PerkinElmer Life and Analytical Sciences, Inc.

PERFORMING ALPHASCREEN cAMP FUNCTIONAL ASSAYS

cAMP ASSAY KITS
CATALOG NUMBERS:
6760625D, 6760625M, 6760625R

For Laboratory Use Only
Research Chemicals for Research Purposes Only

Precautions

- AlphaScreen beads are light sensitive. The cAMP assay must be performed under subdued laboratory lighting (lighting should be under 100 Lux or apply green filters to light fixtures). Any incubation involving the beads should be performed in the dark. Plates can be covered by another microplate to minimize the effect of light.
- The small volumes used in the assay are prone to evaporation. It is recommended that microplates be covered with TopSeal adhesive sealing film to reduce evaporation during incubation. Microplates can be read with the TopSeal in place.
- Beads should be stored in the dark at 4^{o}C. It is recommended not to store reagents containing BSA for longer than 1 day at 4^{o}C.
- Sodium azide concentrations should be limited to 0.005% to avoid any potential interference with the AlphaScreen signal. Proclin-300 (Supelco product number 4-8127 or equivalent) is recommended as an alternative.
- Cells grown in medium containing high concentrations of biotin (ex. RPMI) should be rinsed in an appropriate buffer such as 1x PBS (Gibco BRL® product number 14190 or equivalent) before they are used.
- The cAMP standard contains 0.09% sodium azide, which is a toxic compound.

cAMP Standard

USA: Harmful by contact, ingestion, corrosive to skin; irritating to eyes. Affects central nervous system, lungs and heart.

EN: Contains: Sodium azide – Toxic if swallowed. Contact with acids liberates very toxic gas. Wear suitable gloves. In case of accident or if you feel unwell, seek medical advice immediately.

DE: Enthält: Natriumazide – Giftig beim verschlucken, entwickelt bei berűhrung mit säure sehr giftige gase. Geeignete schutzhandschuhe tragen. Bei unfall oder unwohlsein sofort arzt hinzuziehen (wenn mőglisch dieses etikett vorzeigen).

FR: Contient: Azide de sodium – Toxique en cas d'ingestion. Au contact d'un acide, dégage un gaz très toxique. Porter des gants appropriés. En cas d'accident ou de malaise consulter immédiatement un médecin (si possible lui montrer l'étiquette).

IT: Contiene : Sodio azoturo – Tossico per ingestione. A contatto con acidi libera gas molto tossico. Usare guanti adatti. In caso di incidente o di malessere consultare immediatamente il medico (se possibile, mostrargli l'etichetta).

ES: Contiene : Azida sodica – Toxico por ingestion. Ec contracto con acidos libera gases muy toxicos. Usense guantes adecuados. En caso de accidente o malestar, acudase immediatemente al medico (si es posible, muestresele la etiqueta).

The National Institute for Occupational Safety and Health has issued a bulletin citing the potentially explosive hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide is added at a minimal concentration, it is still recommended that copious amounts of water be used to flush the drain pipeline after disposal of these reagents in the plumbing system. Copper-free and lead-free discharge lines should be used whenever possible. Decontamination procedures should be followed prior to maintenance on drain lines, which have been used for azide-containing reagents. Recommended decontamination procedures are available from PerkinElmer Life Sciences Technical Services.

Performing AlphaScreen™ cAMP Functional Assays

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I. BEFORE STARTING

Receiving the AlphaScreen cAMP Assay Kit

Upon receiving the AlphaScreen cAMP Assay Kit, ensure that it was shipped on blue ice (4ºC) and that the ice packs are not completely melted.

Provided Reagents and Materials

The reagents and materials provided in the AlphaScreen cAMP detection kits are listed in **Table 1**. The following three kit sizes are available:

- 1,000 assay points kit (catalog number # 6760625D) which is meant for assay development and contains 10,000 Units of biotinylated cAMP probe.
- 10,000 assay points kit (catalog number # 6760625M) containing 10,000 Units of biotinylated cAMP probe, streptavidin-Donor and anti-cAMP Acceptor beads.
- 50,000 assay points kit (catalog number # 6760625R) containing 50,000 Units of biotinylated cAMP probe, streptavidin-Donor and anti-cAMP Acceptor beads.

Unit definition: One Unit of biotinylated cAMP probe, Donor-SA beads or Anti-cAMP Acceptor beads corresponds to the amount of material needed to generate an AlphaScreen signal greater than 10,000 cps under the following assay conditions: 1 Unit/well of probe, 1 Unit/well of Donor beads, 1 Unit/well of Acceptor beads, assay buffer: stimulation buffer containing 0.5 mM IBMX and lysis/detection buffer (see composition on Page 13), 1 hour incubation and measurement at 23°C.

Table 1: Listing of components in cAMP detection kits

REAGENTS	6760625D (1,000 assay points)	6760625M (10,000 assay points)	6760625R (50,000 assay points)	Storage conditions
Anti-cAMP Acceptor beads	100 µL @ 10 Units/µL	1 mL @ 10 Units/µL	5 mL @ 10 Units/µL	+2 - 6°C. Do not freeze. Store protected from light
Donor-SA beads	100 µL @ 10 Units/µL	1 mL @ 10 Units/µL	5 mL @ 10 Units/µL	+2 - 6°C. Do not freeze. Store protected from light
Biotinylated cAMP	80 µL @ 133 Units/ µL	80 µL @ 133 Units/ µL	380 µL @ 133 Units/µL	+2 - 6°C. Do not freeze.
cAMP standard	1 vial 1.0 mL @ 50 µM	1 vial 1.0 mL @ 50 µM	1 vial 1.0 mL @ 50 µM	+2 - 6°C. Do not freeze.
10X control buffer	1.5 mL	1.5 mL	1.5 mL	+2 - 6°C. Do not freeze.
3% Tween-20 solution	150 µL	150 µL	150 µL	+2 - 6°C. Do not freeze.

Important Note:

For maximum recovery of products, centrifuge all original vials prior to removing the caps. Resuspend the beads by pipetting before use.

Required Reagents and Materials

The following materials are required for buffers and reagents preparation:

Item	Suggested source*	Catalog #
1 M HEPES	Gibco BRL	15630-106
10% Tween-20	Pierce	28320
BSA	Sigma	A 7030
1x PBS	Gibco BRL	14190
Milli-Q® water		
Forskolin	Sigma®	F 6886
10x HBSS	Gibco BRL	14065-056
IBMX†	Sigma	I 5879
Ethanol 95%	Sigma	E 7148
Versene	Gibco BRL	15040-066
DMSO	Sigma	D 8779
NaOH 1N	Sigma	S 2770
OptiPlate-384 NEW	**PerkinElmer**	**6007290**
TopSeal Adhesive Sealing Film	**PerkinElmer**	**6005185**
Single-channel Pipettors§		

* Equivalent sources can be substituted

† (3-Isobutyl-1-Methylxanthine).

§ For lower volume plate additions (2.5-10 µL), we recommend a pipettor precision ≤ 2%. For higher volume additions (25-1000 µL), we recommend a pipettor precision of ≤ 1%.

II. ALPHASCREEN cAMP ASSAY FAQS

1. What is the minimum receptor expression level required?

There is no minimum receptor expression level required per se. Low levels of endogenous receptors, such as the calcitonin receptors present in CHO cells (15 fmol/mg), were shown to induce the release of high amounts of cAMP. Tight coupling between receptors, G-proteins (Gαi/o or Gαs) and adenylate cyclase is the most critical determinant to yield conditions allowing for robust cAMP assays.

2. Should I use transient or stable transfected cell lines?

Both transient or stable cell lines have been shown to produce good responses upon adenylate cyclase activation.

3. How many cells should I use in my assay?

Cell number will influence cAMP levels prevailing before (basal) and following adenylate cyclase activation. Performing a cell titration will allow one to optimize the signal window by maximizing the difference between the basal and stimulated counts.

4. Is it necessary to perform a time course for cell stimulation?

The stimulation time is critical for reaching optimal detection of cAMP. When determining the optimal cell number for the assay, we recommend stimulating the cells for 30 minutes as a first trial. Once the optimal cell number has been determined, the experimenter should perform a time course experiment starting at 15 minutes up to 120 minutes, using 15-minute intervals. The time of stimulation may vary depending on the cell line, receptor and agonist being studied.

5. How should the cells be handled?

Cells should be at approximately 70-90% confluence and pre-

pared just prior to the assay. Harvest at least 250,000 cells by detaching with **PBS + 5 mM EDTA** (Versene solution, Gibco-BRL product number 15040-066; we do not recommend use of other brand-name cell stripper solutions) for 5 min at 37ºC. Centrifuge the cells for 5 min at 275 X g. Remove supernatant and resuspend the cells with 1.5 mL of 1x PBS. Obtain an accurate cell count and adjust to 10^6 cells / mL with stimulation buffer (see preparation on pages 9-10). Cells should be >95% viable such as determined by Trypan Blue exclusion assay.

6. Can attached cells be used ?

Attached cells can be used as well as detached cells. However, we recommend replacing the cell culture medium by the recommended stimulation buffer for at least 15 minutes prior to assay.

7. Can membrane preparations be used instead of whole cells ?

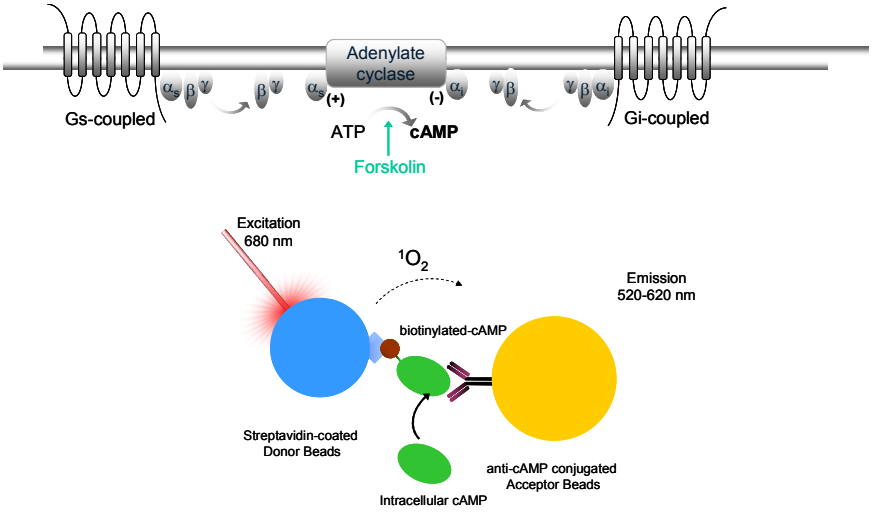
Membranes expressing Gαs-coupled receptors were shown to produce excellent results when the stimulation buffer is supplemented with appropriate ions (ex. 10 mM $MgCl_2$, 1 nM GTP, 10 μM GDP and 100 μM ATP). 1-10 μg membranes are typically used in such assays. We recommend to titer the membranes and all supplemented ions to optimize the performance of membrane based assays. Membranes expressing Gαi-coupled receptors are not suitable since, for an unknown reason, G-protein coupling to the adenylate cyclase is lost following membrane preparation.

8. Does IBMX interfere in the assay ?

IBMX is a well known phosphodiesterase inhibitor. This inhibitory effect of IBMX is due to the structural analogy this compound shares with cAMP. Because of its resemblance with cAMP, IBMX competes with biotinylated cAMP binding to anti-cAMP antibodies such as those coating the acceptor beads. High concentrations of IBMX decrease the AlphaScreen signal without affecting the sensitivity of the assay. We recommend using IBMX final concentrations of 0.2-0.25 mM. At this concentration range, the maximal signal is reduced by approximately 30% (see Graph on Page 23).

III. DESCRIPTION OF THE ASSAY

The AlphaScreen cAMP assay has been designed to directly measure levels of cAMP produced upon modulation of adenylate cyclase activity by GPCRs. The assay is based on the competition between endogenous cAMP and exogenously added biotinylated cAMP. The capture of cAMP is achieved by using a specific antibody conjugated to Acceptor beads. The assay is efficient at measuring both agonist and antagonist activities on Gαi- and Gαs-coupled GPCRs. Gαs and Gαi subunits act through the cAMP pathway by respectively activating or inhibiting adenylate cyclase, an enzyme catalyzing the conversion of ATP to cAMP. Cells are stimulated to either increase or decrease intracellular cAMP levels followed by a combined cell lysis / detection step. For Gαi-coupled receptors, an elevation in intracellular cAMP is stimulated using forskolin, resulting in a decrease in AlphaScreen signal due to an inhibition of association between the beads. However, when forskolin stimulation occurs concurrently with the agonist stimulation of a Gαi-coupled receptor, the resultant decreased signal evoked by forskolin alone is inhibited i.e. there is a signal increase relative to the forskolin alone treatment.



IV. PROTOCOLS FOR BUFFER AND STOCK SOLUTION PREPARATION

For reliable results please use the following protocols for preparing buffers and stock solutions used in the cAMP assay.

Materials & Preparation

Item	Suggested source*	Catalog #
10x control buffer	Kit component	
10x HBSS	Gibco BRL	14065-056
BSA	Sigma	A-7030
IBMX*	Sigma	I 5879
10% Tween-20	Pierce	28320
3% Tween-20	Kit component	
1 M NaOH	Fisher Scientific	S0899
1M HEPES	Gibco BRL	15630-106
Liquid cAMP standard (50 µM)	Kit component	
Forskolin	Sigma	F 6886
Milli-Q H_2O		

* IBMX : 3-Isobutyl-1-Methylxanthine

500 mM IBMX solution

Dissolve 100 mg in 900 µL DMSO to give a 500 mM stock solution.

Aliquot and store at -20°C; use as required.

1x control buffer (for QC purposes only: buffer used for generation of cAMP standard curve in Quality Control Mode; see Section V)

Prepare 10 mL of 1x control buffer pH 7.4 (5 mM HEPES, 50 mM NaCl, 0.03%, Tween-20), as follows:

Reagent	Volume	[Final]
10x buffer	1.00 mL	1x
3% Tween-20	0.10 mL	0.03%
Milli-Q H_20	8.90 mL	-

Adjust pH to 7.4 with 0.1N NaOH.

Note: Prepare fresh prior to assay.

1x HBSS

Prepare 50 mL of 1x HBSS, pH 7.4, as follows:

Reagent	Volume	[Final]
10x HBSS	5 mL	1x
Milli-Q H_20	45 mL	-

Adjust pH to 7.4 with 1N NaOH.

Stimulation buffer (containing IBMX)

Prepare 40 mL of stimulation buffer, pH 7.4, (HBSS containing 0.5 mM IBMX, 5 mM HEPES, 0.1% BSA) as follows:

Reagent	Volume/Amount	[Final]
1x HBSS	39 mL	
BSA	40 mg	0.1%
IBMX	40 µL of 500 mM	0.5 mM
HEPES	200 µL of 1M	5 mM

Adjust pH to 7.4 with NaOH 1N. Complete to 40 mL with HBSS.

Note: Prepare fresh on day of use and keep at room temperature - buffer can be used for either forskolin, agonist or antagonist assays.

Lysis/detection buffer

Prepare 20 mL of lysis/detection buffer, pH 7.4 (5 mM HEPES containing 0.1% BSA and 0.3% Tween-20), as follows:

Reagent	Volume	[Final]
Milli-Q H_20	19 mL	
BSA	20 mg	0.1%
10% Tween-20	600 µL	0.3%
HEPES	100 µL of 1M	5 mM

Adjust pH to 7.4 with NaOH 1N. Complete to 20 mL with Milli-Q H_20.

Note: Prepare fresh on day of use and keep at room temperature

50 µM cAMP standard solution

The liquid cAMP standard (FP1283) is a 50 µM solution.

Vortex at maximum intensity for 5 seconds before use.

Store at 4°C for further use.

50 mM forskolin stock solution

Dissolve 5 mg forskolin in 244 µL of 95% ethanol to make up the required 50 mM stock solution.

Note: Store at -20°C and use as required.

V. cAMP STANDARD CURVE PROCEDURE (QUALITY CONTROL MODE)

25 µL final volume in 384-well white opaque microplate

Assay performed in 1x control buffer containing 0.03% Tween-20

Biotinylated cAMP used at 1 Unit/well

Materials provided:

- Anti-cAMP Acceptor beads (10 Units/µL in PBS)
- Streptavidin-Donor beads (10 Units/µL in PBS)
- Biotinylated cAMP (133 Units/µL in Tris·EDTA)
- Liquid cAMP standard (50 µM)
- 10x control buffer (50 mM HEPES pH 7.4, 500 mM NaCl and 0.05% Proclin-300)
- 3% Tween-20 solution

Additional reagents required (not provided):

Item	Suggested source*	Catalog #
BSA	Sigma	A7030
NaOH	Sigma	S0899

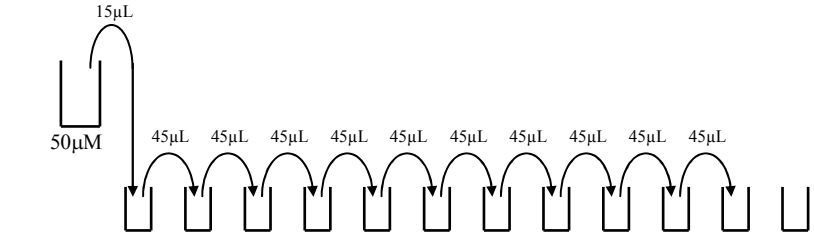
* Equivalent sources can be substituted

Reagent preparation:

1. **Prepare 10 mL of 1x control buffer containing 0.03% Tween-20 as indicated in Section IV (page 13).**

2. **Prepare a Standard cAMP dilution series from the 50 µM cAMP standard solution.**

   Serially dilute to provide a concentration range from $5x10^{-6}$ to $5x10^{-11}$ M in ½ log intervals, as illustrated below. Include a positive control (no cAMP).



Tubes	1	2	3	4	5	6	7	8	9	10	11	12
1x buffer	135µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL
[cAMP] (M)	$5.0x10^{-6}$	$1.5x10^{-6}$	$5.0x10^{-7}$	$1.5x10^{-7}$	$5.0x10^{-8}$	$1.5x10^{-8}$	$5x10^{-9}$	$1.5x10^{-9}$	$5x10^{-10}$	$1.5x10^{-10}$	$5x10^{-11}$	0
[cAMP] (M) Final in 25 µL	$1.0x10^{-6}$	$3x10^{-7}$	$1x10^{-7}$	$3x10^{-8}$	$1x10^{-8}$	$3x10^{-9}$	$1x10^{-9}$	$3x10^{-10}$	$1x10^{-10}$	$3x10^{-11}$	$1x10^{-11}$	0

Note: Prepare fresh prior to assay.

3. Streptavidin Donor beads/biotinylated cAMP detection mix

Add 5 µL of the 133 Units/µL biotinylated cAMP to 195 µL 1x control buffer containing Tween-20 to obtain a 3.3 Units/µL solution. To make 750 µL detection mix, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
1x control buffer/Tween-20	730 µL	**-**	-
Donor beads (10 Units/µL)	5 µL	**1 Unit/15 µL**	1 Unit/25 µL
Biotinylated cAMP (3.3 Units/µL)	15 µL	**1 Unit/15 µL**	1 Unit/25 µL

Note: Prepare fresh and incubate in the dark for at least 30 minutes prior to use.

4. Anti-cAMP Acceptor beads solution

To make 250 µL anti-cAMP Acceptor beads solution, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
1x control buffer/Tween-20	245 µL	**-**	-
Acceptor beads (10 Units/µL)	5 µL	**1 Unit/5 µL**	1 Unit/25 µL

Note: Prepare fresh prior to assay.

Order of addition:

To a 384-well microplate (PerkinElmer Life Sciences OptiPlate-384 NEW product number 6007290**) add in triplicate:**

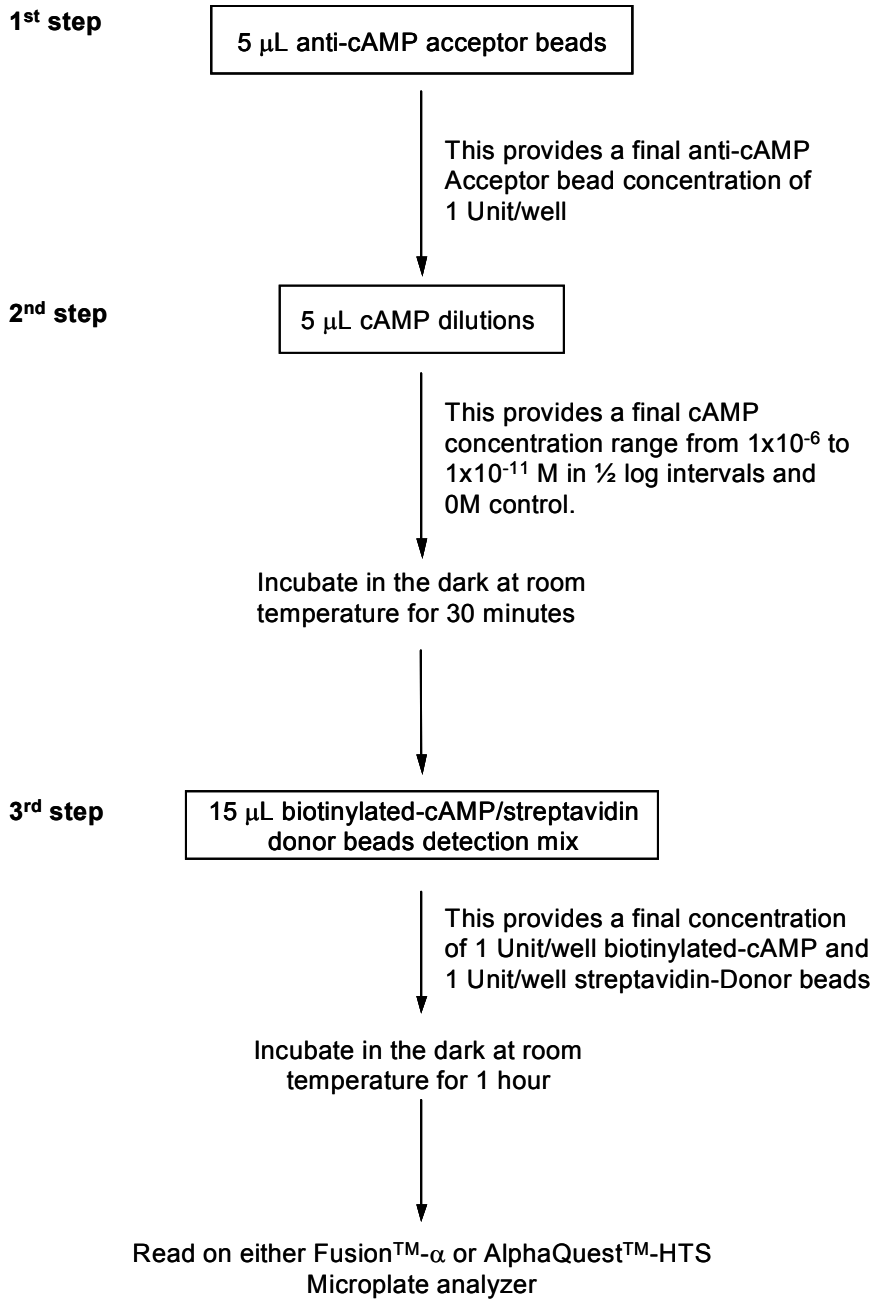
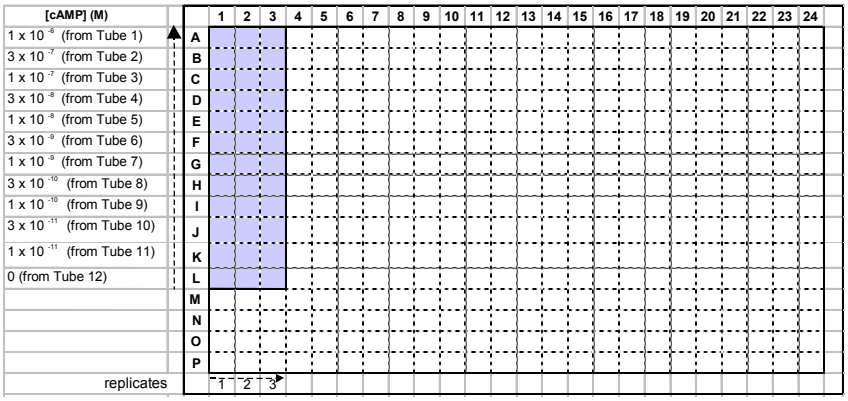


Plate set-up:

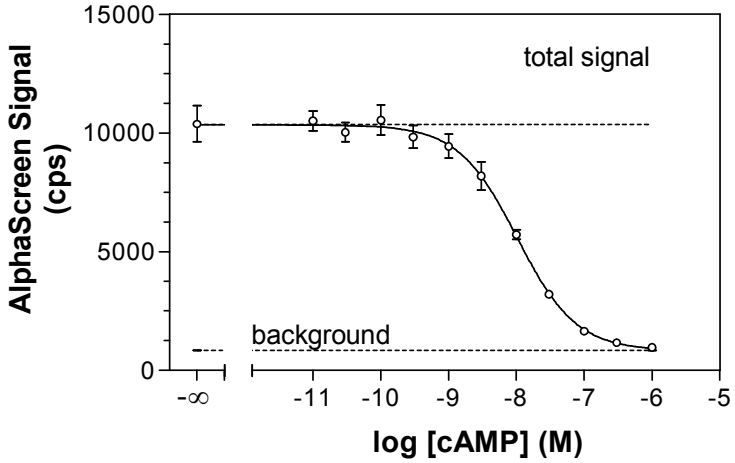


As described under order of addition, to each well add:

5 µL anti-cAMP Acceptor beads solution, 5 µL cAMP dilutions (final concentration/well as indicated) and 15 µL biotinylated cAMP/ streptavidin Donor beads detection mix.

Interpreting the data:

The AlphaScreen signal is plotted as a function of log concentration of cAMP.



Observations:

The above graph illustrates a typical cAMP competition curve performed under QC conditions using 1x control buffer containing 0.03% Tween-20: white opaque 384 well microplate, 25 µL final volume, room temperature (22-23°C) and 1 hour incubation following the last addition step. In these conditions, a typical standard curve provides a signal to background ratio of ~12.5, a 2 log dynamic range (the linear portion of the curve) and an IC_{50} value of ~ 7.5-10 nM. Total signal will vary with ambient temperature and incubation time. For consistent results, the same incubation time and temperature should be used for each plate.

Final concentrations of reagents in the assay are 1 Unit/well for Donor beads, Acceptor beads and biotinylated cAMP analog. Final concentrations of cAMP standard are: 1 µM, 0.3 µM, 0.1 µM, 30 nM, 10 nM, 3 nM, 1 nM, 0.3 nM, 0.1 nM, 0.03 nM. 0.01 nM and 0 M.

VI. cAMP STANDARD CURVE PROCEDURE (FUNCTIONAL ASSAY MODE)

25 µL final volume in 384-well white opaque microplate

Assay performed in 1x stimulation buffer containing 0.5 mM IBMX and lysis/detection buffer

Biotinylated-cAMP used at 1 Unit/well

Materials provided:

Anti-cAMP Acceptor beads (10 Units/µL in PBS)

Streptavidin Donor beads (10 Units/µL in PBS)

Biotinylated cAMP (133 Units/µL in Tris·EDTA)

Liquid cAMP standard (50 µM)

Additional reagents required (not provided):

Item	Suggested source*	Catalog #
BSA	Sigma	A 7030
10% Tween-20	Pierce	28320
1 M HEPES	Gibco BRL	15630-106
HBSS	Gibco BRL	14065-056
IBMX†	Sigma	I 5879
DMSO	Sigma	D 8779
NaOH 1N	Sigma	S 2770

* Equivalent sources can be substituted
† (3-Isobutyl-1-Methylxanthine) Dissolve 100 mg in 900 µL DMSO to yield a 500 mM stock solution. Aliquot and store at –20ºC; use as required.

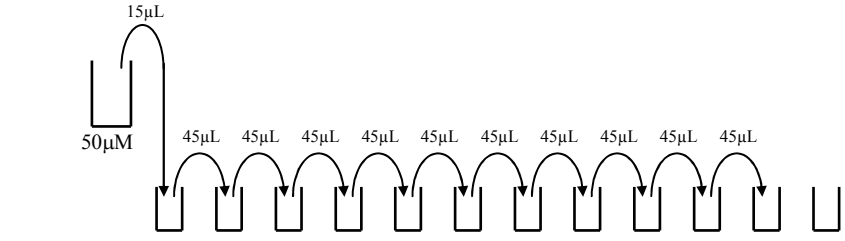
Reagent preparation:

1. **Prepare 40 mL of Stimulation buffer containing IBMX, as described in Section IV Page 13.**

2. **Prepare 20 mL of lysis/detection buffer, as described in Section IV Page 14.**

3. **Prepare a Standard cAMP dilution series from the 50 µM cAMP standard solution:**

   Serially dilute to provide a concentration range from $5x10^{-6}$ to $5x10^{-11}$ M in ½ log intervals. Include a positive control (no cAMP).



Tubes	1	2	3	4	5	6	7	8	9	10	11	12
Stimulation buffer	135µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL	90µL	105µL
[cAMP] (M)	$5.0x10^{-6}$	$1.5x10^{-6}$	$5.0x10^{-7}$	$1.5x10^{-7}$	$5.0x10^{-8}$	$1.5x10^{-8}$	$5x10^{-9}$	$1.5x10^{-9}$	$5x10^{-10}$	$1.5x10^{-10}$	$5x10^{-11}$	0
[cAMP] (M) Final in 25 µL	$1.0x10^{-6}$	$3x10^{-7}$	$1x10^{-7}$	$3x10^{-8}$	$1x10^{-8}$	$3x10^{-9}$	$1x10^{-9}$	$3x10^{-10}$	$1x10^{-10}$	$3x10^{-11}$	$1x10^{-11}$	0

Note: Prepare fresh prior to assay.

4. Streptavidin Donor beads/biotinylated cAMP detection mix

Add 5 µL of the 133 Units/µL biotinylated cAMP to 195 µL lysis/ detection buffer to obtain a 3.3 Units/µL solution. To make 750 µL detection mix, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
Lysis/detection buffer	730 µL	**-**	-
Donor beads (10 Units/µL)	5 µL	**1 Unit/15 µL**	1 Unit/25 µL
Biotinylated cAMP (3.3 Units/µL)	15 µL	**1 Unit/15 µL**	1 Unit/25 µL

Note: Prepare fresh and incubate in the dark for at least 30 minutes prior to use.

5. Anti-cAMP Acceptor beads solution

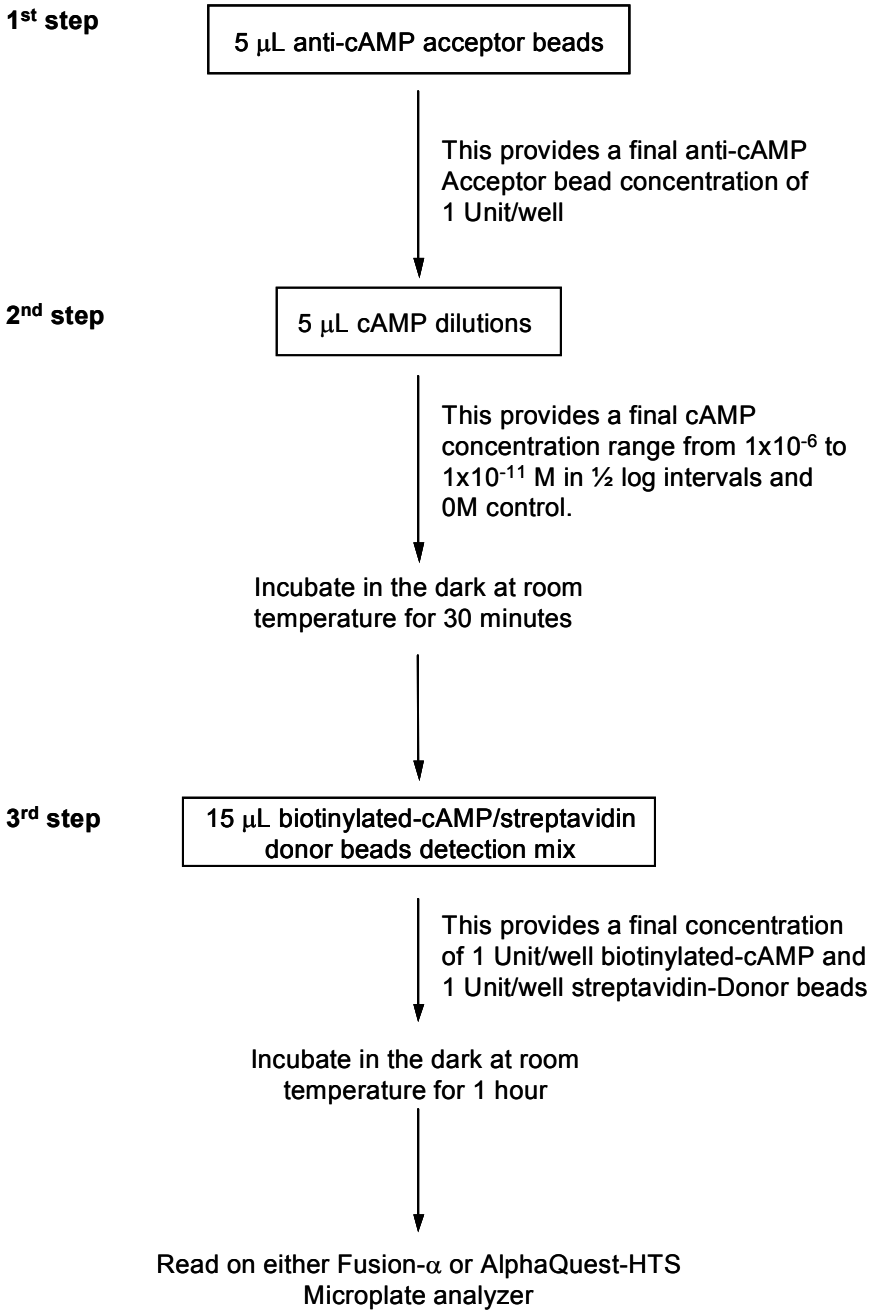
To make 250 µL anti-cAMP Acceptor beads solution, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
Stimulation buffer	245 µL	**-**	-
Acceptor beads (10 Units/µL)	5 µL	**1 Unit/5 µL**	1 Unit/25 µL

Note: Prepare fresh prior to assay.

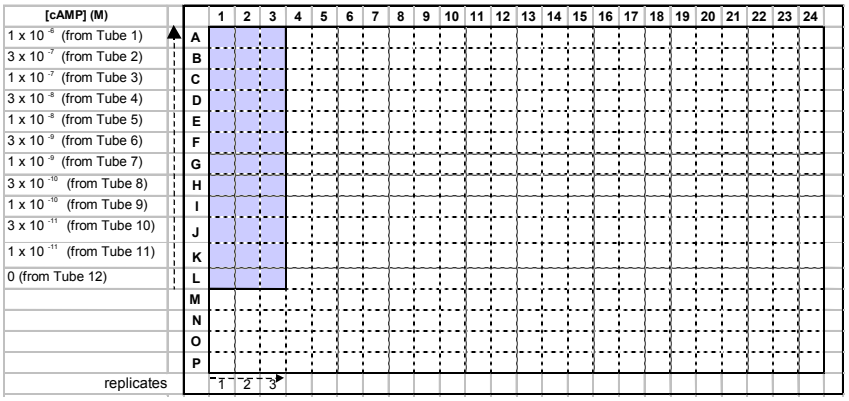
Order of addition:

To a 384-well microplate (PerkinElmer Life Sciences OptiPlate-384 NEW product number 6007290) **add in triplicate:**



Please note that a longer incubation time (after detection mix addition) can be used to increase total counts (see Section IX page 41).

Plate set-up:

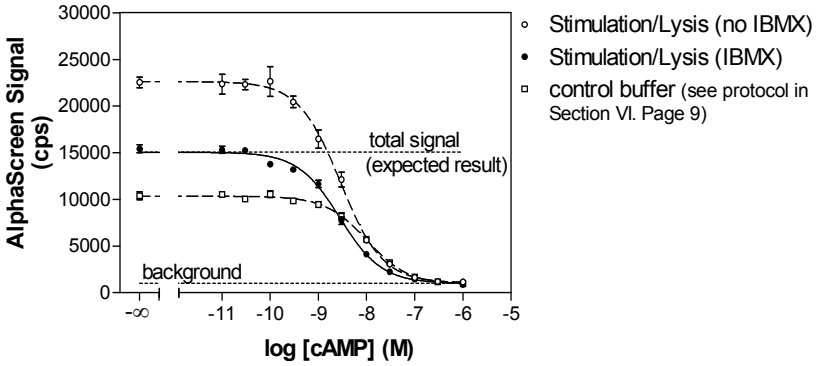


As described under order of addition, to each well add:

5 µL anti-cAMP Acceptor beads solution, 5 µL cAMP dilutions (final concentration/well as indicated) and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

Interpreting the data:

The AlphaScreen signal is plotted as a function of log concentration of cAMP.



Observations:

The above graph illustrates a typical cAMP competition curve performed under functional assay conditions using both stimulation (containing IBMX) and lysis buffers (-●-): white opaque 384 well microplate, 25 µL final volume, room temperature (22-23°C) and 1 hour incubation. In these conditions, typical standard curve provides a signal to background ratio of ~15, a 2 log dynamic range (the linear portion of the curve) and an IC_{50} value lower than 10 nM. Total signal will vary with ambient temperature and incubation time. For consistent results, the same incubation time and temperature should be used for each plate. The sensitivity, or limit of detection, for cAMP detection can be defined from the point of inflection of the upper portion of the sigmoidal curve. For the result using stimulation buffer containing IBMX (conditions one would expect to use in a cell stimulation assay), this occurs at a cAMP concentration of about 0.3 nM. This translates into a cAMP amount equal to 7.5 fmole of cAMP, considering our recommended assay volume of 25 µL. Generally, this kit will detect femtomole levels of cAMP.

Two other cAMP standard curves are presented in this graph for comparison: the first being generated in the 1x control buffer containing 0.03% Tween-20 (-□-) using the specific protocol described in Section V, and the second being generated in a IBMX-free stimulation buffer and lysis/detection buffer (-○-).

Because of its resemblance with cAMP, IBMX competes with cAMP binding to anti-cAMP antibodies such as those coating the acceptor beads. Therefore, in the presence of IBMX, a decrease in AlphaScreen signal is observed but the sensitivity of the assay is not affected. The IBMX concentration should be limited to 0.2-0.25 mM. At this concentration range, the maximal signal is reduced by approximately 30%.

Final concentrations of reagents in the assay are 1 Unit/well for Donor-SA beads, Acceptor beads, and biotinylated cAMP. Final concentrations of cAMP standard are: 1 µM, 0.3 µM, 0.1 µM, 30 nM, 10 nM, 3 nM, 1 nM, 0.3 nM, 0.1 nM, 0.03 nM. 0.01 nM and 0 M.

VII. FORSKOLIN DOSE-RESPONSE / DETERMINATION OF THE OPTIMAL CELL CONCENTRATION

25 µL final volume in 384-well white opaque microplate

Assay performed in 1x stimulation buffer containing 0.5 mM IBMX and lysis/detection buffer

Biotinylated cAMP used at 1 Unit/well

Materials provided:

Anti-cAMP Acceptor beads (10 Units/µL in PBS)

Streptavidin Donor beads (10 Units/µL in PBS)

Biotinylated cAMP (133 Units/µL in Tris·EDTA)

Liquid cAMP standard (50 µM)

Additional reagents required (not provided):

Item	Suggested source*	Catalog #
Forskolin†	Sigma	F 6886
BSA	Sigma	A 7030
10% Tween-20	Pierce	28320
1 M HEPES	Gibco BRL	15630-106
HBSS	Gibco BRL	14065-056
IBMX‡	Sigma	I 5879
Ethanol 95%	Sigma	E 7148
Versene	Gibco BRL	15040-066
DMSO	Sigma	D 8779
1x PBS	Gibco BRL	14190
NaOH 1N	Sigma	S 2770

* Equivalent sources can be substituted
† Dissolve 5 mg in 244 µL ethanol to make a 50 mM stock solution. Aliquot and store at –20ºC; use as required.
‡ (3-Isobutyl-1-Methylxanthine) Dissolve 100 mg in 900 µL DMSO to yield a 500 mM stock solution. Aliquot and store at –20ºC; use as required.

Reagent preparation:

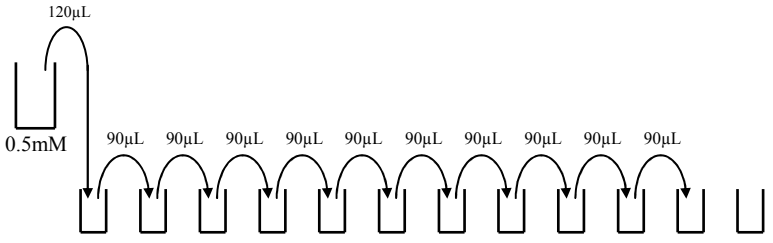
1. **Prepare 40 mL of Stimulation buffer containing IBMX, as described in Section IV Page 13.**

2. **Prepare 20 mL of lysis/detection buffer, as described in Section IV Page 14.**

3. **Prepare a Forskolin dilution series (2X) from 50 mM solution (see Section IV Page 14 for preparation), as follows:**

   Thaw 50 mM forskolin stock solution at room temperature.

   Make an intermediate 500 µM dilution by adding 5 µL of the 50 mM stock solution to 495 µL stimulation buffer containing IBMX. Forskolin dilutions are then prepared in stimulation buffer containing IBMX as 2x concentrated solutions. Serially dilute to provide a concentration range from $2x10^{-4}$ to $2x10^{-9}$ M in ½ log intervals, as illustrated below. Include a "no forskolin" control.



Tubes	1	2	3	4	5	6	7	8	9	10	11	12
Stimulation buffer	180µL	210µL	180µL	210µL	180µL	210µL	180µL	210µL	180µL	210µL	180µL	210µL
[Forskolin](M)	$2x10^{-4}$	$6x10^{-5}$	$2x10^{-5}$	$6x10^{-6}$	$2x10^{-6}$	$6x10^{-7}$	$2x10^{-7}$	$6x10^{-8}$	$2x10^{-8}$	$6x10^{-9}$	$2x10^{-9}$	0
[Forskolin](M) Final in 10 µL reaction	$1x10^{-4}$	$3x10^{-5}$	$1x10^{-5}$	$3x10^{-6}$	$1x10^{-6}$	$3x10^{-7}$	$1x10^{-7}$	$3x10^{-8}$	$1x10^{-8}$	$3x10^{-9}$	$1x10^{-9}$	0

Note: Prepare fresh prior to assay.

4. 5 µM standard cAMP solution

Add 10 µL of the 50 µM cAMP standard solution to 90 µL stimulation buffer to provide the required 5 µM cAMP solution.

Note: Prepare fresh prior to assay.

5. Streptavidin Donor beads/biotinylated cAMP detection mix

Add 5 µL of the 133 Units/µL biotinylated cAMP to 195 µL lysis/detection buffer to obtain a 3.3 Units/µL solution. To make 1950 µL detection mix, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
Lysis/detection buffer	1898 µL	**1x**	1x
Donor beads (10 unit/µL)	13 µL	**1 unit/15 µL**	1 Unit/25 µL
Biotinylated cAMP (3.3 Units/µL)	39 µL	**1 unit/15 µL**	1 Unit/25 µL

Note: Prepare fresh and incubate in the dark for at least 30 minutes prior to use.

Cell preparation:

1. Harvesting cells

For best results, we recommend working with cells ~70-90 % confluent and showing at least 95% viability. Remove growth medium and briefly rinse with Versene. Add fresh Versene and incubate at 37°C for ~5 min to help detach the cells. Collect cells and centrifuge for 5 minutes at 275 x *g*. Decant supernatant and resuspend the pellet in 1x PBS. Determine cell concentration. Re-centrifuge for 5 minutes at 275 x *g* and decant the supernatant. Resuspend the cells in stimulation buffer to a final concentration of 10,000 cells/µL.

Note: Prepare fresh prior to assay.

2. Cells/anti-cAMP Acceptor beads mixes

First, add 16 µL of 10 Units/µL anti-cAMP acceptor beads in 384 µL stimulation buffer containing IBMX to make a bead working solution of 0.4 Unit/µL.

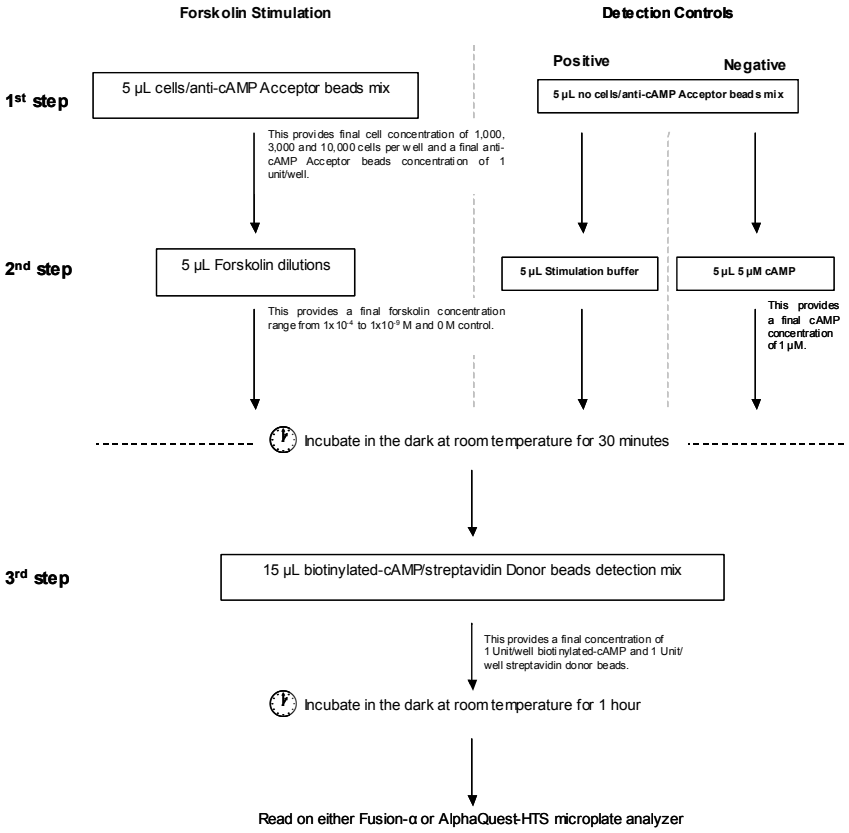
As shown in the table below, dilute the cells in the acceptor bead working solution and complete with stimulation buffer to make 250 µL solutions at intermediate concentrations of 200, 600, 2,000 cells/µL containing 0.2 Unit/µL anti-cAMP Acceptor beads. The 0 cell/well preparation will be used as a detection control.

Cells/well (final)	10,000 cells/µL solution (µL)	Acceptor bead working solution (µL)	Stimulation buffer (µL)	[Intermediate] Cells/µL
10,000	50	125	75	**2,000**
3,000	15	125	115	**600**
1,000	5	125	120	**200**
0 (control)	0	20	20	**0**

Note: Prepare fresh prior to assay.

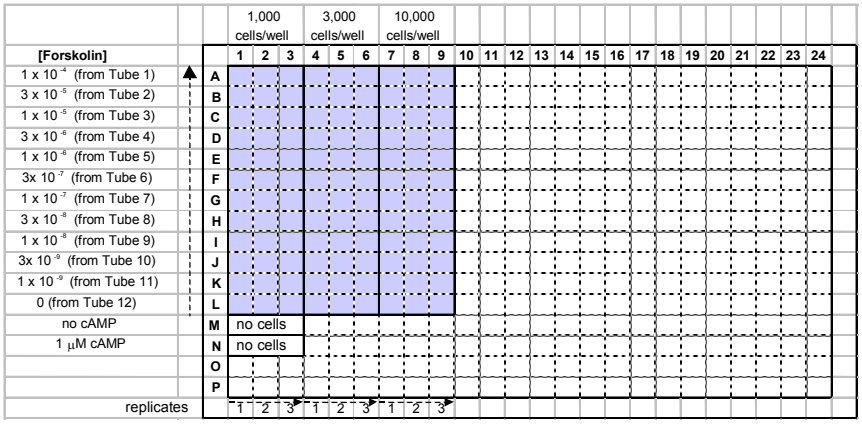
Order of addition:

To a 384-well microplate (PerkinElmer Life Sciences OptiPlate-384 NEW product number 6007290) **add in triplicate:**



Please note that a longer incubation time (after detection mix addition) can be used to increase total counts (see Section IX page 41).

Plate set-up:



As described under order of addition, to each well add:

1. Forskolin stimulation:

   5 µL cells/anti-cAMP Acceptor beads mix, 5 µL forskolin dilutions (final concentration/well as indicated) and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

2. Detection control - no cAMP:

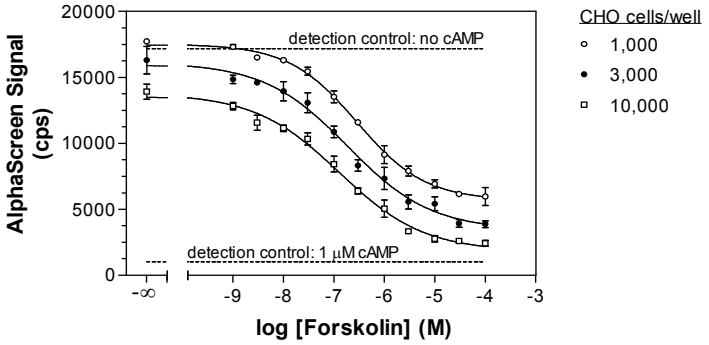
   5 µL no cell (control)/anti-cAMP Acceptor beads mix, 5 µL stimulation buffer and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

3. Detection control - 1 µM cAMP:

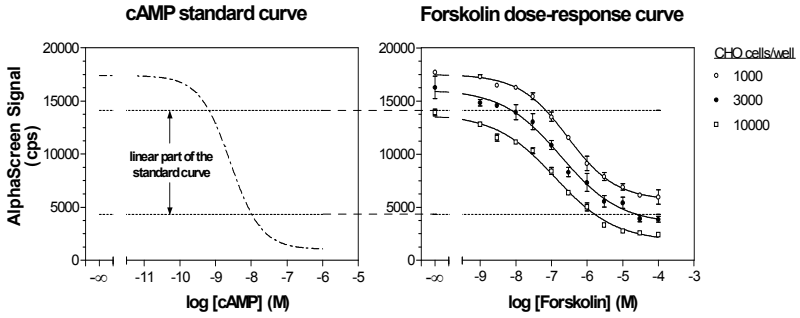
   5 µL no cells/anti-cAMP Acceptor beads mix, 5 µL 5 µM cAMP and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

Interpreting forskolin stimulation data:

The AlphaScreen signal is plotted as a function of the log of the concentration of forskolin for each of the 3 cell concentrations. The control signals are plotted to provide the upper and lower values of the possible signal range.



The forskolin dose-response curves obtained with CHO cells are related to the cAMP standard curve in order to establish what cell concentration provides a response that occupies most of the linear region of the cAMP standard curve with the highest S/B ratio.



Observations:

From the above example, the preferred cell concentration for subsequent experiments (ex. agonist effect determinations) would be 3,000 cells/well as most of the forskolin dose-response curve at this cell concentration falls within the linear region of the cAMP standard curve.

VIII. $G\alpha_i$-COUPLED RECEPTOR STIMULATION / DETERMINATION OF Z' VALUES FOR ANTAGONIST

25 µL final volume in 384-well white opaque microplate

Assay performed in 1x stimulation buffer containing 0.5 mM IBMX and lysis/detection buffer

Biotinylated cAMP used at 1 Unit/well

Materials provided:

Anti-cAMP Acceptor beads (10 Units/µL in PBS)

Streptavidin Donor beads (10 Units/µL in PBS)

Biotinylated cAMP (133 Units/µL in Tris.EDTA)

Liquid cAMP standard (50 µM)

Additional reagents required (not provided):

Item	Suggested source*	Catalog #
Forskolin†	Sigma	F 6886
BSA	Sigma	A 7030
10% Tween-20	Pierce	28320
1 M HEPES	Gibco BRL	15630-106
HBSS	Gibco BRL	14065-056
IBMX‡	Sigma	I 5879
Ethanol 95%	Sigma	E 7148
Versene	Gibco BRL	15040-066
DMSO	Sigma	D 8779
1x PBS	Gibco BRL	14190
NaOH 1N	Sigma	S 2770
Spiperone	RBI	D-050
8-OH-DPAT	Sigma	H-140

* Equivalent sources can be substituted
† Dissolve 5 mg in 244 µL ethanol to make a 50 mM stock solution. Aliquot and store at –20ºC; use as required.
‡ (3-Isobutyl-1-Methylxanthine) Dissolve 100 mg in 900 µL DMSO to yield a 500 mM stock solution. Aliquot and store at –20ºC; use as required.

Reagent preparation:

The example included in this section is based on the stimulation of **$G\alpha_i$-coupled serotoninergic 5-HT1a receptors expressed in CHO-K1 cells.** Forskolin stimulates the adenylate cyclase whereas the *selective agonist 8-OH-DPAT* is used to reverse the effect of Forskolin. The non-selective antagonist *spiperone* reversed the effect of 8-OH DPAT. The table below describes some typical pharma codynamic parameters obtained with this system at PerkinElmer LAS (BioSignal).

Compound	EC_{50} (μM)	EC_{80} (μM)
Forskolin	1.5-3.0	10-20
8-OH DPAT	0.06-0.12	0.3-0.6

Note: We recommend preparing all reagents fresh before use and keep them at room temperature.

1. **Prepare 40 mL of Stimulation buffer containing IBMX, as described in Section IV Page 13.**

2. **Prepare 20 mL of lysis/detection buffer, as described in Section IV Page 14.**

3. **Prepare a 50 mM forskolin stock solution as described in Section IV Page 14.**

4. **Prepare Antagonist solution, as follows:**

   Antagonists as well as library compounds are typically tested at a final concentration of 10 μM. The final concentration of these compounds is calculated based on the volume prevailing during cell stimulation (10 μL). We suggest preparing a 4x (40 μM) antagonist solution in stimulation buffer. Since most library compounds are generally dissolved in 100% DMSO, we recommend limiting the DMSO concentration to 2% v/v during cell stimulation to ensure maximum cell viability and responsive-ness. Proceed to the final antagonist dilution as follows:

500 µL 4x antagonist solution for performing 200 assay points:

Reagent	Volume	**[4x]**	[Final]
Stimulation buffer/IBMX	480 µL	-	-
1 mM Antagonist	20 µL	**40 µM**	10 µM

5. **Prepare Forskolin alone or Forskolin/Gα_i agonist solutions, as follows:**

Forskolin is prepared in stimulation buffer as a 4x concentrated solution. The final concentration of Forskolin is calculated based on the volume prevailing during cell stimulation (10 µL). Forskolin is typically used at a concentration producing either 50% (EC_{50}) or 80% (EC_{80}) of the maximum adenylate cyclase activation. Assays performed at the EC_{50} will be more sensitive to weak agonists concentrations compared to those performed at the EC_{80}. On the other hand, assays performed using a forskolin concentration equivalent to its EC_{80}, allows an enhanced signal window. In this particular example, EC_{80} concentrations of forskolin were used.

Forskolin alone: Thaw 50 mM forskolin stock solution at room temperature. Make an intermediate 0.5 mM dilution by adding 5 µL of the 50 mM stock solution to 495 µL stimulation buffer containing IBMX. Making 500 µL of 4x forskolin solution allows one to perform 200 assay points. Proceed to the final dilution as follows:

Reagent	Volume	**[4x]**	[Final]
Stimulation buffer/IBMX	460 µL	-	-
0.5 mM Forskolin	40 µL	**40 µM**	10 µM

Forskolin/ Gα_i agonist solution is prepared in stimulation buffer as a 4x concentrated solution. The final concentration of agonist is calculated based on the volume prevailing during cell stimulation (10 µL). Agonist is typically used at a concentration producing 50% (EC_{50}) or 80% (EC_{80}) of the maximum inhibition of forskolin-induced adenylate cyclase activation. Assays performed at the EC_{50} will be more sensi-

tive to weak antagonists compared to those performed at the EC_{80}. Assays performed using an agonist concentration equivalent to its EC_{80}, allows an enhanced signal window. In this particular example, EC_{50} concentrations of agonist were used.

Agonist: From a 1 mM stock solution of agonist, make an intermediate 100 µM dilution by adding 5 µl of the stock solution to 45 µL of stimulation buffer/IBMX. Making 500 µL of 4x agonist solution allows one to perform 200 assay points. Proceed to the final dilution as follows:

Reagent	Volume	**[4x]**	[Final]
Stimulation buffer/IBMX	440 µL	-	-
100 µM agonist	20 µL	**4 µM**	1 µM
0.5 mM Forskolin	40 µL	**40 µM**	10 µM

6. **Prepare a 5 µM cAMP standard solution, as follows:**

Exogenous cAMP is used as a detection control. Add 10 µL of the 50 µM standard solution to 90 µL stimulation buffer/IBMX to provide the required 5 µM cAMP solution.

7. Streptavidin Donor beads/biotinylated cAMP detection mix

Add 5 µL of the 133 Units/µL biotinylated cAMP to 195 µL lysis buffer to obtain a 3.3 Units/µL solution. To make 3000 µL detection mix, add reagents as follows:

Reagent	Volume	**[Intermediate]**	[Final]
Lysis/detection buffer	2920 µL	**1x**	1x
Donor beads (10 Units/µL)	20 µL	**1 Unit/15 µL**	1 Unit/25 µL
Biotinylated cAMP (3.3 Units/µL)	60 µL	**1 Unit/15 µL**	1 Unit/25 µL

Note: Prepare fresh and incubate in the dark for at least 30 minutes prior to use.

Cell preparation: See Section VII Pages 27-28

Order of addition:

To a 384-well microplate (PerkinElmer Life Sciences OptiPlate-384 NEW product number 6007290) **add in triplicate:**

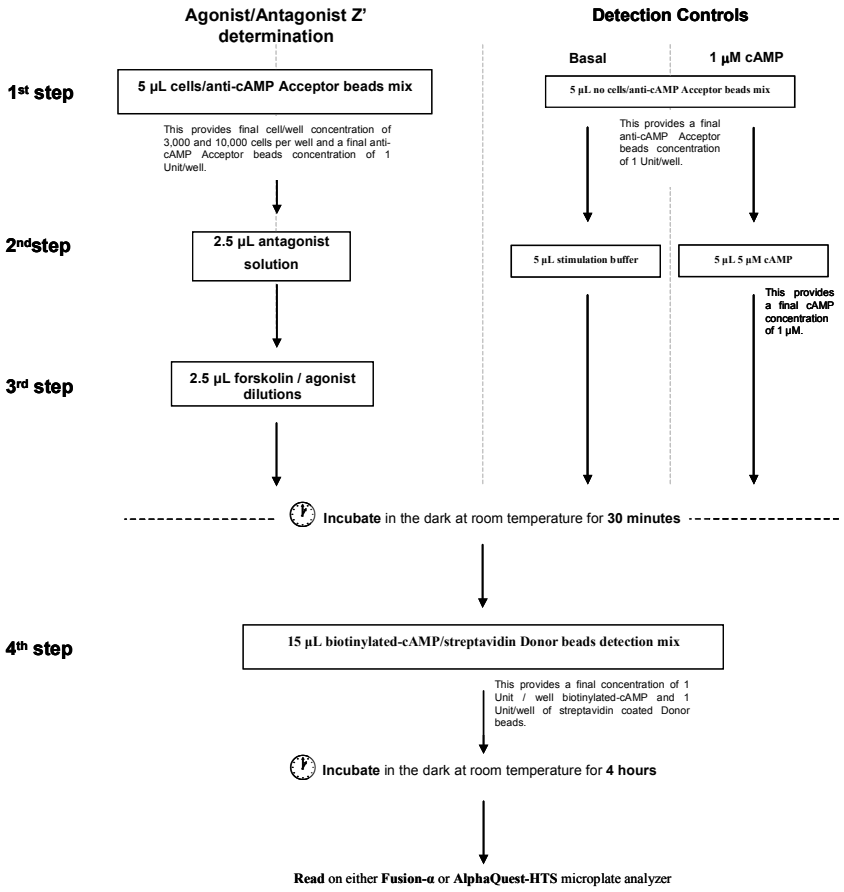
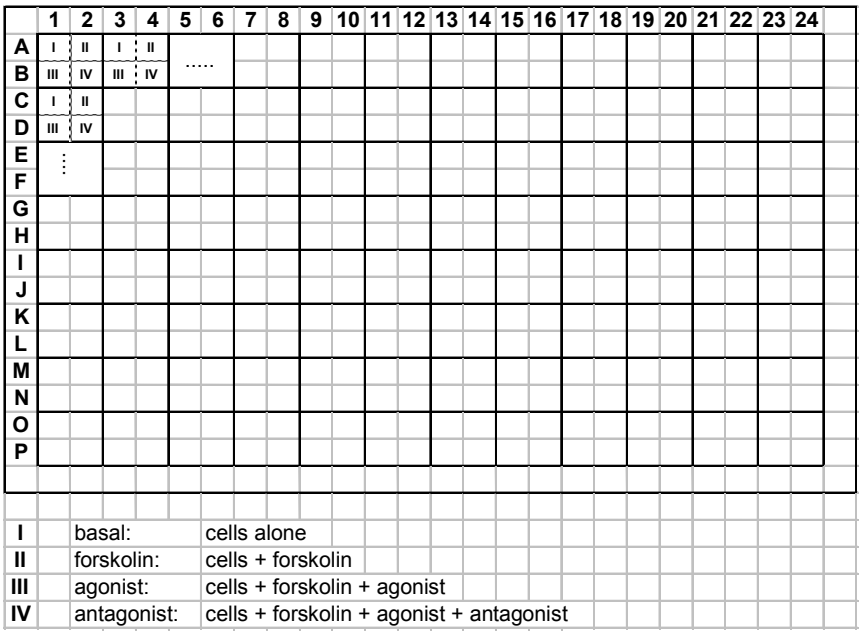


Plate set-up (chessboard pattern)**:**

We recommend distributing the reagents using a "chessboard pattern" to take into account the variability inherent to the assay plate and the detection system.



As described under order of addition, to each well add:

1. Basal:

   5 µL cells/anti-cAMP Acceptor beads mix, 5 µL stimulation buffer and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

2. Forskolin stimulation:

   5 µL cells/anti-cAMP Acceptor beads mix, 2.5 µL stimulation buffer, 2.5 µL forskolin dilution and 15 µL biotinylated cAMP/ streptavidin Donor beads detection mix.

3. Agonist stimulation:

   5 µL cells/anti-cAMP Acceptor beads mix, 2.5 µL stimulation buffer, 2.5 µL forskolin/agonist and 15 µL biotinylated cAMP/ streptavidin Donor beads detection mix.

4. Antagonist inhibition:

   5 µL cells/anti-cAMP Acceptor beads mix, 2.5 µL antagonist dilution, 2.5 µL forskolin/agonist and 15 µL biotinylated cAMP/ streptavidin Donor beads detection mix.

We also recommend to include the following controls:

No cell- no cAMP:

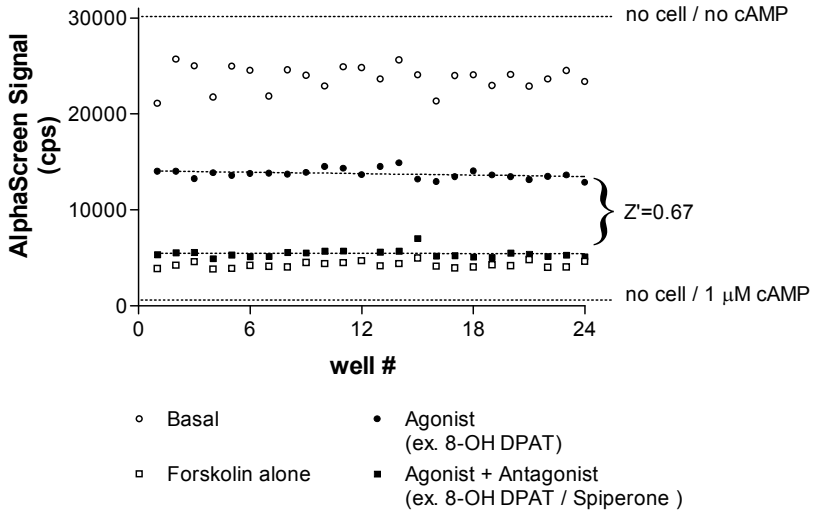
5 µL no cell (control)/anti-cAMP Acceptor beads mix, 5 µL stimulation buffer and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

No cell- 1 µM cAMP:

5 µL no cells/anti-cAMP Acceptor beads mix, 5 µL 5 µM cAMP and 15 µL biotinylated cAMP/streptavidin Donor beads detection mix.

Interpreting the data

The scatter plot presented below shows typical results obtained from the experiments presented in this section (4 hour-incubation after streptavidin-Donor/biotinylated cAMP detection mix addition).



Metrics	Basal	Forskolin	Agonist	Agonist + Antagonist
Mean	23781	4284	13754	5448
SD	1284	311	503	416
CV%	5.4	7.3	3.7	7.6

Z' coefficient determination:

Calculating the Z' coefficient (Zhang et al., JBS, 4:67, 1999) is an easy and well accepted method to measure the statistical difference prevailing between two data populations such as those generated by the experiment suggested in this section. The formula described below is used to estimate the Z' coefficient:

$$Z' = 1 - \frac{(3SD_1 + 3SD_2)}{|X_1 - X_2|}$$

where:

X_1 and SD_1 = mean and standard deviation of data population 1

X_2 and SD_2 = mean and standard deviation of data population 2

Using the formula and the metrics described above, one can determine the robustness of the assay by measuring the statistical difference prevailing between the "Agonist" and "Agonist + Antagonist" treatments:

$$\mathbf{Z'} = 1 - \frac{(3 \times 503 + 3 \times 416)}{|13754-5448|} = 1 - \frac{2757}{8306} = 1 - 0.33 = \mathbf{0.67}$$

Key observations:

Internal controls performed in the absence of cells (see scatter plot) indicate that the overall signal window is exploitable using the protocol described in this section.

Addition of cells alone (basal) leads to a signal reduction produced by the endogenous cAMP already present in the cells prior to stimulation.

Forskolin stimulation leads to a significant signal decrease, which brings the level of counts close to that measured following the addition of 1 μM exogenous cAMP.

Treating cells with agonist reversed the effect of forskolin and brought the level of counts back to that observed with "basal".

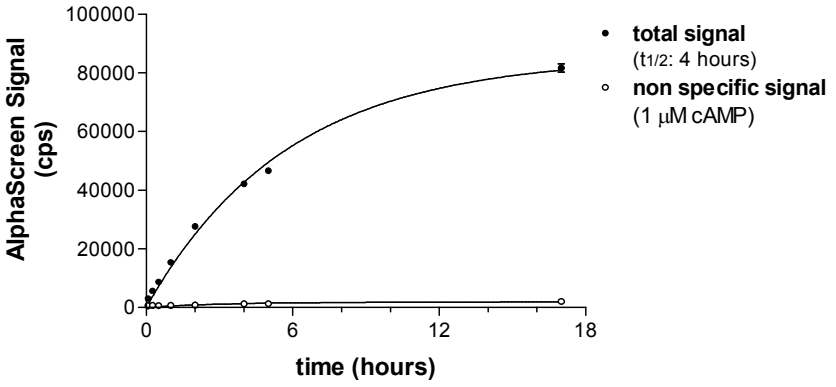
Antagonist treatment inhibited the effect of the agonist and restored the level of counts close to those measured following cell stimulation with forskolin.

Z' values higher than 0.5 indicates that the assay is robust enough to screen putative antagonists in singlet with a high level of statistical confidence ($p < 0.005$).

IX. TIME COURSE OF cAMP DETECTION

As shown in the figure below, robust counts (15,000 cps S/B = 20) are obtained after only one hour incubation following detection mix addition (assay performed in stimulation buffer containing IBMX using 1U/well biotinylated cAMP) and higher signals can be produced with longer incubation times. If needed, a 5-fold signal increase could be obtained following an overnight incubation (75-80,000 cps). The signal at 4 hours is approximately half that achieved at 17 hours at 23°C. At the recommended probe concentration of 1U/well, precision of the assay is not affected by long incubation times. Although a higher signal may be obtained at 17 hours incubation, this higher signal is associated with a shift in IC_{50} value towards a decrease in sensitivity.

Thus the AlphaScreen cAMP kit offers the option of using short incubation times (1 hour) to achieve satisfactory performance, or achieve higher performance by using longer incubation times.



X. TROUBLESHOOTING GUIDE

Low Counts

- Quick spin the tubes of AlphaScreen beads using a micro-centrifuge in "Pulse" mode to ensure that all the beads are recovered in the bottom of the tube.
- Ensure the right amount of biotinylated-cAMP analog was used.
- AlphaScreen beads are light sensitive. Pipet in a subdued light environment (~100 lux).
- Ensure the right amount of Acceptor and Donor beads were used.
- Check that the buffers are at the correct pH.
- Ensure that white opaque 384 white opaque microplates are being used.
- Check the cell concentration. Abnormally high cell numbers/well will decrease the signal due to the high sensitivity of the kit, which allows quantitation of endogenous levels of cAMP within the cell before stimulation. Perform a cell titration.
- Incubation temperature much lower than 23°C.
- Low temperature prevailing in the room where the Fusion α / AlphaQuest reader is located. All data presented here were performed at 22-23ºC.

High Background

- Ensure that Tween-20 was used at the recommended final concentration.

Poor Assay Sensitivity

- Ensure that the right amount of biotinylated-cAMP analog was used.
- Ensure that the right amount of Acceptor and Donor beads were used.

- Check that the buffers are at the correct pH.

Poor Cell Stimulation

- Check the cell concentration. Sub optimum number of cells will decrease the signal. Perform a cell titration.
- Check the stimulation time. Perform a time course.
- Check cell harvesting conditions. Use Versene (PBS-EDTA) for cell stripping.
- Ensure cell confluence is between 70-90% and cell viability is at least 95%.
- Check time between harvesting and cell stimulation (should be less than 30 minutes).
- Ensure that receptor is coupled to Gαi/o or Gαs and cAMP levels are modulated by receptor stimulation.
- Ensure that stimulant (Gαs agonist or forskolin) is not degraded. Prepare fresh prior to assay.

Day-to-day Variation

- Check harvesting conditions. Use a standard protocol.
- Check room temperature. Variations in temperature will cause variations in signal.
- Work in subdued light environment (~100 lux). Exposure of Donor beads to bright light will reduce signal.
- Ensure that the incubation times do not vary between experiments.

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