upstate

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cAMP HTS Immunoassay Kit, 5X (Chemiluminescent)

Cat. No. 17-518

960 Wells

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Not for use in diagnostic procedures

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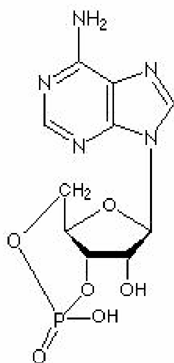
Introduction

Cyclic AMP (cAMP, adenosine 3′, 5′-cyclic monophosphate) is a nucleotide which acts as a key second messenger in multiple signal transduction pathways [1]. It is synthesized from ATP by the action of adenylate cyclase, and is inactivated by hydrolysis to 5′-AMP by the actions of phosphodiesterases [2]. All receptors that act via cAMP are coupled to a stimulatory G protein, which activates adenylate cyclase upon ligand binding [3]. Many different drugs, neurotransmitters and hormones exert their cellular effects by modulating adenylate cyclase activity and thus raising or lowering intracellular cAMP concentrations [3,4].

cAMP regulates many cellular functions, such as metabolism, cell growth and differentiation, gene transcription, ion transport and ion channel function [3,4]. These cAMP effects, mediated primarily by cAMP-dependent protein kinase (PKA), result in cAMP being responsible for the regulation of many physiological processes, including cardiovascular, endocrine, neuronal, glandular, kidney, and immune functions, as well as general metabolism [5-11]. Consequently, agents which increase or decrease intracellular cAMP levels are of major interest in drug discovery [6,12].



Upstate®'s cAMP HTS immunoassay kit is a competitive immunoassay for *in vitro* quantitative detection of cAMP in mammalian cell lysates and supernatants, which has been optimized for use in high throughput screening applications. To facilitate this, the kit provides ten ready to use 96 well pre-coated assay microplates and all reagents required to perform the assay.

Figure 1: Structure of cAMP

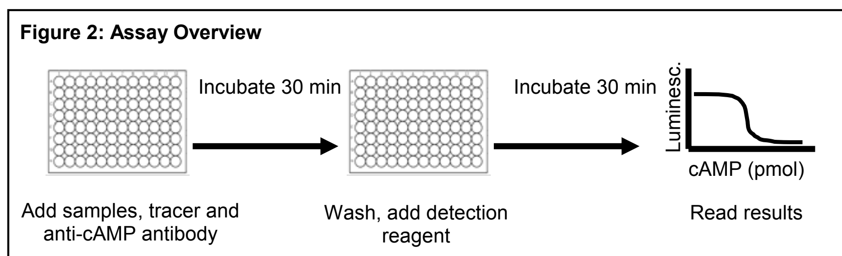
Test Principle

Upstate®'s cAMP HTS Immunoassay kit is a competitive immunoassay for highly sensitive and rapid chemiluminescent quantitation of cyclic AMP (cAMP, adenosine 3´, 5´-cyclic monophosphate) from cell extracts of any species. The kit comprises a specific anti-cAMP antibody that recognizes all species, an alkaline phosphatase (AP)-labeled cAMP conjugate, ten 96 well microplates pre-coated with an anti-Rabbit antibody, cAMP standard, and an Alkaline Phosphatase chemiluminescence substrate.

Competitive ELISAs differ from traditional sandwich ELISAs in that a competition between labeled and unlabeled antigen for available antibody binding sites occurs. In order to utilize a competitive ELISA, one reagent must be conjugated to a detection enzyme, such as alkaline phosphatase. In Upstate®'s cAMP HTS Immunoassay kit, standards or unknown samples are mixed with an alkaline phosphatase (AP)-labeled cAMP conjugate and a highly specific anti-cyclic AMP antibody, and incubated in wells of a 96 well microtiter plate pre-coated with a capture antibody. The AP-labeled cAMP conjugate will bind to the anti-cAMP antibody wherever its binding sites are not already occupied by unlabeled cAMP. Thus, the more cAMP in the sample or standard, the lower the amount of AP-labeled cAMP-conjugate that is bound. Upon plate development, the chemiluminescence intensity is inversely proportional to the amount of cAMP in a sample or standard.

This kit contains antibody coated plates and all reagents required to perform 960 assays.

Figure 2: Assay Overview



Application

Upstate®'s cAMP HTS immunoassay kit is designed to measure the amount of cAMP in cell culture supernatants, tissue homogenates and biological fluid (serum, plasma, and serum-free) samples from any species. Sufficient reagents are included in this kit for ten 96-well immunoassay plates. Running triplicate wells for samples and standards is recommended.

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Kit Components

1. White 96-Well anti-Rabbit Coated Plate: (Part No. 2004070) Ten 96-well immuno-plates pre-coated with anti-Rabbit polyclonal antibody, individually sealed in a foil pouch.
2. Rabbit anti-cAMP antibody: (Part No. CS200431) One 60 µL vial.
3. cAMP Standard: (Part No. CS200418) One 10 mL bottle.
4. cAMP Alkaline Phosphatase Conjugated Tracer: (Part No. CS200417) One 100 µL vial.
5. 2x Assay Diluent: (Part No. CS200419) One 125 mL bottle.
6. 5x Wash Buffer: (Part No. CS200420) One 600 mL bottle.
7. Lysis Buffer: (Part No. CS200421) One 500 mL bottle.
8. 6x Alkaline Phosphatase Substrate: (Part No. CS200422) One 25 mL bottle.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Pipettors & tips capable of accurately measuring 10-1000 µL
3. Plate shaker (optional)
4. Automated Liquid Handler (optional)
5. Plate Washer (optional)
6. Luminometer capable of reading 96-well microplates.
7. Test tubes for standard and sample dilutions
8. Mechanical vortex

Storage of Kit Components

Maintain the unopened kit at 2° to 8°C until the expiration date indicated on the label. After opening the kit, maintain the White 96-Well anti-Rabbit Coated Plates, cAMP Alkaline Phosphatase Conjugate Tracer, cAMP Standard, 5x Wash Buffer, 2x Assay Diluent, Lysis Buffer, and Alkaline Phosphatase Substrate at 2° to 8°C until the expiration date indicated on the label. For long-term storage, maintain the Rabbit anti-cAMP Polyclonal Antibody at -20°C.

Precautions

- The White 96-Well anti-Rabbit Coated Plates, 5x Wash Buffer, 2x Assay Diluent, and Lysis Buffer contain thimerosal. Thimerosal is highly toxic by inhalation, contact with skin, or if swallowed. Thimerosal is a possible mutagen and should be handled accordingly.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.

Technical Hints

- Allow samples and all assay reagents to reach room temperature before use.
- Standards should be made in either glass, or polypropylene tubes; avoid polystyrene.
- Mix samples thoroughly before use; avoid excessive foaming.
- Pipet the sample / standard to the bottom of the well.
- To avoid contamination, add additional assay reagents to the side of the well.
- The use of plate sealers is recommended to reduce the possibility of well-to-well contamination.
- During the incubation time, the use of a plate shaker may improve assay sensitivity.
- Minimize contamination by endogenous alkaline phosphatase. Care should be taken to not touch pipet tips or other reagents with bare hands.

- Ensure that no residual wash buffer remains in the wells. Be sure to completely pat dry the plate on paper towels following the last wash step.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values.
- **Recommended Method for Manual Plate Washing:**
  1. Remove existing fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  2. Pipet 250 μL of diluted 1x Wash Buffer into each well with a multi-channel pipet.
  3. Remove the Wash Buffer from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels to remove excess fluid.
  4. Repeat washing and flicking 5 times.

Preparation of Reagents

1. **Wash Buffer**

   Add the entire contents of the 5x Wash Buffer to an appropriate container, and adjust volume to 3000 mL with deionized water. Stir to homogeneity.

2. **Assay Diluent**

   Add the entire contents of the 2x Assay Diluent to an appropriate container, and adjust volume to 250 mL with deionized water. Vortex or stir thoroughly. Use this 1x solution for diluting the Rabbit anti-cAMP Antibody and cAMP Alkaline Phosphatase Conjugate Tracer.

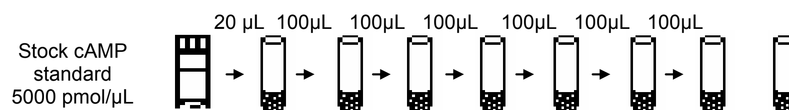
3. **cAMP Standard**

   The cAMP Standard is provided at a concentration of 5000 pmol/μL. This stock material is then used to generate a standard curve. Use Lysis Buffer to make cAMP Standard dilutions. A suggested dilution scheme is as follows:

   a) Label 8 test tubes #1-8. Add 900 μL of Lysis Buffer to test tubes #2-8.

   b) Add 980 μL of Lysis Buffer to tube #1. Remove 20 μL of the cAMP Standard solution from the stock bottle and add it to tube #1. Vortex the tube thoroughly. This is Standard tube #1 with a concentration of 100 pmol/μL.

   c) Standards #2-8 are then prepared by performing a 1:10 dilution of the preceding standard. Refer to Figure 3 below. For example, to make Standard #2, remove 100 μL of Standard #1 and add it to tube #2 and vortex and so on. Do not add any cAMP Standard to the #8 or "0.0 pmol/μL Concentration" Standard tube.



Standard Number:	#1	#2	#3	#4	#5	#6	#7	#8
Initial Volume (µL):	980	900	900	900	900	900	900	900
Concentration (pmol/µL):	100	10	1.0	0.1	0.01	0.001	0.0001	0.0

Figure 3: Serial Dilution of cAMP Standard

Note: *The cAMP Standard curve can be set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern. For example, if working within a narrower range of cAMP concentrations than in the typical standard curve shown in the dilution series above, the cAMP standard provided may be diluted within a narrower range. An example is shown in Figure 5 below.*

4. **Rabbit anti-cAMP Antibody**

   Immediately before use, dilute a sufficient amount of the Rabbit anti-cAMP polyclonal antibody 1:2,000 with 1x Assay Diluent. Mix thoroughly.

5. **cAMP Alkaline Phosphatase Conjugate Tracer**

   Immediately before use, dilute a sufficient amount of the cAMP Alkaline Phosphatase Conjugate Tracer 1:3000 with 1x Assay Diluent. Mix thoroughly.

6. **Alkaline Phosphatase Substrate**

   Immediately before use, dilute a sufficient amount of the Alkaline Phosphatase Substrate 1:6 with 1x Wash Buffer. Mix thoroughly.

Preparation of Cell Lysates

a. For Measurement of Intracellular cAMP

1. Chill Lysis Buffer on ice or by refrigeration.

2. Remove tissue culture media or assay buffer and add 200 µL of Lysis Buffer to each well. (200 µL volume of lysate is recommended for preparation of triplicate 50 µL samples in 96 well plates. If using larger wells, the user may increase volume of lysis buffer proportionately. Alternatively, if triplicate samples are not required, volume of lysis buffer may be reduced to 50 µL per well of 96 well plate).

3. Incubate for 10 minutes at room temperature. Use of a plate shaker during this step may facilitate cell lysis.

4. Triturate the sample several times with successive pipetting. Transfer 50 µL of the sample to the assay plate. Follow the protocol given in the Assay Instructions below.

b. For Measurement of Total cAMP (intracellular and extracellular)

1. Chill Lysis Buffer on ice or by refrigeration.

2. Do NOT remove the tissue culture media or assay buffer. Add 100 µL of Lysis Buffer to each well.

3. Incubate for 10 minutes at room temperature. Use of a plate shaker during this step may facilitate cell lysis.

4. Triturate the sample several times with successive pipetting. Transfer 50 µL of the sample to the assay plate. Follow the protocol given in the Assay Instructions below.

Notes:

1. *Best results will be obtained with freshly prepared samples.*
2. *Optimal sample dilution may vary between different cell types. Appropriate sample dilutions should be established by each investigator. If desired, samples can be diluted with 1x Assay Diluent or Lysis Buffer.*

Assay Instructions

1. Equilibrate assay reagents to room temperature before use.
2. Remove White 96-Well anti-Rabbit Coated Plate/s from foil pouch.
3. Add 50 µL of cAMP Standards 1 through 8 or prepared samples to wells. It is recommended that standards and samples be run in triplicate.

   Note: *A standard curve must be performed for each assay.*
4. Add 25 µL of the diluted cAMP Alkaline Phosphatase Conjugate Tracer dilution to all wells being tested.
5. Add 50 µL of the diluted Rabbit anti-cAMP Antibody to all wells being tested.
6. Cover or seal the plate with a plate sealer. Incubate the plate for 30 minutes at room temperature (on a shaker if possible).
7. Remove the fluid from the wells with an automated plate washer or by inverting the plate over a sink.
8. Carefully wash the wells 5 times with 1x Wash Buffer.

   For users of mechanical plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. This may result in poor precision and an unsuitable standard curve. For best results, we recommend at least 5 wash cycles.
9. Add 100 µL of the diluted Alkaline Phosphatase Substrate. Cover or seal the plate and incubate at room temperature for 30 minutes (on shaker if possible).
10. Read the plate for 1.0 second with a luminometer.

    CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to reading plate.*

Calculation of Results

Plate Reader/PC Interface: Enter the data into a computer program curve fitting software such as GraphPad Prizm®. A good fit can be obtained with a log regression analysis. Some data points at the top or bottom of the range tested may need to be dropped to get a good fit. Spreadsheet software packages can also perform such plotting. The standard curve should result in a graph that shows an inverse relationship between cAMP concentrations and the corresponding luminescence. Therefore, the greater the concentration of unconjugated, or "free" cAMP in the sample, the lower the Relative Light Units.

Manual Plotting: A more traditional method is to plot the standard curve on semi-log graph paper. Known concentrations of cAMP are plotted on the X-axis and the corresponding RLUs (Relative Light Units) on the Y-axis.

Sample Results

The Upstate® cAMP HTS Immunoassay is a competitive ELISA, thus low levels of free cAMP are indicated by a high signal, while high levels of free cAMP are

indicated by a low signal. An example of a typical standard curve is shown in Figure 4 below.

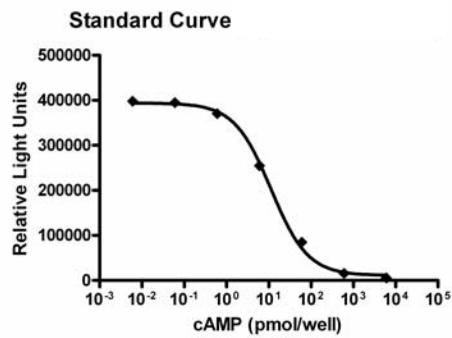


Figure 4. The provided cAMP standard containing 1 mM cAMP (5000 pmol/µL) was used in this experiment. Seven serial 1:10 dilutions were prepared in Lysis buffer. 50 µL of each standard was used to generate the standard curve in a range from 0.01 to 5000 pmol cAMP/well.

The cAMP Standard curve can be set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern. For example, if working within a narrower range of cAMP concentrations than in the typical standard curve shown in the dilution series in Figure 4 above, the cAMP standard provided may be diluted within a narrower range. An example is shown in Figure 5 below.

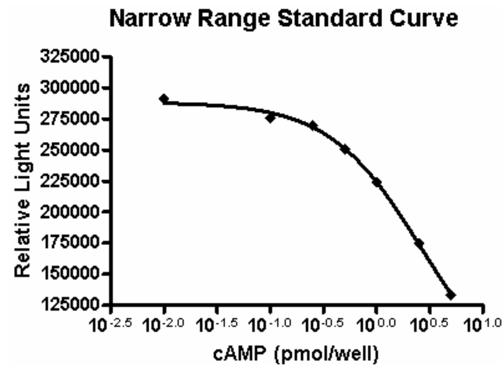


Figure 5. The provided cAMP standard containing 1 mM cAMP (5000 pmol/µL) was used in this experiment. Seven serial dilutions were prepared in Lysis buffer. 50 µL of each standard was used to generate the standard curve in a range from 0.01 to 5 pmol cAMP/well.

An example of an experimental data set is shown in Figure 6 below. This figure compares data obtained using the Upstate® cAMP HTS Immunoassay Kit with data obtained using a leading competitor's kit.

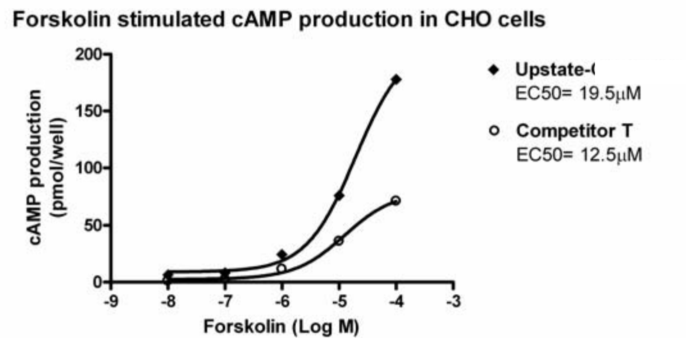


Figure 6. CHO wt cells were seeded at 30,000 cells per well in a 96-well plate 24 hr before the assay. Cells were pre-incubated with 1 mM IBMX for 5 minutes, then various concentrations of forskolin were added for an additional 15 minute incubation at 37ºC. Cells were lysed using Lysis Buffer provided, according to the recommended protocol. 50 μL of lysate was used for cAMP analysis.

Millipore's cloned human ChemiScreen™ β2 Adrenoceptor-expressing cell line (Catalog Number HTS073C) supports high levels of recombinant β2 expression on the cell surface and contains high levels of the promiscuous G protein Gα16 to couple the receptor to the calcium signaling pathway. Thus, the cell line is an ideal tool for screening for agonists and antagonists of β2. An example is shown below.

ChemiScreen™ β2 Adrenoceptor Dose-Response

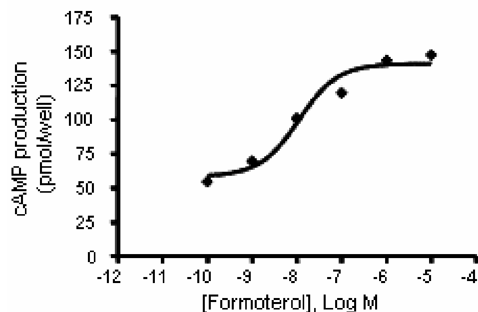


Figure 7. ChemiScreen™ β2 cells were spun down and resuspended in HBSS buffer with Ca^{++} and Mg^{++} containing 1mM IBMX at $2x10^6$ cells/mL. 100,000 cells/well (50 µL of cell suspension) were distributed into 96-well plates and preincubated for 5 minutes at 37ºC. Various concentrations of formoterol were added for an additional 15 minute incubation at 37ºC. Cells were then lysed using 100 µL Lysis Buffer, according to the recommended protocol. 50 µL of lysate was used for cAMP analysis.

Table 1. Cross-reactivity of rabbit anti-cAMP polyclonal antibody

cAMP	100%	CTP	<0.01%
cGMP	<0.1%	AMP	<0.01%
GTP	<0.01%	ADP	<0.01%
GMP	<0.01%	ATP	<0.01%

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