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## **Product Manual**

# **Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA Kit**

**Catalog No. 80201**

**96 Well Kit**

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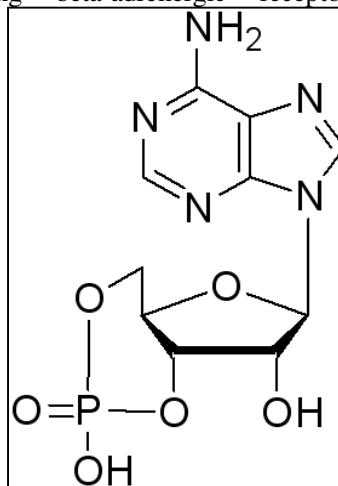
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## **Product Description**

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.

### **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<sup>2+</sup>/calmodulin (1). There is also one soluble AC which could be modulated by bicarbonate and/or Ca<sup>2+</sup> (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.
3. **cAMP Direct Antibody, 6 mL, Catalog No. 26002**  
A solution of a mouse monoclonal antibody to cAMP.
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, 0.5 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.**

10. **Triethylamine, 2 mL, Catalog No. 30112**  
**CAUTION: Lachrymator, Harmful Vapor, Flammable.**
11. **Acetic Anhydride, 1 mL, Catalog No. 30113**  
**CAUTION: Lachrymator, Corrosive, Flammable.**
12. **0.1 M HCl, 27 ml, Catalog No. 30114**

### **Storage**

All components of this kit are stable at 4°C until the kit's expiration date. **For long-term best results, store standard and conjugate at -20°C.**

### **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. Please refer to the Sample Recovery recommendations for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their samples. We have provided reagents to acetylate samples and standards for samples with very low levels of cAMP.

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 acetylation or 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**

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1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or polypropylene tubes; **avoid polystyrene.**
4. **Pre-rinse the pipet tip with reagent**, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. 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.
8. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells.**

### **Reagent Preparation**

#### **1. Acetylation Reagent**

Prepare the Acetylating Reagent by adding 0.5 mL of acetic anhydride to 1 mL triethylamine. **Use the prepared reagent within 60 minutes of preparation.**

#### **2. cAMP Standard - Acetylated Version**

Allow the 5,000 pmol/mL cAMP standard solution to warm to room temperature. Label six (or more) 12 x 75 mm glass tubes #1 through #6. Pipet 950 µL 0.1M HCl into tube #1 and 800 µL 0.1M HCl into tubes #2-6. Add 50 µL of the 5,000 pmol/mL

standard to tube #1. Vortex thoroughly. Add 200  $\mu$ L 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.**

**Acetylate all standards and samples by adding 15  $\mu$ L of the Acetylating Reagent for each 300  $\mu$ L of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds immediately after the addition.**

Label one 12 x 17 mm glass tube as the Zero Standard/NSB tube. Pipet 1 mL 0.1M HCl into this tube. Add 50  $\mu$ L of the Acetylating Reagent to the Zero Standard/NSB tube and use in Step 3 and 6, on Page 7. **Failure to acetylate the NSB and Zero standard will result in inaccurate OD or B/Bo values.**

**Use the acetylated standards or samples within 30 minutes.**

**3. 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 (Acetylated version is recommended)**

**Bring all reagents to room temperature for at least 30 minutes prior to**

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**opening. All standards and samples should be run in duplicate. If Acetylated Version of the kit is to be run, acetylate all standards and samples by adding 15  $\mu$ L of the Acetylating Reagent for each 300  $\mu$ L of standard or sample. Add 50  $\mu$ L of the Acetylating Reagent to the Zero Standard/NSB tube (Refer to Step 3 on Page 6) and use in Steps 3 and 6 below (Failure to acetylate the NSB and Zero standard will result in inaccurate OD or B/Bo values). Add the reagent directly to the samples and vortex for 2 seconds immediately after the addition. Use the acetylated standards or samples within 30 minutes.**

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  $^{\circ}$ C.
2. Pipet 50  $\mu$ L of the Neutralizing Reagent into each well, except the TA and Blank wells.
3. Pipet 100  $\mu$ L of 0.1M HCl (acetylated) into the NSB and the Bo (0 pmol/mL Standard) wells.
4. Pipet 100  $\mu$ L of Standards into the appropriate wells.
5. Pipet 100  $\mu$ L of the Samples into the appropriate wells.
6. Pipet 50  $\mu$ L of 0.1 M HCl (acetylated) into the NSB wells.
7. Pipet 50  $\mu$ L of Conjugate into each well **except** the TA and Blank wells.
8. Pipet 50  $\mu$ L 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  $\mu$ L 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  $\mu$ L of the Conjugate to the TA wells.
13. Add 200  $\mu$ L of the Substrate solution to every well. Incubate at room temperature for 1 hour without shaking. (**Substrate A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light.**)
14. Add 50  $\mu$ L 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:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

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

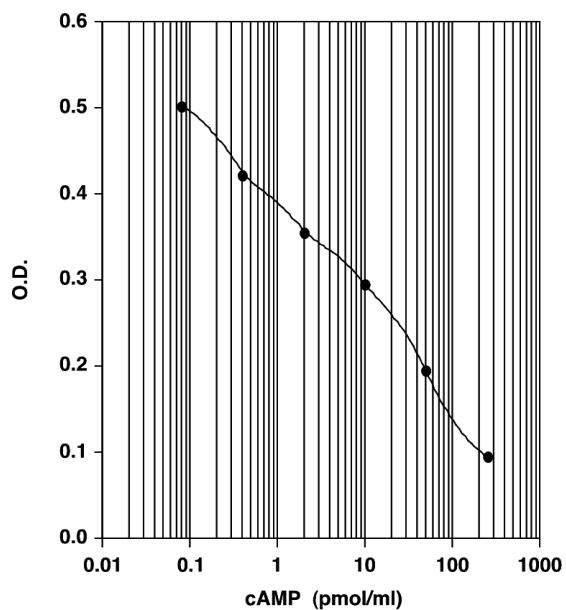
$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

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**

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

#### **Acetylated Version**



### **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 in the Non-Acetylated and Standard #5 in the Acetylated Formats. 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

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Mean OD for Bo =	0.326 ± 0.012
Mean OD for Standard #5 =	0.295 ± 0.013
Delta Optical Density (0-0.8 pmol/mL) =	0.031
2 SD's of the Zero Standard =	0.024
Sensitivity = $\frac{0.024}{0.031} \times 0.8$ pmol/mL =	<b>619 fmol/mL</b>

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#### Acetylated Version

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Mean OD for Bo =	0.565	± 0.007
Mean OD for Standard #6 =	0.500	± 0.006
Delta Optical Density (0-0.08 pmol/mL) =	0.065	
2 SD's of the Zero Standard =	0.014	
Sensitivity = $\frac{0.014}{0.065} \times 0.08$ pmol/mL =	<b>17 fmol/mL</b>	

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## **Linearity**

### **Acetylated Version**

A sample containing 16.0 pmol/mL cAMP was serially diluted 7 times 1:2 in the 0.1M HCl supplied in the kit and measured in the Acetylated 2 Hour Format of the assay. 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.

<u>Compound</u>	<u>Cross Reactivity</u>
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**

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**suitable protection.**

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