New Standard and Conjugate Storage Temperatures

Format A Cyclic AMP "PLUS" Enzyme Immunoassay Kit

Catalog No. BML-AK215-0001
480 Well (5 by 96 Well) Kit

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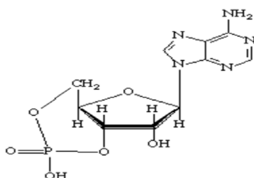
Description

The Enzo Life Sciences Format A cyclic AMP “PLUS” EIA kit is a competitive immunoassay for the quantitative determination of cyclic AMP in samples treated with 0.1M HCl. 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 Chard[1] or Tijssen[2].

Introduction

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, nervous system, and 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



Precautions

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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 Mg^{2+} and Zn^{2+} 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.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, Five Plates of 96 Wells, Catalog No. 80-0060**
   A plate using break-apart strips coated with goat antibody to rabbit IgG.
2. **cAMP Direct Conjugate, 25 mL, Catalog No. 80-0195**
   A blue solution of alkaline phosphatase conjugated with cAMP.
3. **cAMP EIA Antibody, 25 mL, Catalog No. 80-0606**
   A yellow solution of a rabbit polyclonal antibody to cAMP.
4. **0.1M HCl, 100 mL, Catalog No. 80-0082**
   0.1M hydrochloric acid in water. **CAUTION: Acid, wear suitable protective clothing.**
5. **Neutralizing Reagent, 27 mL, Catalog No. 80-1476**
6. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
7. **Cyclic AMP Standard, 3 x 0.5 mL, Catalog No. 80-0056**
   A solution of 2,000 pmol/mL cAMP.
8. **p-Npp Substrate, 100 mL, Catalog No. 80-0076**
   A solution of p-nitrophenyl phosphate.
9. **Stop Solution, 27 mL, Catalog No. 80-0248**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution**: Caustic.**
10. **Triethylamine, 2 x 2 mL, Catalog No. 80-0063**
    CAUTION: Lachrymator, Harmful Vapor, Flammable.
11. **Acetic Anhydride, 2 x 1 mL, Catalog No. 80-0064**
    CAUTION: Lachrymator, Corrosive, Flammable.
12. **cAMP Assay Layout Sheet, 1 each, Catalog No. 30-0116**
13. **Plate Sealer, 5 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Conjugate and Standard**, are stable at 4°C until the kit's expiration date. The Conjugate and Standard should be stored at -20°C upon receipt.

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

The Enzo Life Sciences Format A cAMP "PLUS" 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 on page 11 for details of sugested 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 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 0.1M HCl. Centrifuge at ≥ 600 x g at room temperature. The samples can then be diluted in the 0.1M HCl provided for the assay.

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

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 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. **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 µL 0.1M HCl into tube #1 and 750 µL of 0.1M HCl into tubes #2-5. Add 100 µL of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 250 µL 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 Format A cAMP "PLUS" 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 µL 0.1M HCl into tube #1 and 750 µL 0.1M HCl into tubes #2-5. Add 10 µL of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 250 µL 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 Direct cAMP Assay Layout Sheet for dilution details.

   Acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL 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 µL of the Acetylating Reagent to the Zero Standard/NSB tube and use in Step 3 and 6, 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 3 and 6 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 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°C.
2. Pipet 50 µL of the Neutralizing Reagent into each well, except the Total Activity (TA) and Blank wells.
3. Pipet 100 µL of 0.1M HCl into the NSB and the Bo (0 pmol/mL Standard) wells.
4. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.
5. Pipet 100 µL of the Samples into the appropriate wells.
6. Pipet 50 µL of 0.1M HCl into the NSB wells.
7. Pipet 50 µL of blue Conjugate into each well except the TA and Blank wells.
8. 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.

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 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 blue Conjugate to the TA wells.
13. Add 200 µL of the pNpp 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 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:

   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 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

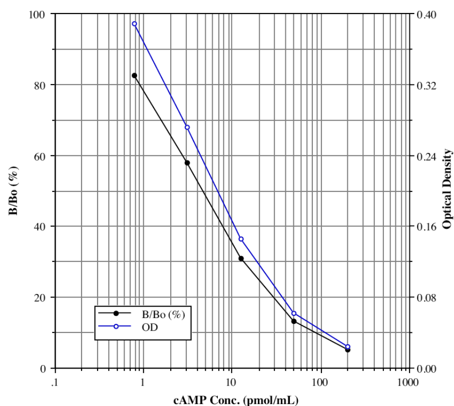
The results shown below are for illustration only and **should not** be used to calculate results from another assay.

	Non-Acetylated Version			Acetylated Version		
Sample	Mean OD (-Blank)	Percent Bound	**cAMP (pmol/mL)**	Mean OD (-Blank)	Percent Bound	**cAMP (pmol/mL)**
Blank OD	(0.081)			(0.083)		
TA	0.486			0.437		
NSB	-0.001	-0.18%		0.003	0.93%	
Bo	0.471	100%	**0**	0.331	100%	**0**
S1	0.024	5.1%	**200**	0.017	5.2%	**20**
S2	0.062	13.1%	**50**	0.068	20.5%	**5**
S3	0.145	30.9%	**12.5**	0.127	38.5%	**1.25**
S4	0.272	57.9%	**3.125**	0.229	69.1%	**0.312**
S5	0.388	82.5%	**0.781**	0.301	90.9%	**0.078**
Unknown 1	0.125	26.6%	**16.21**	0.125	37.8%	**1.45**
Unknown 2	0.291	61.8%	**2.57**	0.276	83.5%	**0.13**

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

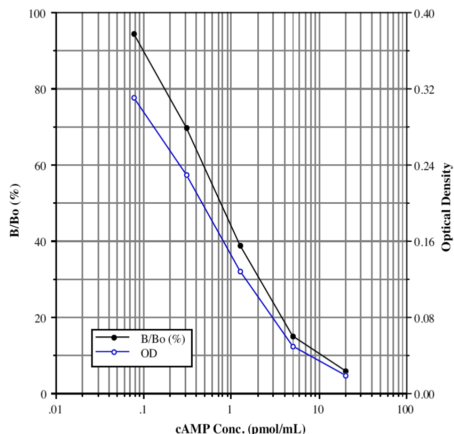


Typical Quality Control Parameters

Total Activity Added = 0.486 x 10 = 4.86
%NSB = 0.0%
%Bo/TA = 9.7%
Quality of Fit = 1.000
(Calculated from 4 parameter logistics curve fit)

20% Intercept = 26.1 pmol/mL
50% Intercept = 4.6 pmol/mL
80% Intercept = 0.9 pmol/mL

Acetylated Version



Typical Quality Control Parameters

Total Activity Added = 0.437 x 10 = 4.37
%NSB = 0.1%
%Bo/TA = 7.6%
Quality of Fit = 0.998
(Calculated from 4 parameter logistics curve fit)

20% Intercept = 4.5 pmol/mL
50% Intercept = 0.8 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 Protocols[15].

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 =	0.340 ± 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 the Zero Standard =	0.024
Sensitivity = $\frac{0.024}{0.048}$ x 0.78 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 the Zero Standard =	0.008
Sensitivity = $\frac{0.008}{0.017}$ x 0.078 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 0.1M 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 0.1M 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 differing concentrations of cAMP and running these samples multiple times (n≥24) in the same assay. Inter-assay precision was determined by measuring samples with differing 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			Acetylated Version		
	cAMP (pmol/mL)	Intra-assay (%CV)	Inter-assay (%CV)	cAMP (pmol/mL)	Intra-assay (%CV)	Inter-assay (%CV)
Low	1.24	8.9		0.679	4.6	
Medium	6.31	4.3		3.58	8.4	
High	35.29	8.3				
Low	1.18		13.1	1.29		13.6
Medium	5.52		4.2	5.62		7.8
High	30.36		11.6			

Cross Reactivities

The cross reactivities for a number of related compounds were determined by using the Enzo Life Sciences "PLUS" EIA cAMP kit, Catalog Number AK-255, 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

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

cAMP concentrations were measured in tissue culture media. cAMP was spiked into the sample which was diluted with the kit 0.1M HCl and then assayed in the kit. The following result was obtained:

	Non-Acetylated Version		Acetylated Version	
Sample	**% Recovery**	**Recommended Dilution***	**% Recovery**	**Recommended Dilution***
Tissue Culture Media	94.8	1:4	95.2	1:4

* See Sample Handling instructions on page 4 for details.

References

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USE OF PRODUCT

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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Enzo Life Sciences International, Inc.
5120 Butler Pike
Plymouth Meeting, PA 19462

Phone: (800) 942-0430
(610) 941-0430
Fax: (610) 941-9252
email: info-us@enzolifesciences.com

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