	2	10
Substrate Reagent 2 (mL)	0.4	2
cAMP Solution D (mL)	10	50
cAMP Solution A (mL)	16	80
Forskolin Kit (dried powder) (2 x 0.25 mg/vial)	1	2
AssayComplete™ Cell Plating Reagent 2	1 x 100 mL	2 x 100 mL
Cell Assay Buffer	1 x 50 mL	2 x 50 mL
Control Ligand, Peptide YY (No. of vials)	1	1
96-Well White, Flat-Bottom, TC-Treated, Sterile Plates with Lid (No. of Plates)	2	10

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. The manufacturer of the cryovial recommends storing the vials in the vapor phase above the liquid N_2. Upon thawing, if liquid N_2 is present in the cryovial, it rapidly converts back to its gas phase which can result in the explosion of the vial upon its removal from the freezer.

Additional Materials Required

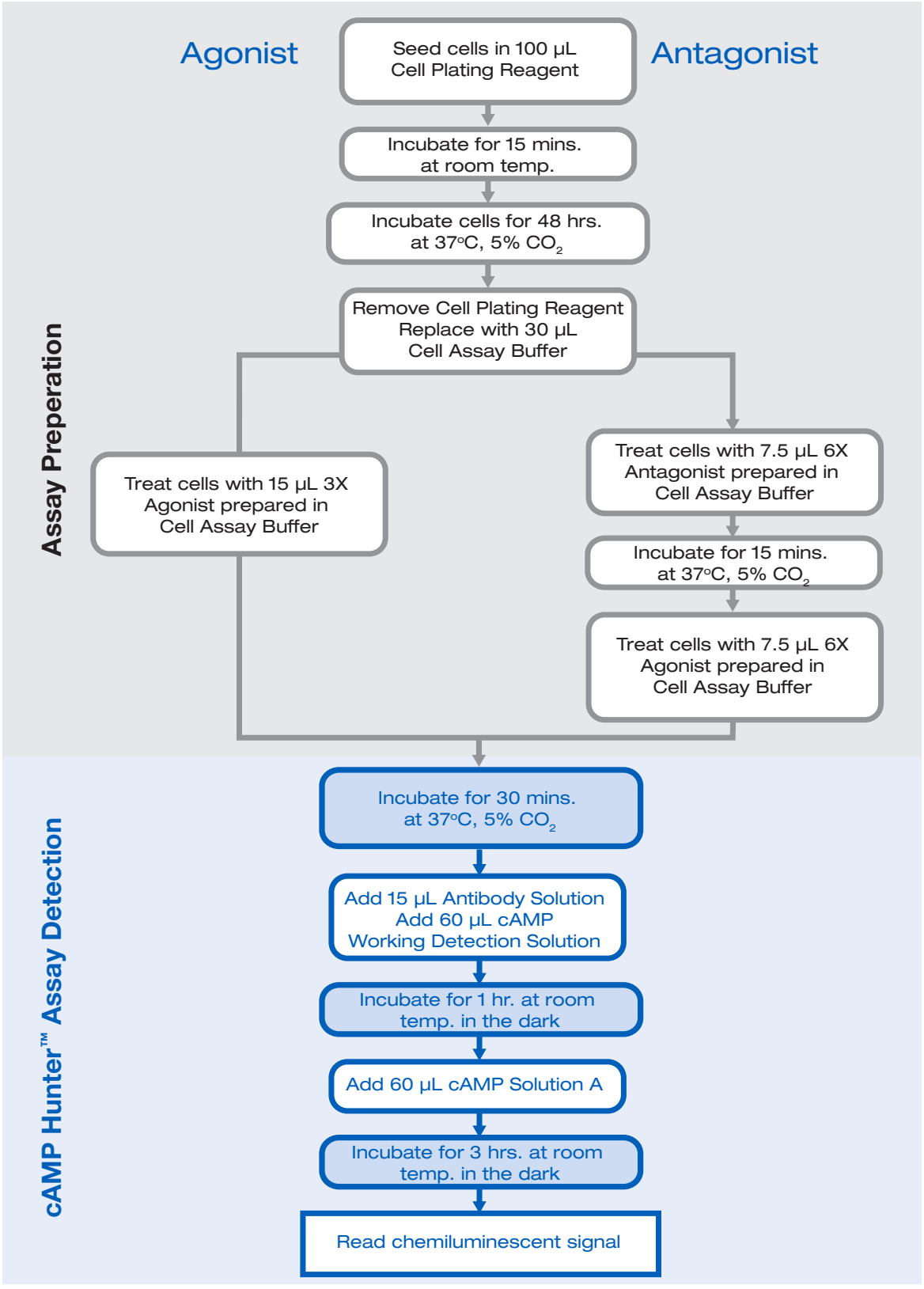
Additional Materials Required
Single and multichannel micro-pipettors and pipette tips
Multimode or luminescence plate reader
Disposable reagent reservoir (Thermo Scientific, Prod. No. 8094 or similar)

Recommended Materials, Reagents and Equipment	Ordering Information
Green V-bottom ligand dilution plates (10 plates/pack)	DiscoveRx Prod. No. 92-0011

Instrument Compatibility Chart	
Compatible with any luminometer. Select examples indicated below.	
Berthold Technologies: Mithras LB940, CentroLIApc	**Molecular Devices:** FLIPR, SpectraMax M3/ M4/M5/M5e, FlexStation 3, SpectraMax L
Biotek: Synergy 2	**Perkin Elmer:** TopCount, Victor 2 or V, Fusion, LumiCount, Envision, Micro-beta (Trilux), Viewlux, Northstar, EnSpire
BMG: PheraStar, Cytostar, LumiStar	**Promega:** GloMax systems
Caliper: LabChip 3000 & EZ Reader	**Tecan:** Ultra, Evolution
GE: LEAD seeker, Farcyte	**Thermo Scientific:** Luminoskan Ascent
Hamamatzu: FDS6000, FDSS/RayCatcher	**Turner BioSystems:** Modulus Microplate

Protocol Schematic

Quick-Start Procedure: In a white-walled 96-well tissue culture treated plate perform the following:



Detailed Protocol (Agonist, 96-well)

The following detailed protocol is specific to detecting cAMP in cells stimulated by peptide YY in a 96-well format plate.

Refer to the appendix for variations of this protocol (e.g. antagonist, 384-well).

Step 1: Cell Thawing Method

a. Pre-warm the AssayComplete Cell Plating Reagent 2 in a 37°C water bath for 15 mins. to equilibrate temperature.

b. Remove the cAMP CHO-K1 NPY2R Bioassay cryovials from -80°C or liquid N_2 vapor storage and place them immediately on dry ice prior to thawing. (see Safety Warning)

Do not expose vials to room temperature

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid N_2 storage, use tongs and place immediately on dry ice in a covered container. Wait at least 1 minute for any liquid N_2 inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

c. Decontaminate the vial by wiping with 70% ethanol.

d. Add 0.5 mL of pre-warmed AssayComplete Cell Plating Reagent 2 to the cell vial to thaw the cells. Pipette up and down gently several times to ensure that cells are evenly distributed.

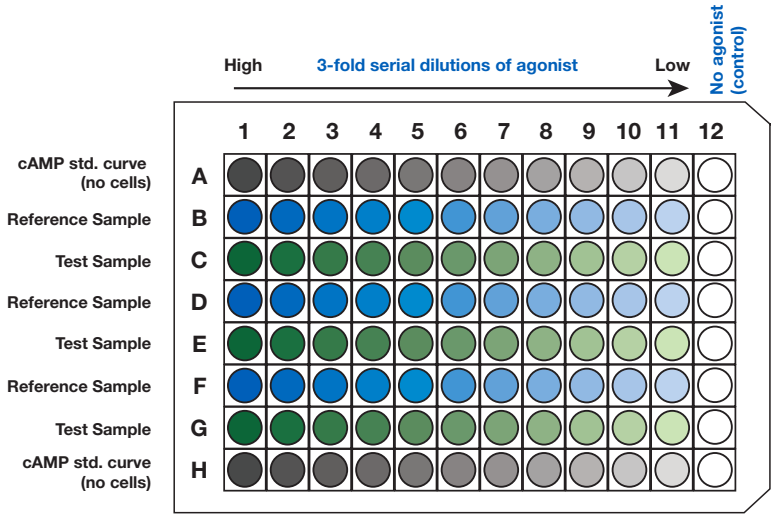
Do not thaw in a 37°C water bath or centrifuge.

e. Immediately transfer the cells to 11.5 mL of pre-warmed AssayComplete Cell Plating Reagent 2. Mix and pour into a disposable reagent reservoir.

f. Plate 100 µL of cells into each well of the provided 96-well tissue culture treated plate as shown in the example assay plate map below. Leave the first and last rows (labeled A and H) of the assay plate empty (with no cells) to allow for an 11-point cAMP standard curve in duplicate. Use the remaining rows to create a profile map alternating reference and test samples (e.g. agonist) in triplicate. Optionally, samples can be run on different plates or in other variations.

g. Incubate assay plate for 15 mins. at room temperature.

h. Place the seeded plate in a 37°C, 5% CO_2 humidified incubator for 48 hours prior to testing. For optimal inter-assay reproducibility, use the same incubation time from assay to assay.



Agonist assay plate map: 11-point curves with cAMP standard curve in duplicate and alternating reference and test samples in triplicate.

Step 2: Assay Plate Preparation

a. Completely remove the cell media from assay plate wells by aspiration.

b. Immediately add 30 µL of Cell Assay Buffer to all empty wells in the assay plate.

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

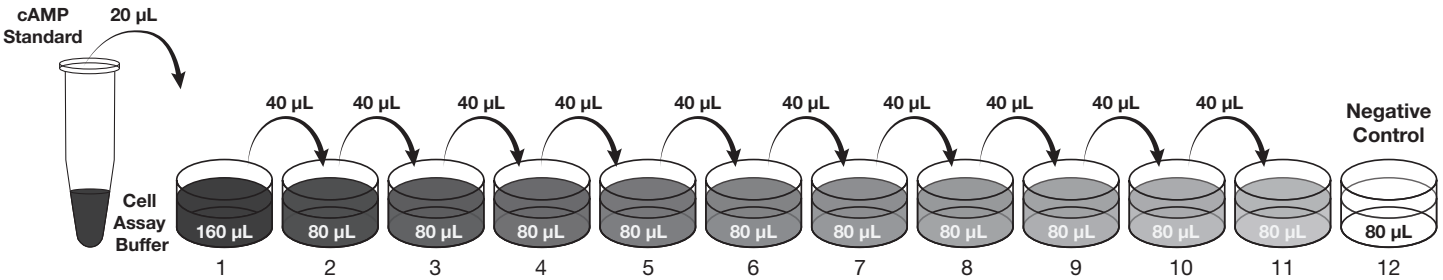
Step 3: cAMP Standard Preparation

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 left-shift. To avoid this situation, the cell number per well should be optimized. cAMP standard can be prepared fresh before agonist compound addition.

a. Prepare cAMP standard serial dilutions in a separate plate by diluting the cAMP standard (2.5 x 10^{-4}M) in a 1:9 ratio (i.e. 1 part cAMP standard plus 8 parts Cell Assay Buffer). This dilution ratio corresponds to the highest standard concentration (well No. 1) at 2.31 x 10^{-6}M in an assay volume of 180 µL. Make 10 additional 3-fold serial dilutions, using the Cell Assay Buffer, with the last well as the negative control as shown below.

   1. Using a separate dilution plate (or polypropylene tubes), label the wells No. 1 to No. 12.
   2. Add 160 µL of Cell Assay Buffer and 20 µL of cAMP standard to well No. 1 (this will be your highest concentration of cAMP standard).
   3. Add 80 µL of Cell Assay Buffer to dilution wells No. 2 to No. 12. This is enough volume for more than 4 rows.
   4. Remove 40 µL from well No. 1 and add it to well No. 2. Mix gently.
   5. With clean tip, remove 40 µL from well No. 2 and add it to well No. 3. Mix gently.
   6. Repeat this process until well No. 11. *Do not* add cAMP standard to well No. 12 since this is your negative control well (only Cell Assay Buffer).

b. Add 15 µL of cAMP standard dilutions in duplicate to the first and last rows (rows A and H) of the assay plate as shown in the previously described assay plate map.



	1	2	3	4	5	6	7	8	9	10	11	12
Final Volume:	140 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	80 µL	120 µL	80 µL
Sequential Dilution Ratio:	1:9	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	1:3	
~Final Assay Concentration (M):	$2.31x10^{-6}$	$7.70 x10^{-7}$	$2.57 x10^{-7}$	$8.56 x10^{-8}$	$2.85 x10^{-8}$	$9.51 x10^{-9}$	$3.17 x10^{-9}$	$1.06 x10^{-9}$	$3.52 x10^{-10}$	$1.17 x10^{-10}$	$3.91 x10^{-11}$	

Step 4: Peptide YY Reference and Test Sample Addition

a. For the reference and test samples, prepare agonist serial dilutions in a separate dilution plate in a 11-point series of 3X (3-fold) dilutions of sample in Cell Assay Buffer. Use the assay plate map for guidance. The final concentration of each dilution should be prepared at 3X of the final screening concentration.

For other ligand variations (e.g. antagonists), refer to the appendix section.

1. For each agonist (including the control ligand), label the wells of a separate dilution plate (or polypropylene tubes) No. 1 to No. 12.
2. Add 80 µL of Cell Assay Buffer containing 45 µM forskolin (3X the final concentration of 15 µM) to dilution wells No. 2 to No. 12. This is enough volume for over 4 rows, so the dilution volume may need to be adjusted according to the number of wells desired.
3. Prepare a 200 µM stock concentration of peptide YY by adding 580 µL of reconstitution buffer (H_20) to the lyophilized powder.
4. In a separate tube, dilute the stock concentration of peptide YY to make a 10 µM peptide YY concentration. Do this by adding 50 µL of the stock concentration of peptide YY to 950 µL of Cell Assay Buffer.
5. To well No. 1, dilute the 10 µM concentration of peptide YY to make a 0.3 µM peptide YY concentration (3X the final concentration of 0.1 µM). Do this by adding 6 µL of the 10 µM concentration of peptide YY to 194 µL of Cell Assay Buffer containing 45 µM forskolin (3X the final concentration of 15 µM). Mix gently.
6. Remove 40 µL from well No. 1 and add it to well No. 2 (3-fold dilution). Mix gently.
7. With clean tip, remove 40 µL from well No. 2 and add it to well No. 3 (3-fold dilution). Mix gently.
8. Repeat the 3-fold dilution process until well No. 11. *Do not* add agonist to well No. 12 since this is your negative control well.
9. Set up additional serial dilutions for additional agonists in a similar manner.

b. Add 15 µL of each 3X agonist serial dilution in triplicate to the designated agonist rows (e.g. rows B, D, and F for the reference sample, and rows C, E and G for test sample) of the assay plate as indicated on the previously described assay plate map.

c. Incubate assay plate for 30 mins. at 37°C.

Step 5: Antibody and Working Solution Addition

a. Following agonist incubation, add 15 µL of cAMP Antibody Reagent to all wells.

b. Prepare a stock of cAMP Working Detection Solution in a separate 15 ml polypropylene tube, by mixing 19 parts of cAMP Lysis Buffer, 5 parts of Substrate Reagent 1 and 1 part Substrate Reagent 2. Mix gently, then add 25 parts of cAMP Solution D.

c. Add 60 µL of cAMP Working Detection Solution to all wells of the assay plate. Do not pipette up and down in the vial to mix or vortex plates.

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

Make stock within 8 hours of use.

cAMP Working Detection Solution		
Components	**Volume ratio**	**Volume per plate**
cAMP Lysis Buffer	19	3.8 ml
Substrate Reagent 1	5	1.0 mL
Substrate Reagent 2	1	0.2 mL
cAMP Solution D	25	5.0 mL
Total Volume		**10 mL**

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

Step 6: Enzyme Acceptor Addition

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

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

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

Step 7: Assay Plate Reading

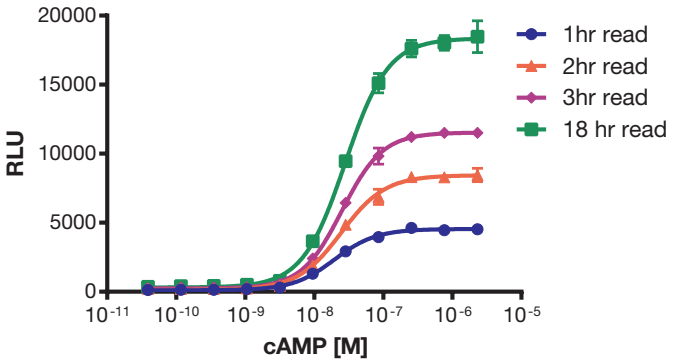
a. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for PMT readers or 5-10 seconds for imager. The plate may be incubated overnight and the signal may be measured the next day. In general, the signal continues to increase and reaches a maximum approximately 3-6 hours after the step 6a. The actual signal characteristics over time are affected by lab conditions such as temperature and the user should establish an optimal read time. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturer may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

b. Data analysis can be performed using your choice of statistical analysis software (e.g. GraphPad Prism, Molecular Devices Softmax Pro, Biotek Instruments Gen5, Microsoft Excel, etc.).

Typical Results

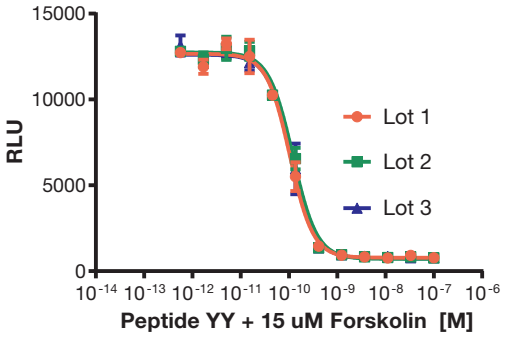
Typical results of the cAMP standard curve analysis (left) and cAMP bioassay using the cAMP CHO-K1 NPY2R Bioassay cells (right) are shown below. Note for the cAMP standard curve graph, the signal continues to increase over time and plates can be read the following day.

cAMP Standard Curve (96 well)



	1 hr. read	2 hr. read	3 hr. read	18 hr. read
EC_{50}	$1.96x10^{-8}$	$2.47x10^{-8}$	$2.53x10^{-8}$	$1.82x10^{-8}$
S/B	37.6	42.2	43.1	50.4

cAMP Bioassay NPY2R $G\alpha_i$ Cells



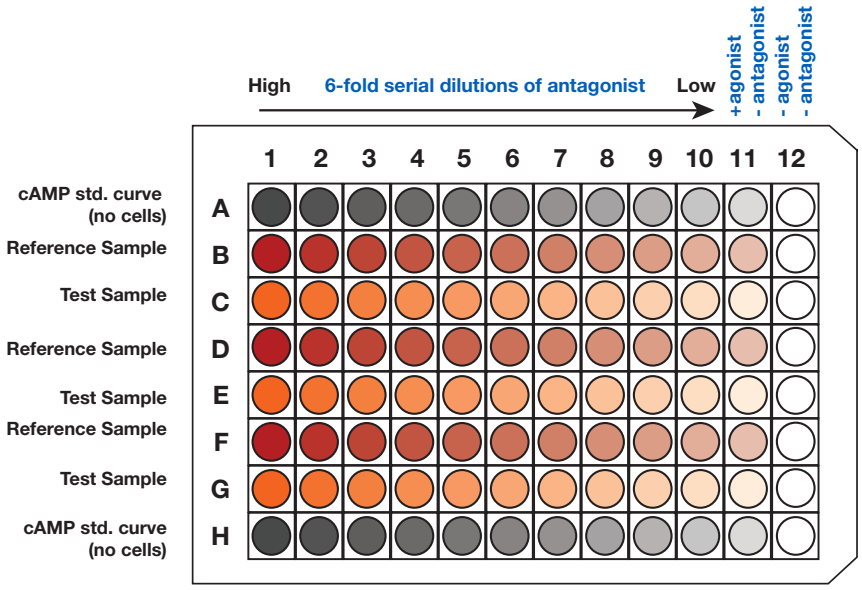
	Lot 1	Lot 2	Lot 3
EC_{50}	0.1 nM	0.12 nM	0.11 nM
S/B	16.3	16.6	16.8

Appendix

Antagonist Protocol

Protocol Modification

1. Antagonist serial dilution: Similar to the agonist step 4a, prepare a 10-point series of 6X dilutions of antagonist in Cell Assay Buffer. The concentration of each dilution should be prepared at 6X (6-fold) of the final screening concentration. See plate map below for an antagonist set-up example.



Antagonist assay plate map: 10-point curves with cAMP standard curve in duplicate and alternating reference and test samples in triplicate.

2. Antagonist/Agonist addition: Replace step 4b of the agonist detailed protocol with the following directions.

   a. Add 7.5 µl of each 6X antagonist serial dilution to the designated antagonist rows of the assay plate.

   b. Incubate assay plate for 15-30 minutes at 37°C. For best results, the optimal incubation time should be empirically determined.

   c. For the agonist challenge, add 7.5 µL of agonist at 6X the EC_{80} (i.e. 1.38 nM peptide YY, which is 6X the final EC_{80} concentration of 0.23 nM) in Cell Assay Buffer containing 90 µM forskolin (6X the final concentration of 15 µM).

   d. Continue with step 4c (incubation) of the agonist detailed protocol.

Crude Biologic Samples

The cAMP Hunter NPY2R Bioassay can be run in the presence of high levels of serum or plasma without significantly impacting assay performance (Ryding *et al.* 2013). Therefore, standard curves and 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.

Adjusted Volumes for 384-well Format

Use the following table to adjust the component volumes per well for 384-well plates.

Assay Format		
Assay Reagents	**96 plate (per well)**	**384 plate (per well)**
No. of Cells	**10,000**	**2,500**
AssayComplete Cell Plating Reagent 2	100 µL *	20 µL *
Cell Assay Buffer	30 µL	10 µL
Ligand (e.g. Agonist)	15 µL	5 µL
cAMP Antibody Reagent	15 µL	5 µL
cAMP Working Detection Solution	60 µL	20 µL
cAMP Solution A	60 µL	20 µL
Final Assay Volume	180 µL	**60 µL**

* The AssayComplete Cell Plating Reagent volume is used to plate cells and this volume is removed during first step.

FAQs

What if there is no or low signal?

- If plated on the included white-bottom assay plates, visually inspect the cells on the day of the assay 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.
- Make sure the proper assay mode is used (agonist mode 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 the ligand is prepared properly, take extra care to observe ligand solubility.
- Make sure DMSO and/or other solvents are not too high.
- 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.

What if the variability between replicates is high?

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

For additional information or Technical Support, contact SupportUS@discoverx.com or SupportEurope@discoverx.com

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