Rat cyclic adenosine monophosphate (cAMP) ELISA Kit

Catalog Number. CSB-E07298r

For the quantitative determination of rat cyclic adenosine monophosphate (cAMP) concentrations in serum, plasma, cell lysis, tissue lysis.

This package insert must be read in its entirety before using this product.

If You Have Problems

Technical Service Contact information

Phone: 86-27-87582341
Fax: 86-27-87196150
Email: tech@cusabio.com
Web: www.cusabio.com

In order to obtain higher efficiency service, please ready to supply the lot number of the kit to us (found on the outside of the box).

PRINCIPLE OF THE ASSAY

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with a goat-anti-mouse IgG. Standards or samples are added to the appropriate microtiter plate wells with an antibody specific for cAMP and Horseradish Peroxidase (HRP) conjugated cAMP. The competitive inhibition reaction is launched between with HRP labeled cAMP and unlabeled cAMP with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of cAMP in the sample. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE

0.08 pmol/ml-250 pmol/ml.

PRECISION

Intra-assay Precision (Precision within an assay): CV%<15%
Three samples of known concentration were tested twenty times on one plate to assess.

Inter-assay Precision (Precision between assays): CV%<15%
Three samples of known concentration were tested in twenty assays to assess.

LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples and repeat the assay.

- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Reagents	Quantity
Assay plate	1(96 wells)
Standard (5000pmol/ml)	1 x 0.25 ml
Antibody (1000 x concentrate)	1
HRP-conjugate (1000 x concentrate)	1
Antibody Diluent	1 x 6 ml
HRP-conjugate Diluent	1 x 6 ml
Neutralizing Reagent	1 x 6 ml
Wash Buffer (10 x concentrate)	1 x 15 ml
Substrate A	1 x 10 ml
Substrate B	1 x 10 ml
Stop Solution	1 x 6 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

STORAGE

All other components of this kit are stable at 4°C until the kit's expiration date. For long-term best results, store stocks of the **Antibody** and **HRP-conjugate** at -80°C upon receipt. Unused wells must be kept desiccated at 4°C in the sealed bag provided.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 570 nm or 590 nm.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent paper for blotting the microtiter plate.
- 100ml and 500ml graduated cylinders.
- Deionized or distilled water.
- Pipettes and pipette tips.
- Test tubes for dilution.
- Concentrated HCl.
- Adsorbent paper for blotting.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

This ELISA is compatible with cAMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity. Samples in this matrix can be measured directly without evaporation or further treatment.

- **Serum** Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g, 2 - 8°C. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.
- **Plasma** Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g, 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.
- **Tissue Lysis** Tissue samples should be frozen in liquid nitrogen. The tissue should be ground to a fine powder under liquid nitrogen in a stainless steel mortar. After the liquid nitrogen has evaporated, weigh the frozen tissue and homogenize in 10 volumes of 0.1M HCl. Centrifuge at > 600 x g at room temperature. The samples can then be diluted in the 0.1M HCl.
- **Cell Lysis** Cells grown in tissue culture media can be treated with 0.1M HCl after first removing the media. Incubate for 10 minutes and visually inspect the cells to verify cell lysis. If adequate lysis has not occurred incubate for a further 10 minutes and inspect. Centrifuge at 600 x g at room temperature, then use the supernatant directly in the assay.
- Cell or tissue lysis can be enhanced by adding 0.1% to 1% Triton x-100 to the 0.1M HCl prior to use. When used in this concentration range, the detergent will not interfere with the binding portion of the assay, however there will be a modest increase in the optical density. Samples containing

Triton should be evaluated against a standard curve diluted in the same for the most accurate determination. Cyclic AMP in the media can be measured after treating 1 mL of the supernatant media with 10µL of concentrated hydrochloric acid. Centrifuge at 600 x g at room temperature. The supernatants can then be used directly in the assay.

Note:

1. CUSABIO is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2month) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
9. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

REAGENT PREPARATION

Note:

- **Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.**
- Bring all reagents to room temperature (18-25°C) before use for 30min.
- Making serial dilution in the wells directly is not permitted.
- To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μl for once pipetting.
- Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

1. **Antibody (1x) -** Centrifuge the vial before opening.
   Antibody requires a 1000-fold dilution. A suggested 1000-fold dilution is 6 μl of **Antibody** + 6 ml of **Antibody Diluent**.

2. **HRP-conjugate (1x) -** Centrifuge the vial before opening.
   HRP-conjugate requires a 1000-fold dilution. A suggested 1000-fold dilution is 6 μl of **HRP-conjugate** + 6 ml of **HRP-conjugate Diluent**.

3. **Wash Buffer(1x)-** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 15 ml of Wash Buffer Concentrate (10 x) into deionized or distilled water to prepare 150 ml of Wash Buffer (1 x).

4. **Standard**

Centrifuge the standard vial at 6000-10000rpm for 30s.

Allow the **5000 pmol/ml** cAMP standard solution to warm to room temperature. Label six tubes #1 through #6. Pipet 475 µl 0.1M HCl into tube #1 and 400 µl 0.1M HCl into tubes #2-6. Add 25 µl of the 5000 pmol/ml standard to tube #1. Vortex thoroughly. Add 100 µl of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #6.

The concentration of cAMP in tubes #1 through #6 will be **250, 50, 10, 2, 0.4,** and **0.08** pmol/ml, respectively. Diluted standards should be used within 30 minutes of preparation.

Label one tube as the Zero Standard/NSB tube. Pipet 600µl 0.1M HCl into this tube.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Pipet 50 µl of the Neutralizing Reagent into each well, except the TA(Total Activity) and Blank wells.
4. Pipet 100 µl of 0.1M HCl into the NSB(None Specific Binding) and the Bo (0 pmol/ml Standard) wells.
5. Pipet 100 µl of **Standards** into the appropriate wells.
6. Pipet 100 µl of the **Samples** into the appropriate wells.
7. Pipet 50 µl of 0.1 M HCl into the NSB wells.
8. Pipet 50 µl of **HRP-conjugate(1x)** into each well **except** the TA and Blank wells.
9. Pipet 50 µl of **Antibody(1x)** into each well, **except** the Blank, TA and NSB wells.
10. Incubate the plate at room temperature for 2 hours on a plate shaker at 250~500 rpm.
11. Empty the contents of the wells and wash by adding 400 µl of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
12. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
13. Add 5 µl of the **HRP-conjugate(1x)** to the TA wells.
14. Add 200 µl of the **Substrate solution** to every well. Incubate at room temperature for 5~30 minutes without shaking. A gradient of blue color should become visible during the incubation period. (Substrate A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light.)

15. Add 50 µl of **Stop Solution** to every well. This stops the reaction and the plate should be read immediately.
16. Blank the plate reader against the Blank wells, read the optical density at 450 nm (for HRP), preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard. Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of cAMP in the samples. The X-axis is the concentration of cAMP for the standards. The Y-axis is either the Average Net Optical Density or the Percent Bound.

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD － Average NSB OD

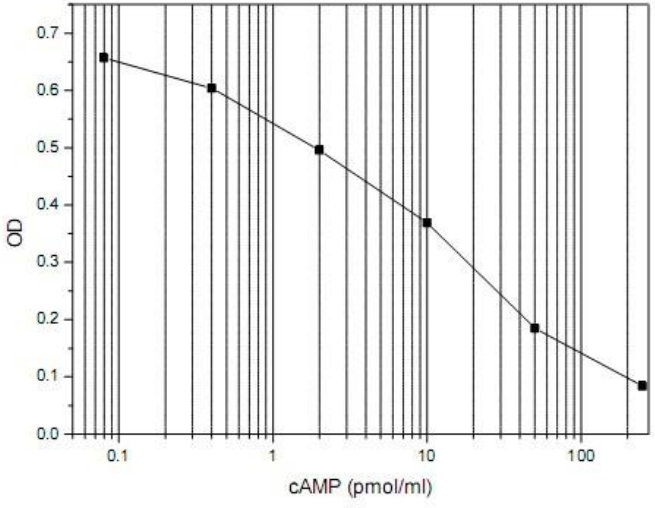
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using Logit-Log paper plot Average Net OD or Percent Bound (B/Bo) versus concentration of cAMP for the standards. The concentration of cAMP in the unknowns can be determined by interpolation.

TYPICAL STANDARD CURVES

These curves must not be used to calculate cAMP concentrations; each user must run a standard curve for each assay and version used.



SENSITIVITY

Sensitivity was calculated by determining the average optical density bound for ten wells run with the Bo, and comparing to the average optical density for ten wells run with Standard #5. The detection limit was determined as the concentration of cAMP measured at two standard deviations from the zero along the standard curve.

Non-Acetylated Version	
Mean OD for Bo =	0.685±0.003
Mean OD for Standard #5 =	0.604±0.010
Delta Optical Density(0-0.4pmol/ml) =	0.081
2 SD's of the Zero Standard =	0.006
Sensitivity =0.006/0.081×0.4pmol/ml=	29.6 fmol/ml

LINEARITY

A sample containing 16.0 pmol/ml cAMP was serially diluted 7 times 1:2 in the 0.1M HCl and measured. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 1.000 with a correlation coefficient of 0.999.

CROSS REACTIVITIES

The cross reactivities for a number of related compounds were determined by competition ELISA assays. Potential cross reactants were dissolved in the kit Assay Buffer at concentrations from 500000 to 500 pmol/ml. These samples were then measured in the cAMP assay, and the measured cAMP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
cAMP	100%
AMP	<0.0001%
ATP	<0.0001%
cGMP	<0.0001%
GMP	<0.0001%
GTP	<0.0001%
cUMP	<0.0001%
CTP	<0.0001%