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# cAMP HTS Immunoassay Kit, 384 well (Chemiluminescent)

**Cat. No. 17-416**

**768 Wells**

**FOR RESEARCH USE ONLY**
**Not for use in diagnostic procedures**

USA & Canada
Phone: +1(800) 437-7500 • Fax: +1 (951) 676-9209 • Europe +44 (0) 23 8026 2233
Australia +61 3 9839 2000 • Germany +49-6192-207300 • ISO Registered Worldwide
www.chemicon.com • custserv@chemicon.com • techserv@chemicon.com

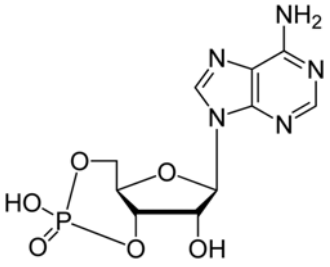
## Introduction

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, 384 well is a competitive immunoassay for quantitative chemiluminescence-based detection of cAMP in cell lysates and supernatants, which has been optimized for use in high throughput screening applications. The assay is reproducible and robust, with a Z' value of 0.7 and a S/B ratio of 92.4. Results can be obtained in as little as 1.5 hrs. Sensitivity of the assay is 1.07 fmol/well, and the assay has a large dynamic range, detecting cAMP concentrations from 0.0003 pmol/well to 3,000 pmol/well. The polyclonal antibody used is highly specific for cAMP and shows minimal cross-reactivity with other nucleotides. The kit provides two ready to use 384 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, 384 well 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 rabbit anti-cAMP antibody that recognizes all species, an alkaline phosphatase (AP)-labeled cAMP conjugate, two 384 well microplates pre-coated with an anti-Rabbit capture 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, 384 well, 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 384 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 768 assays.

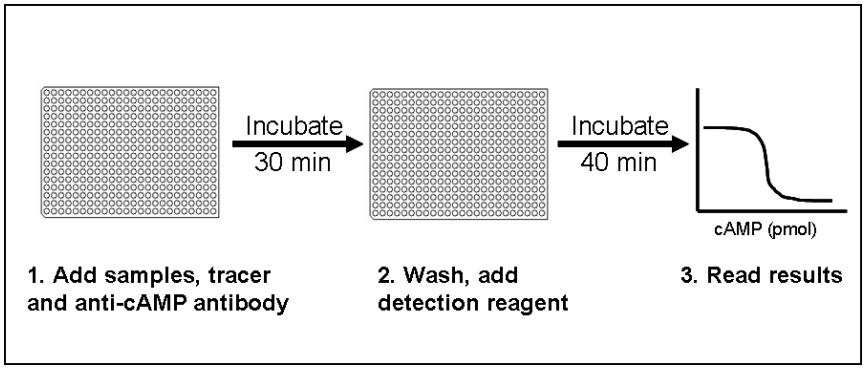



**Figure 2**: Assay Overview

## Application

Upstate®'s cAMP HTS immunoassay kit, 384 well 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 two 384-well immunoassay plates. Running triplicate wells for samples and standards is recommended.

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## Kit Components

1. White 384-Well anti-Rabbit Coated Plate: (Part No. CS200427) Two 384-well immuno-plates pre-coated with anti-Rabbit polyclonal antibody, sealed in a foil pouch.
2. Rabbit anti-cAMP Antibody: (Part No. CS200428) One 20 µL vial.
3. cAMP Standard: (Part No. 2004073) One 2 mL bottle.
4. cAMP Alkaline Phosphatase Conjugated Tracer: (Part No. 2004072) One 20 µL vial.
5. 2x Assay Diluent: (Part No. CS200419) One 125 mL bottle.
6. 5x Wash Buffer: (Part No. CS200445) One 125 mL bottle.
7. Lysis Buffer: (Part No. CS200416) One 100 mL bottle.
8. 6x Alkaline Phosphatase Substrate: (Part No. CS200430) One 8 mL bottle.
9. Plate Sealers: (Part No. 2007518) 6 each.

## Materials Not Supplied

1. Pipettors & tips capable of accurately measuring 10-1000 µL
2. Plate shaker (optional)
3. Automated Liquid Handler (optional)
4. Plate Washer (optional)
5. Luminometer capable of reading 384-well microplates.
6. Test tubes for standard and sample dilutions

7. 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 384-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 384-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 blot 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 80 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 625 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. Stir thoroughly. Use this 1x solution for diluting the Rabbit anti-cAMP Antibody and cAMP Alkaline Phosphatase Conjugate Tracer.

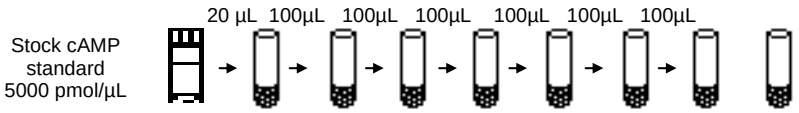
3. **cAMP Standard**

   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.*

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 100 µL of Lysis Buffer to each well. (100 µL volume of lysate is recommended for preparation of triplicate 30 µL samples in 384 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 30 µL per well of 384 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 30 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 a sufficient volume of lysis buffer to give a final well volume of 100 µL e.g. add 50 µL of Lysis Buffer to a well containing 50 µL of tissue culture media.

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 30 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 384-Well anti-Rabbit Coated Plate/s from foil pouch.
3. Add 30 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 15 µL of the diluted cAMP Alkaline Phosphatase Conjugate Tracer dilution to all wells being tested.
5. Add 30 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 30 µL of the diluted Alkaline Phosphatase Substrate. Cover or seal the plate and incubate at room temperature for 40 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, 384 well 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.

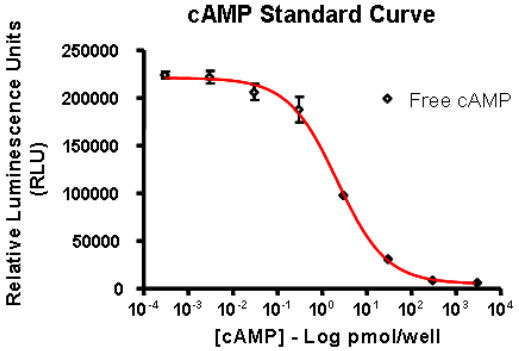



**Figure 4.** cAMP Standard Curve. The provided cAMP standard containing 5 mM cAMP (5,000 pmol/µL) was used in this experiment. Seven serial dilutions were prepared in Lysis buffer. 30 µL of each standard was used to generate the standard curve in a range from 0.0003 to 3,000 pmol cAMP/well. This represents a typical Standard Curve.

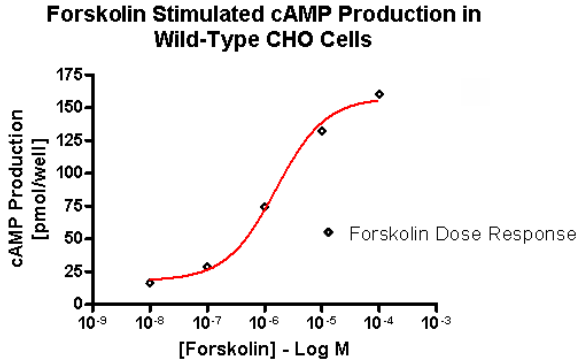



**Figure 5.** Forskolin dose-response. CHO wt cells were seeded at 10,000 cells per well in a 384-well plate 24 hr before the assay. Cells were pre-incubated with 1 mM IBMX for 5 minutes, then various concentrations of forskolin were added for an additional 15 minute incubation at 37ºC. The reaction was terminated by the addition of cold cell lysis buffer (provided in the kit). 30 µL of the sample was used for cAMP analysis.

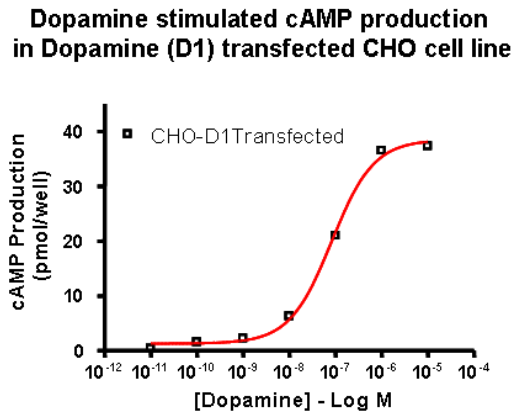



**Figure 6.** Validation of assay for structure-activity relationship (SAR) studies. CHO cells transfected with a Gs-protein coupled receptor Dopamine1 (D1) were seeded at 10,000 cells per well in a 384-well plate 24 hr before the assay. A dose response range of the natural ligand Dopamine was added to induce the production of cAMP.

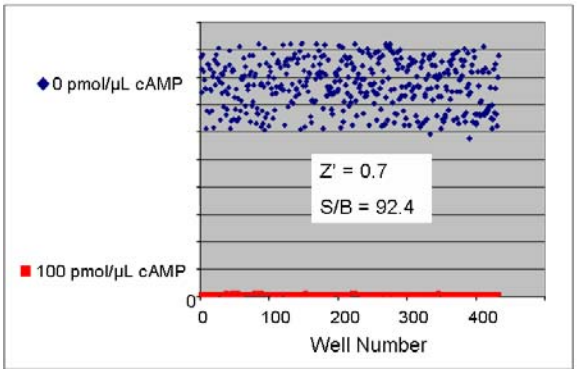



**Figure 7.** Validation of assay for screening studies. Assay performance was assessed by calculation of the Z' value. A Z' value of 0.7 was obtained from multiple data points of minimum (100 pmol/µL cAMP) and maximum luminescence (0 pmol/µL cAMP). Signal/Background (SB) ratio was determined as 92.4 by calculating the ratio between the mean max signal and the mean minimum signal. This ratio describes the dynamic range of the assay.

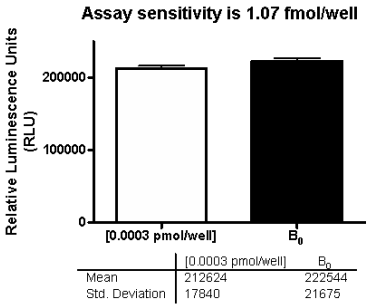



**Figure 8.** Calculation of assay sensitivity. Sensitivity of 1.07 fmol/well was calculated by determining the average RLU for 24 wells run with the Bo, and comparing to the average RLU for 24 wells run with 0.0003 pmol/well. The detection limit was determined as the concentration of cAMP measured at two standard deviations from the zero along the standard curve.

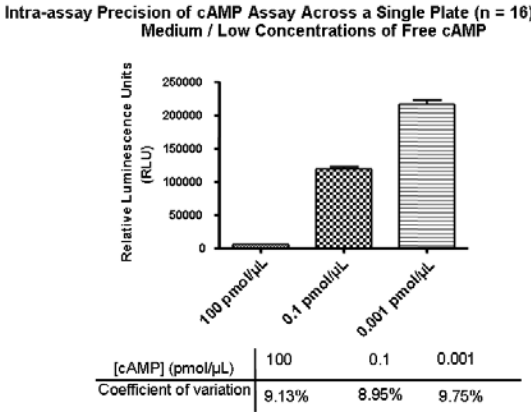



**Figure 9.** Calculation of intra-assay precision. Intra-assay precision was determined by taking samples containing differing concentrations of cAMP and running these samples multiple times (n = 16) in the same assay. The precision numbers shown above represent the percent coefficient of variation for the concentrations of cAMP determined in these assays as calculated by curve fitting software.

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

| | | | |
|---|---|---|---|
| cAMP | 100% | CTP | <0.01% |
| cGMP | <0.1% | AMP | <0.01% |
| GTP | <0.01% | ADP | <0.01% |

GMP <0.01% ATP <0.01%

## References: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## Warranty

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