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Product Manual

Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA Kit (New Non-acetylated Version)

Catalog No. 80203
96 Well Kit

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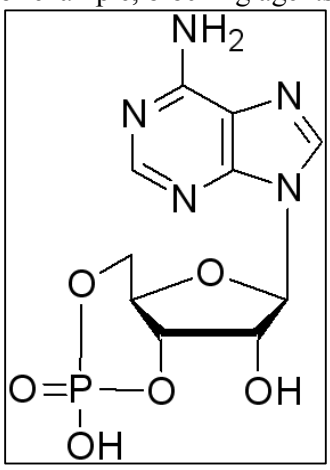
Product Description

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) modulates various physiological functions such as cardiovascular biology, learning and memory, olfaction, immune response, asthma and kidney function (1,2). cAMP is produced from ATP by adenylyl cyclases and is degraded by phosphodiesterases. Stimulation of adenylyl cyclases or inhibition of phosphodiesterases can increase cellular cAMP concentrations. Blockers of adenylyl cyclase-activating receptors and inhibitors of the cAMP-specific phosphodiesterases are used for treating human diseases. For example, blocking agents for cAMP-increasing beta-adrenergic receptors (beta-blockers) are used for treating abnormal heart rhythms, high blood pressure (hypertension), myocardial infarction and heart failure. Inhibitors of cAMP specific phosphodiesterase types 2 and 4 are being tested for cognition enhancement.



To screen for inhibitors or stimulators of cellular cAMP levels, it is essential to have a sensitive, selective and reproducible method to measure the cAMP concentrations. This is especially true for the initial screenings given the possible weaker effects of larger pools of compounds.

Currently available other ELISA kits measuring cAMP levels are based on the non-affinity-purified polyclonal anti-cAMP antibody. Despite the claimed selectivity, these polyclonal anti-cAMP antibodies display certain cross-reactivity with ATP. Given that ATP is the substrate for the cAMP production, it is very desirable to have an antibody with high specificity towards cAMP over ATP.

NewEast Biosciences cAMP ELISA kit is based on the **unique mouse monoclonal anti-cAMP antibody**. This monoclonal anti-cAMP antibody displays $>10^8$ fold of selectivity over ATP, cGMP, and other nucleoside analogues. NewEast Biosciences cAMP ELISA kit provides significantly **improved sensitivity and selectivity** over other kits based on polyclonal anti-cAMP antibodies. Our monoclonal anti-cAMP antibody-based ELISA kit also avoids the batch-to-batch variations associated with polyclonal antibody productions from animals, thus providing the reproducibility in the long run.

Furthermore, while polyclonal anti-cAMP antibodies used in other ELISA kits have higher affinity for acetylated cAMP than non-acetylated cAMP, NewEast Biosciences monoclonal anti-cAMP antibody has similar affinities to non-acetylated and acetylated cAMP molecules. Therefore, acetylation treatments of samples and standards are not needed in NewEast Biosciences cAMP ELISA kit. This significantly **reduces the time for the assay**. The avoidance of organic reagents used in the acetylation process provides **a safe and healthy work environment**.

Principle Outline

NewEast Biosciences cAMP ELISA Kit is a competitive immunoassay to measure the cAMP levels, either from cell extracts or from in vitro adenylyl cyclase assays. Briefly, multi-well plates are coated with goat-anti-mouse serum. cAMP in cell extracts or in in vitro adenylyl cyclase assays will competitively bind to the monoclonal anti-cAMP antibody in the presence of fixed amounts of cAMP-conjugated horse-radish peroxidase or alkaline phosphatase. Known amounts of cAMP are used as standards to generate the calculation curve. After a short incubation, the excess reagents are washed away and substrate is added. The multiwell plates are then read on a microplate reader at 450 nm or 405

nm. The intensity of the yellow color is inversely proportional to the concentration of cAMP in samples. The measured optical density is used to calculate the concentration of cAMP in samples based on the curve from the cAMP standards.

Background

cAMP is a ubiquitous second messenger mediating cellular responses to various exogenous and endogenous signaling molecules. cAMP regulates physiological processes by activating protein kinases, gating specific ion channels, modulating cellular cyclic nucleotide concentrations through phosphodiesterases, and activating Epac (exchange protein directly activated by cAMP) (3-6). The conversion of ATP to cAMP is catalyzed by adenylyl cyclases (ACs). The major family of ACs in mammals is the transmembrane ACs which have nine isoforms and could be activated by G protein Gs and/or Ca^{2+}/calmodulin (1). There is also one soluble AC which could be modulated by bicarbonate and/or Ca^{2+} (7-9).

Materials Supplied

1. **Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 30101** A plate using break-apart strips coated with goat antibody specific to mouse IgG.
2. **cAMP Direct Conjugate, 6 mL, Catalog No. 30202**
   A solution of horse radish peroxidase conjugated with cAMP (a 1000 X stock solution and dilution solution are provided).

Note: For long-term best results, store conjugate at -80℃upon receipt. Add 6μL conjugate into 6mL dilution solution and mix the solution gently before use. Please avoid repeated thawing and freezing after mixing.

3. **cAMP Direct Antibody, 6 mL, Catalog No. 26002-2**
   A solution of a mouse monoclonal antibody to cAMP(a 1000 X stock solution and dilution solution are provided).

Note: For long-term best results, store conjugate at -80℃upon receipt. Add 6μL Antibody into 6mL dilution solution and mix the solution gently before use. Please avoid repeated thawing and freezing after mixing.

4. **Neutralizing Reagent, 6 mL, Catalog No. 30103**
5. **10X Wash Buffer Concentrate, 15 mL, Catalog No. 30106**
   Phosphate buffered saline containing detergents.
6. **Cyclic AMP Standard, 0.25 mL, Catalog No. 30203**
   A solution of 5,000 pmol/mL cAMP.
7. **Substrate A, 12 mL, Catalog No. 30107**
8. **Substrate B, 12 mL, Catalog No. 30108**
9. **Stop Solution, 6 mL, Catalog No. 30110**
   A solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic.**

Storage

All components of this kit are stable at 4°C until the kit's expiration date. **For long-term best results, store 1000 X stock solutions at -80℃upon receipt.**

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Concentrated HCl.
3. Precision pipets for volumes between 5 µL and 1,000 µL.
4. Repeater pipets for dispensing 50 µL and 200 µL.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. A microplate shaker.
8. Adsorbent paper for blotting.
9. Microplate reader capable of reading at 450 nm, preferably with correction between 570 and 590 nm.

Sample Handling

NewEast Biosciences EIA 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.

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.

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 10 µL of **concentrated** hydrochloric acid. Centrifuge at 600 x g at room temperature. The supernatants can then be used directly in the assay.

Procedural Notes

1. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
2. **Pre-rinse the pipet tip with reagent**, use fresh pipet tips for each sample, standard and reagent.
3. Pipet standards and samples to the bottom of the wells.
4. Add the reagents to the side of the well to avoid contamination.

5. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.

6. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. cAMP Standard – Non-Acetylated Version

Allow the 5,000 pmol/mL cAMP standard solution to warm to room temperature. Label six (or more) 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 5,000 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. See Direct cAMP Assay Layout Sheet for dilution details. Diluted standards should be used within 30 minutes of preparation.

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

2. Wash Buffer

Prepare the Wash Buffer by diluting 15 mL of the supplied concentrate with 135 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.
Add the reagent directly to the samples and vortex for 2 seconds immediately after the addition.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 50 µL of the Neutralizing Reagent into each well, except the TA (Total Activity) and Blank wells.
3. Pipet 100 µL of 0.1M HCl into the NSB (None Specific Binding) and the Bo (0 pmol/mL Standard) wells.
4. Pipet 100 µL of Standards into the appropriate wells.
5. Pipet 100 µL of the Samples into the appropriate wells.
6. Pipet 50 µL of 0.1 M HCl into the NSB wells.

7. Pipet 50 µL of Conjugate into each well **except** the TA and Blank wells.

8. Pipet 50 µL of Antibody into each well, **except** the Blank, TA and NSB wells.

9. Incubate the plate at room temperature for 2 hours on a plate shaker at 250~500 rpm.

10. 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.**

11. 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.

12. Add 5 µL of the Conjugate to the TA wells.

13. Add 200 µL of the Substrate solution to every well. Incubate at room temperature for 5~30 minutes without shaking. (**Substrate A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light.)**

14. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

15. Blank the plate reader against the Blank wells, read the optical density at 450 nm, 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.

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

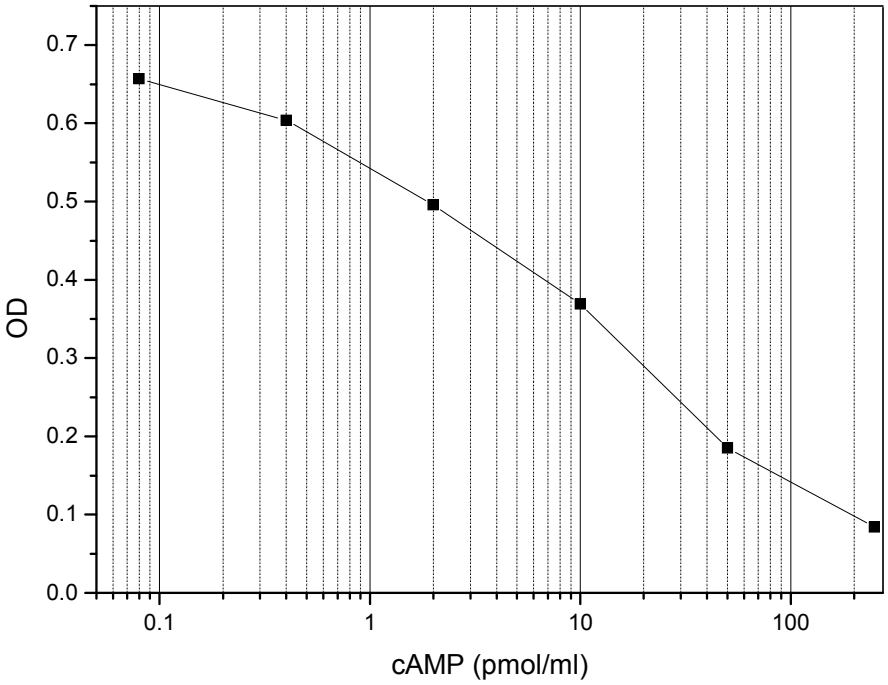
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

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

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

Acetylated Version	
Mean OD for Bo =	0.685 ± 0.003
Mean OD for Standard #5 =	0.604 ± 0.010
Delta Optical Density (0-0.08 pmol/mL) =	0.081
2 SD's of the Zero Standard =	0.006
Sensitivity = $\frac{0.006}{0.081}$ × 0.4 pmol/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 500,000 to 500 pmol/mL. These samples were then acetylated and 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%

CAUTION: Some components of this kit contain chemicals that are lachrymators, corrosive and flammable. Use with caution and wear suitable protection.

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Direct cAMP Assay Layout Sheet

- **Table of Dilutions for Making Standards:**

Non-Acetylated Version

Std.	0.1M HCl Vol. (µL)	Vol. Added (µL)	cAMP Conc. (pmol/mL)
1		_____, stock (5000 pmol/mL)	
2		_____, Std. 1	
3		_____, Std. 2	
4		_____, Std. 3	
5		_____, Std. 4	
6		_____, Std. 5	
7		_____, Std. 6	
0			

- **Assay Flow Chart**

Well I.D.	Blank	TA	NSB	Bo.	Stds.	Samples
Neutralizing Reagent	---	---	50 µL	50 µL	50 µL	50 µL
0.1 M HCl	---	---	150 µL	100 µL	---	---
Std. and/or Sample	---	---	---	---	100 µL	100 µL
Conjugate	---	---	50 µL	50 µL	50 µL	50 µL
Antibody	---	---	---	50 µL	50 µL	50 µL
Incub. 2 hours @ RT, shaking						
Asp. & Wash 3@ 200 µL						
Conjugate	---	5 µL	---	---	---	---
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incub. 1 hour @ RT						
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Direct cAMP Plate Layout:

A1 Bo	A2 Bo	A3 Blank	A4 Blank	A5	A6	A7	A8	A9	A10	A11	A12
B1 Std 1	B2 Std 1	B3 TA	B4 TA	B5	B6	B7	B8	B9	B10	B11	B12
C1 Std 2	C2 Std 2	C3 NSB	C4 NSB	C5	C6	C7	C8	C9	C10	C11	C12
D1 Std 3	D2 Std 3	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 Std 4	E2 Std 4	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 Std 5	F2 Std 5	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Std 6	G2 Std 6	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Kit Lot No. _____ Exp. Date _____ Date _____ Tech. _____

1st Incub: Start Time _____ Temp. _____ Note: _____
End Time _____ Temp. _____ _____

2nd Incub: Start Time _____ Temp. _____ _____
End Time _____ Temp. _____ _____