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Product Information

cAMP Enzyme Immunoassay Kit, Direct

Catalog Number **CA200**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

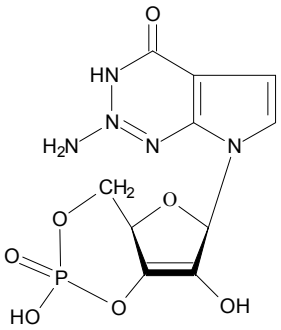
The EIA Direct cyclic AMP kit is a competitive immunoassay for the quantitative determination of cyclic AMP in samples treated with 0.1 M HCl. The kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the sample or an alkaline phosphatase molecule that has cAMP covalently attached to it. Samples or standards, alkaline phosphatase conjugate, and antibody are simultaneously incubated at room temperature in a secondary antibody coated multiwell plate. The excess reagents are then washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a multiwell plate reader at 405 nm. The intensity of the yellow color is inversely proportional to the concentration of cAMP in either the standards or the samples. The measured optical density is used to calculate the concentration of cAMP. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard or Tijssen.[1,2]

The cAMP Direct EIA may be used to assay cAMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity. Samples in this matrix can be read directly without evaporation or further treatment. Samples with very low levels of cAMP may be acetylated. Acetylation of the samples increases the sensitivity of the assay.

Adenosine 3′,5′-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important "second messengers" involved as a modulator of physiological processes.[3-7] cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune, and other functions and actions.[8-11] A number of hormones are known to activate cAMP through the action of the enzyme adenylate cyclase which converts ATP to cAMP.

These hormones include a variety of anterior pituitary peptide hormones such as corticotropin (ACTH), glucagon, calcitonin, thyroid stimulating hormone (TSH), and luteinizing hormone (LH). cAMP has been shown to be involved in the cardiovascular and nervous systems, immune mechanisms, cell growth and differentiation, and general metabolism.[12-14] There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of the physiology and pathology of many disease states.

Cyclic AMP



Components

Sufficient reagents are supplied for 96 assays.

Goat Anti-Rabbit IgG Coated 96 Well Multiwell Plate - break-apart strips coated with goat antibody specific to rabbit IgG (Catalog Number M3683)	1 each
cAMP-Alkaline Phosphatase Conjugate A blue solution of alkaline phosphatase conjugated with cAMP (Catalog Number C7351)	5 ml
cAMP EIA Antibody Rabbit Anti-cAMP A yellow solution of a polyclonal rabbit antibody to cAMP (Catalog Number C7226)	5 ml

0.1 M Hydrochloric Acid 0.1 M HCl in water Caution: acid (Catalog Number H5159)	30 ml
Neutralizing Reagent (Catalog Number N7533)	6 ml
Wash Buffer Concentrate Tris buffered saline containing detergents and sodium azide as preservative. (Catalog Number W1265)	30 ml
Cyclic AMP Standard A solution of 2,000 pmol/ml cAMP (Catalog Number C7601)	0.5 ml
p-Nitrophenyl Phosphate Substrate Solution A solution of *p*-nitrophenyl phosphate in buffer Ready-to-use (Catalog Number N7408)	20 ml
Stop Solution A solution of trisodium phosphate in water Keep tightly capped. Caution: caustic (Catalog Number S2436)	5 ml
Triethylamine Caution: lachrymator, harmful vapor, flammable (Catalog Number T7441)	2 ml
Acetic Anhydride Caution: lachrymator, corrosive, flammable (Catalog Number A5344)	1 ml
Plate Sealer (Catalog Number P2107)	1 each

Equipment and Reagents Required but Not Provided

- Deionized or distilled water. No difference in assay results is seen with distilled water.
- Precision pipettes for volumes between 5 µl and 1,000 µl
- Repeater pipettes for dispensing 50 µl and 200 µl
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A multiwell plate shaker
- Adsorbent paper for blotting
- Multiwell plate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- 5 Cycle Log-Log Paper

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, diagnostic, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Caution: Some components of this kit contain chemicals that are acidic, caustic, lachrymators, corrosive, and flammable. Use with caution and wear suitable protection. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.

Preparation Instructions

Reagent Preparation

Note: Standards can be made up in either glass or polypropylene tubes. Avoid polystyrene tubes.

1. cAMP Standards (Non-Acetylated Version) – Allow the Cyclic AMP Standard (2,000 pmol/ml) to warm to room temperature. Label five 12 × 75 mm tubes 1 through 5. Pipette 900 µl of 0.1 M HCl into tube 1 and 750 µl of 0.1 M HCl into tubes 2-5. Add 100 µl of the Cyclic AMP Standard to tube 1. Vortex thoroughly. Add 250 µl of tube 1 to tube 2 and vortex thoroughly. Continue for tubes 3 through 5. The concentration of cAMP in tubes 1 through 5 will be 200, 50, 12.5, 3.12, and 0.78 pmol/ml, respectively. Diluted standards should be used within 60 minutes of preparation.

2. Acetylation Reagent – Prepare the Acetylation Reagent by adding 0.5 ml of acetic anhydride to 1 ml of triethylamine. Use the prepared reagent within 60 minutes of preparation.

3. cAMP Standards (Acetylated Version) – Allow the Cyclic AMP Standard (2,000 pmol/ml) to warm to room temperature. Label five 12 × 75 mm tubes 1 through 5. Pipette 990 µl of 0.1 M HCl into tube 1 and 750 µl of 0.1 M HCl into tubes 2-5. Add 10 µl of the Cyclic AMP Standard to tube 1. Vortex thoroughly. Add 250 µl of tube 1 to tube 2 and vortex thoroughly. Continue for tubes 3 through 5.

   Label one 12 × 17 mm glass tube as the Zero Standard/NSB tube. Pipette 1 ml of 0.1 M HCl into this tube for use in Assay Procedure, step 3. The concentration of cAMP in tubes 1 through 5 will be 20, 5, 1.25, 0.312, and 0.078 pmol/ml, respectively.

Acetylate all standards and samples by adding 10 µl of the Acetylation Reagent for each 200 µl of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds. Add 50 µl of the Acetylation Reagent to the Zero Standard/NSB tube and use in Assay Procedure, step 3. (Failure to acetylate the NSB and Zero will result in inaccurate B/Bo values).

Use the acetylated standards or samples within 30 minutes.

4. 1× Wash Buffer – Prepare 1× Wash Buffer by diluting 10 ml of the Wash Buffer Concentrate with 90 ml of deionized water. This can be stored at room temperature for 3 months.

Sample Handling

The Direct cAMP Enzyme Immunoassay is compatible with cAMP samples that have been treated with hydrochloric acid to stop endogenous phospho-diesterase activity. Samples in this matrix can be read directly without evaporation or further treatment. If samples with very low levels of cAMP are to be measured, reagents are provided to acetylate samples and standards. Acetylation of the samples increases the sensitivity of the assay. Please refer to references 15–21 for further methods of extraction of cAMP from samples.

Serum samples should be adjusted to make the serum 0.1 M in hydrochloric acid by the addition of ~10 µl of **concentrated** hydrochloric acid per 1 ml of serum. The serum should be incubated for 15 minutes at room temperature and then centrifuged at 600 × *g* at room temperature. The supernatants can then be diluted in the 0.1 M HCl provided with the kit. In experiments with serum samples diluted greater than 1:2 in 0.1 M HCl, recoveries of cAMP of greater than 96% were seen. Note: EDTA plasma is not a suitable matrix for the acetylated procedure since it tends to precipitate.

Tissue samples frozen in liquid nitrogen 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 0.1 M HCl. Centrifuge at 600 × *g* at room temperature. The samples can then be diluted in the 0.1 M HCl provided for the assay.

Cells grown in tissue culture medium can be treated with 0.1 M HCl after first removing the medium. 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 × *g* at room temperature, then use the supernatant directly in the assay. Cyclic AMP in the medium can be measured after treating the supernatant medium with **concentrated** hydrochloric acid as described for serum. Centrifuge at 600 × *g* at room temperature. The supernatants can then be used directly in the assay. In experiments with tissue culture medium samples diluted greater than 1:2 in 0.1 M HCl, recoveries of cAMP of 98% were seen.

Storage/Stability

The kit ships on wet ice and storage at –20 °C is recommended.

Procedure

The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.

The kit's performance has been tested with a variety of samples; however, it is possible that high levels of interfering substances may cause variation in assay results.

Allow all reagents to warm to room temperature for at least 30 minutes before opening.

Standards can be made up in either glass or polypropylene tubes. Avoid polystyrene tubes.

Keep unused plate strips sealed in bag with desiccant.

Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard, and reagent.

Pipette standards and samples to the bottom of the wells.

Add the reagents to the side of the well to avoid contamination.

This kit uses break-apart multiwell strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 2–8 °C in the sealed foil bag. The wells should be used in the frame provided.

Care must be taken to **minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipette tips and other items that are used in the assay with bare hands.

Assay Procedure

Allow all reagents to warm to room temperature for at least 30 minutes before opening.

All standards and samples should be run in duplicate.

If the Acetylated Version of the kit is to be run, acetylate all standards and samples by adding 10 µl of the Acetylation Reagent for each 200 µl of standard or sample. Add 50 µl of the Acetylation Reagent to the Zero Standard/NSB tube (refer to Reagent Preparation, step 3) and use in steps 3 and 6 in this procedure. Add the reagent directly to the samples and vortex for 2 seconds. Use the acetylated standards or samples within 30 minutes.

1. Determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal. Store unused wells at 2–8 °C.
2. Pipette 50 µl of the Neutralizing Reagent into each well, except the TA and Blank wells.
3. Pipette 100 µl of the 0.1 M HCl into the NSB and the Bo (0 pmol/ml Standard) wells.
4. Pipette 100 µl of Standards 1 through 5 into the appropriate wells.
5. Pipette 100 µl of the samples into the appropriate wells.
6. Pipette 50 µl of the 0.1 M HCl into the NSB wells.
7. Pipette 50 µl of blue cAMP-Alkaline Phosphatase Conjugate into each well except the TA and Blank wells.
8. Pipette 50 µl of yellow cAMP EIA Antibody into each well, except the Blank, TA (total activity), and NSB (non specific binding) wells.
   Note: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.
9. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
10. Empty the contents of the wells and wash by adding 200 µl of 1× Wash Buffer 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.
    Note: Prior to addition of substrate, ensure that there is no residual Wash Buffer in the wells. Any remaining Wash Buffer in the wells may cause variation in assay results.
12. Add 5 µl of the blue cAMP-Alkaline Phosphatase Conjugate to the TA wells.
13. Add 200 µl of the *p*-Nitrophenyl Phosphate Substrate Solution to every well. Incubate at room temperature for 1 hour without shaking.
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 405 nm, preferably with correction between 570 and 590 nm. If the plate reader cannot be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Results

Several options are available for the calculation of the concentration of cAMP in the samples. It is recommended that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program such as "AssayZap" (www.biosoft.com). If this type of data reduction software is not readily available, the concentration of cAMP can be calculated as follows:

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 the Logit-Log paper plot Percent Bound (B/Bo) versus Concentration of cAMP for the standards. Approximate a straight line through the points. The concentration of cAMP in the unknowns can be determined by interpolation.

Product Profile

Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from other assays.

Non-Acetylated Version

Sample	Net OD	Percent Bound	**cAMP (pmol/ml)**
Blank OD	(0.072)		
TA	1.563		
NSB	0.001	0.06%	
Bo	0.335	100%	**0**
S1	0.023	6.88%	**200**
S2	0.050	14.95%	**50**
S3	0.114	34.08%	**12.5**
S4	0.212	63.38%	**3.125**
S5	0.294	87.74%	**0.781**
Unknown1	0.093	27.76%	**17.89**
Unknown 2	0.224	66.87%	**2.64**

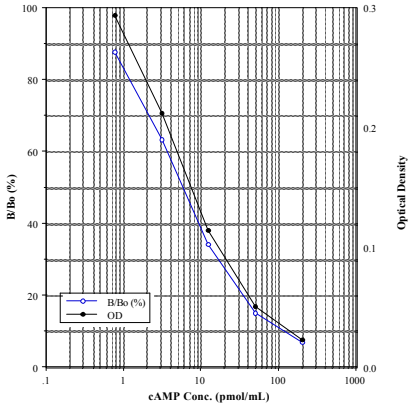
Acetylated Version

Sample	Net OD	Percent Bound	**cAMP (pmol/ml)**
Blank OD	(0.073)		
TA	1.691		
NSB	0.000	0%	
Bo	0.257	100%	**0**
S1	0.029	11.31%	**20**
S2	0.064	24.95%	**5**
S3	0.129	50.29%	**1.25**
S4	0.206	80.12%	**0.312**
S5	0.252	98.05%	**0.078**
Unknown1	0.076	29.57%	**3.73**
Unknown 2	0.210	81.71%	**0.27**

Typical Standard Curves

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

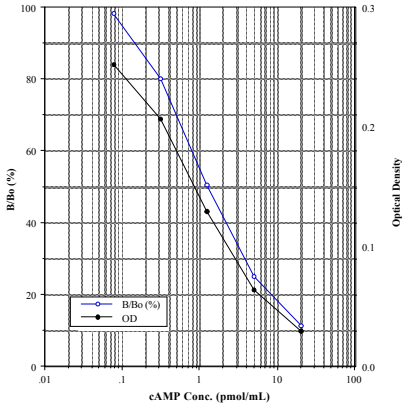
Non-Acetylated Version



Typical Quality Control Parameters

Total Activity Added	=	1.563 × 10 = 15.63
%NSB	=	0.001%
%Bo/TA	=	2.14%
Quality of Fit	=	0.99989
20% Intercept	=	28.8 pmol/ml
50% Intercept	=	5.4 pmol/ml
80% Intercept	=	1.1 pmol/ml

Acetylated Version



Typical Quality Control Parameters

Total Activity Added	=	1.691 × 10 = 16.91
%NSB	=	0.0%
%Bo/TA	=	1.52%
Quality of Fit	=	0.99993
20% Intercept	=	5.9 pmol/ml
50% Intercept	=	1.0 pmol/ml
80% Intercept	=	0.2 pmol/ml

Specificity

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.[22]

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard 5. The detection limit was determined as the concentration of cAMP measured at two (2) standard deviations from the zero along the standard curve.

Non-Acetylated Version

Mean OD for Bo = 0.340 ± 0.012 (3.4%). Mean OD for Standard 5 = 0.293 ± 0.007 (2.5%).
Delta Optical Density (0-0.78 pmol/ml) = 0.340 – 0.293 = 0.047. 2 SD's of Bo = 0.024
Sensitivity = $\frac{0.024}{0.048} \times 0.7$ pmol/ml = **0.39 pmol/ml**

Acetylated Version

Mean OD for Bo = 0.313 ± 0.004 (1.24%). Mean OD for Standard 5 = 0.295 ± 0.009 (2.9%).
Delta Optical Density (0-0.078 pmol/ml) = 0.313 – 0.295 = 0.017. 2 SD's of Bo = 0.008
Sensitivity = $\frac{0.008}{0.017} \times 0.07$ pmol/ml = **0.037 pmol/ml**

Linearity

Non-Acetylated Version

A sample containing 15.44 pmol/ml cAMP was serially diluted 4 times 1:2 in the 0.1 M HCl supplied in the kit and measured in the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 0.98 with a correlation coefficient of 0.988.

Acetylated Version

A sample containing 3.41 pmol/ml cAMP was serially diluted 4 times 1:2 in the 0.1 M HCl supplied in the kit and measured in the Acetylated version of the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 1.226 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium, and high concentrations of cAMP and running these samples multiple times (n ≥24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium, and high concentrations of cAMP in multiple assays (n ≥8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of cAMP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

Non-Acetylated Version

	cAMP (pmol/ml)	Intra Assay (%CV)	Inter Assay (%CV)
Low	1.24	8.9	
Medium	6.31	4.3	
High	35.29	8.3	
Low	1.18		13.1
Medium	5.52		4.2
High	30.36		11.6

Acetylated Version

	cAMP (pmol/ml)	Intra Assay (%CV)	Inter Assay (%CV)
Low	0.679	4.6	
Medium	3.58	8.4	
High			
Low	1.29		13.6
Medium	5.62		7.8
High			

Cross Reactivities

The cross reactivities for a number of related compounds were determined by using the EIA cAMP kit, Catalog Number CA201, which uses the same antibody and conjugate as this kit. Potential cross reactants were dissolved in the kit Assay Buffer at concentrations from 2,000 to 2 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	0.33%
ATP	0.12%
cGMP	<0.001%
GMP	<0.001%
GTP	<0.001%
cUMP	<0.001%
CTP	<0.001%

Sample Recoveries

cAMP concentrations were measured in a variety of different samples including tissue culture media, human saliva, serum, and urine. For all of the samples, cAMP was spiked into the undiluted samples, which were diluted with the 0.1 M HCl supplied with the kit and then assayed in the kit. Recovery values were not obtained with urine samples because the endogenous levels of cAMP are so high. The following results were obtained:

Non-Acetylated Version

Sample	% Recovery	Recommended Dilution*
Tissue Culture Media	95.9	none
Human Serum	108.4	none
Human EDTA Plasma	96.8	none

Acetylated Version

Sample	% Recovery	Recommended Dilution*
Tissue Culture Media	86.8	none
Human Serum	89.4	none
Human EDTA Plasma	70.3	none

* See Sample Handling instructions for details

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