



Correlate-EIA™
Cyclic AMP
Enzyme Immunoassay Kit

Catalog No. 900-067

96 Well Kit

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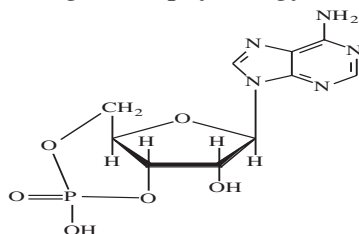
Description

Assay Designs' Correlate-EIA™ cyclic AMP kit is a competitive immunoassay for the quantitative determination of cyclic AMP in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the standards or sample or an alkaline phosphatase molecule which has cAMP covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of cAMP in either standards or 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 Chard¹ or Tijssen².

Introduction

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important "second messengers" involved as a modulator of physiological processes³⁻⁷. cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune and other functions and actions⁸⁻¹¹. 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, nervous system, and immune mechanisms, cell growth and differentiation, and general metabolism¹²⁻¹⁴. 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



Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

1. 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.
2. Some solutions supplied in this kit are caustic; care should be taken with their use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The cyclic AMP Standard provided, Catalog No. 80-0056, is supplied in ethanolic buffer at a pH optimized to maintain cAMP integrity. Care should be taken in handling this material because of the known and unknown effects of cAMP.

CAUTION: Some components of this kit contain chemicals that are lachrymators, corrosive and flammable. Use with caution and wear suitable protection. Refer to the Material Safety Data Sheets available on our Web site or by fax (See Page 12).

Materials Supplied

- 1. Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0060**
A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
- 2. cAMP EIA Conjugate, 5 mL, Catalog No. 80-0053**
A blue solution of alkaline phosphatase conjugated with cAMP.
- 3. cAMP EIA Antibody, 5 mL, Catalog No. 80-0604**
A yellow solution of a rabbit polyclonal antibody to cAMP.
- 4. Assay Buffer 2, 27 mL, Catalog No. 80-0055**
Sodium acetate buffer containing proteins and sodium azide as preservative.
- 5. Wash Buffer Concentrate, 27 mL, Catalog No. 80-1286**
Tris buffered saline containing detergents.
- 6. Cyclic AMP Standard, 0.5 mL, Catalog No. 80-0056**
A solution of 2,000 pmol/mL cAMP.
- 7. p-Npp Substrate, 20 mL, Catalog No. 80-0075**
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
- 8. Stop Solution, 5 mL, Catalog No. 80-0247**
A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
- 9. Triethylamine, 2 mL, Catalog No. 80-0063**
CAUTION: Lachrymator, Harmful Vapor, Flammable.
- 10. Acetic Anhydride, 1 mL, Catalog No. 80-0064**
CAUTION: Lachrymator, Corrosive, Flammable.
- 11. cAMP Assay Layout Sheet, 1 each, Catalog No. 30-0106**
- 12. Plate Sealer, 1 each, Catalog No. 30-0012**

Storage

All components of this kit are stable at 4 °C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

Assay Designs' Correlate-EIA™ is compatible with cAMP samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 2 can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user must verify that the recommended dilutions are appropriate for their sample. **Samples containing rabbit IgG may interfere with the assay.** Samples with very low levels of cAMP can be measured when acetylated with the provided reagents. Please refer to References 15-21 for methods to extract cAMP from samples.

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer 2. There will be a small change in the binding associated with running the standards and samples in Media. Users should only use standard curves generated in media or buffer to calculate concentrations of cAMP in the appropriate matrix.

Urine samples should not be used with this assay. Serum samples should be assayed immediately or frozen below -20 °C. Note: EDTA plasma is not a suitable matrix for the acetylated procedure since it tends to precipitate. Tissue samples should be rapidly frozen in liquid nitrogen. Grind the frozen tissue to a fine powder under liquid nitrogen in a stainless steel mortar. Weigh the frozen tissue and homogenize in 10 volumes of cold 5% TCA in a glass-Teflon tissue grinder. Centrifuge at $\geq 600 \times g$ for 10 minutes. Extract the supernatants with 3 volumes of water-saturated ether. Dry the aqueous extracts and run the reconstituted samples directly in the assay.

For cells adhering to glass or plastic, add 0.1M HCl to the samples and let sit for 10 minutes. Visually inspect the cells to verify cell lysis. If adequate lysis has not occurred incubate for a further 10 minutes and inspect. Take off the 0.1M HCl lysate and centrifuge at $\geq 600 \times g$ for 10 minutes. The supernatants may be assayed directly in our Direct cAMP kit, Catalog Numbers 900-066 or 901-066, or dried down prior to reconstitution in Assay Buffer 2 and run in the kit.

Procedural Notes

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 plastic tubes. Avoid polystyrene.
4. Pre-rinse the pipet tip with each 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. **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 pipet tips and other items that are used in the assay with bare hands.
9. **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 2,000 pmol/mL cAMP standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 900 μ L Standard Diluent (Assay Buffer 2 or Tissue Culture Media) into tube #1. Pipet 750 μ L Standard Diluent into tubes #2-#5. Add 100 μ L of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Continue this 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. See cAMP Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

2. 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.

3. cAMP Standard - Acetylated Version

Allow the 2,000 pmol/mL cAMP standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 990 μ L Standard Diluent (Assay Buffer 2 or Tissue Culture Media) into tube #1 and 750 μ L Standard Diluent into tubes #2-5. Add 10 μ L of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.

The concentration of cAMP in tubes #1 through #5 will be 20, 5, 1.25, 0.312 and 0.078 pmol/mL respectively. See cAMP Assay Layout Sheet for dilution details.

Acetylate all standards and samples by adding 10 μ L of the Acetylating Reagent for each 200 μ L of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds.

Label one 12 x 17 mm glass tube as the Zero Standard/NSB tube. Pipet 1 mL Standard Diluent into this tube. Add 50 μ L of the Acetylating Reagent to the Zero Standard/NSB tube and use in Step 2 on Page 6.

Failure to acetylate the NSB and Zero standard will result in inaccurate B/Bo values.

Use the acetylated standards or samples within 30 minutes.

4. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration, 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.

If Acetylated Version of the kit is to be run, acetylate all standards and samples by adding 10 μL of the Acetylating Reagent for each 200 μL of standard or sample. Add 50 μL of the Acetylating Reagent to the Zero Standard/NSB tube (Refer to Step 3 on Page 5) and use in Steps 2 and 5 below (Failure to acetylate the NSB and Zero standard will result in inaccurate B/Bo values). Add the reagent directly to the samples and vortex for 2 seconds. 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 °C.
2. Pipet 100 μL of Standard Diluent (Assay Buffer 2 or Tissue Culture Media) into the NSB and the Bo (0 pmol/mL Standard) wells.
3. Pipet 100 μL of Standards #1 through #5 into the appropriate wells.
4. Pipet 100 μL of the Samples into the appropriate wells.
5. Pipet 50 μL of Assay Buffer 2 into the NSB wells.
6. Pipet 50 μL of blue Conjugate into each well except the TA and Blank wells.
7. Pipet 50 μL of yellow Antibody into each well, except the Blank, TA and NSB 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.

8. 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.
9. 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**.
10. 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.
11. Add 5 μL of the blue Conjugate to the TA wells.
12. Add 200 μL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.
13. Add 50 μL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. 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 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. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program. 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:

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

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 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.

Typical Results

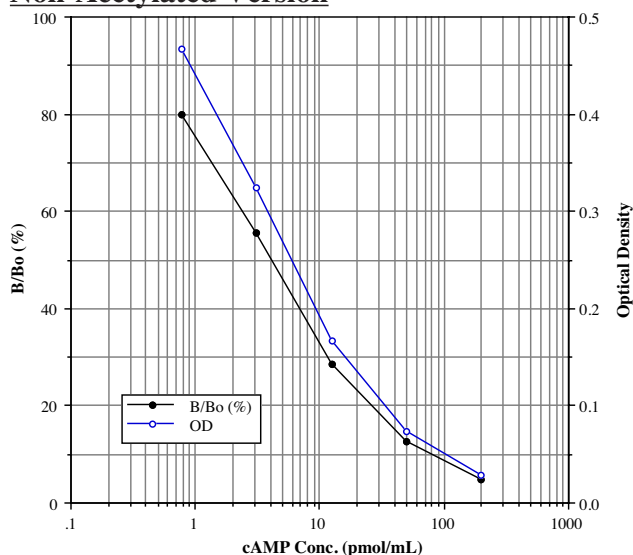
The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	<u>Non-Acetylated Version</u>			<u>Acetylated Version</u>		
	<u>Average Net OD</u>	<u>Percent Bound</u>	<u>cAMP (pmol/mL)</u>	<u>Average Net OD</u>	<u>Percent Bound</u>	<u>cAMP (pmol/mL)</u>
Blank OD	(0.085)			(0.086)		
TA	0.494			0.435		
NSB	0.000	0.03%		0.002	0.53%	
Bo	0.585	100%	0	0.403	100%	0
S1	0.029	4.9%	200	0.034	8.5%	20
S2	0.074	12.7%	50	0.087	21.6%	5
S3	0.167	28.5%	12.5	0.183	45.5%	1.25
S4	0.325	55.6%	3.125	0.296	73.4%	0.3125
S5	0.467	79.9%	0.781	0.362	89.8%	0.0781
Unknown 1	0.130	22.3%	19.39	0.061	15.1%	8.53
Unknown 2	0.398	68.0%	1.63	0.142	35.3%	2.12

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.

Non-Acetylated Version



Typical Quality Control Parameters

Total Activity Added = $0.494 \times 10 = 4.94$

%NSB = 0.0%

%Bo/TA = 11.8%

Quality of Fit = 1.000

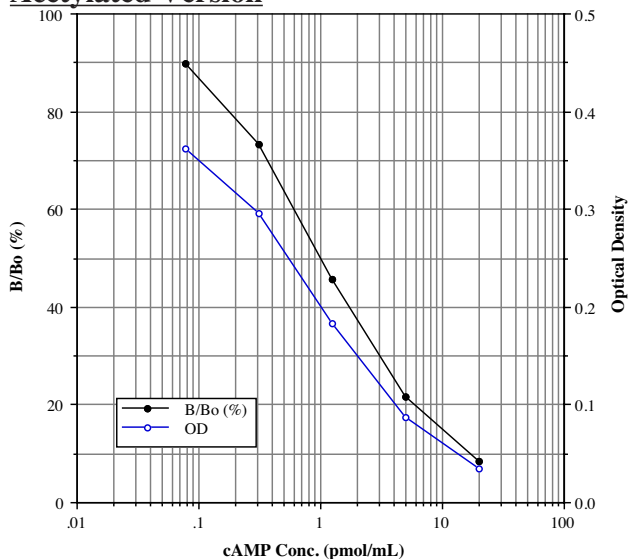
(Calculated from 4 parameter logistics curve fit)

20% Intercept = 23.1 pmol/mL

50% Intercept = 4.1 pmol/mL

80% Intercept = 0.8 pmol/mL

Acetylated Version



Typical Quality Control Parameters

Total Activity Added = $0.435 \times 10 = 4.35$

%NSB = 0.0%

%Bo/TA = 9.3%

Quality of Fit = 1.000

(Calculated from 4 parameter logistics curve fit)

20% Intercept = 5.5 pmol/mL

50% Intercept = 1.0 pmol/mL

80% Intercept = 0.2 pmol/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols²².

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with the 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 = 0.658 ± 0.025 (3.8%)

Mean OD for Standard #5 = 0.531 ± 0.011 (2.1%)

Delta Optical Density (0-0.78 pmol/mL) = 0.658 - 0.531 = 0.127

2 SD's of the Zero Standard = 0.050

Sensitivity = $\frac{0.050}{0.127} \times 0.78 \text{ pmol/mL} = \mathbf{0.30 \text{ pmol/mL}}$

Acetylated Version

Mean OD for Bo = 0.469 ± 0.011 (2.3%)

Mean OD for Standard #5 = 0.425 ± 0.009 (2.2%)

Delta Optical Density (0-0.078 pmol/mL) = 0.469 - 0.425 = 0.044

2 SD's of the Zero Standard = 0.022

Sensitivity = $\frac{0.022}{0.044} \times 0.078 \text{ pmol/mL} = \mathbf{0.039 \text{ pmol/mL}}$

Linearity

Non-Acetylated Version

A sample containing 49.19 pmol/mL cAMP was serially diluted 5 times 1:2 in the Assay Buffer 2 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 0.936 with a correlation coefficient of 0.995.

Acetylated Version

A sample containing 5.42 pmol/mL cAMP was serially diluted 5 times 1:2 in the Assay Buffer 2 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 1.082 with a correlation coefficient of 0.997.

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.

	<u>Non-Acetylated Version</u>			<u>Acetylated Version</u>		
	<u>cAMP</u> (pmol/mL)	<u>Intra-assay</u> (%CV)	<u>Inter-assay</u> (%CV)	<u>cAMP</u> (pmol/mL)	<u>Intra-assay</u> (%CV)	<u>Inter-assay</u> (%CV)
Low	1.18	10.5		0.40	7.4	
Medium	5.96	2.5		0.90	6.8	
High	18.6	2.9		5.58	7.7	
Low	1.13		13.7	0.46		11.2
Medium	4.95		11.2	0.98		11.2
High	19.18		8.4	4.75		7.9

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer 2 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.

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

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

cAMP concentrations were measured in a variety of different samples including tissue culture media, porcine serum and human saliva. cAMP was spiked into the samples which were diluted with the appropriate diluent and then assayed in the kit. Recovery values were not obtained with urine samples due to high endogenous levels of cAMP. The following results were obtained:

Sample	Non-Acetylated Version		Acetylated Version	
	% Recovery	Recommended Dilution*	% Recovery	Recommended Dilution*
Tissue Culture Media	96.2	None	101.2	None
Human Serum	101.5	1:10	117.8	1:64
Human Saliva	103.2	1:4	94.9	1:4
Human Urine	Do Not Use		Do Not Use	

* See Sample Handling instructions on page 4 for details.

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LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

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For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

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