



cAMP ELISA

For the quantitative determination of extracellular cyclic AMP in human saliva, serum and culture supernatants.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number:	74-AMPHU-E01
Size:	96 Wells
Version:	01/28/2015 – ALPCO July 15, 2016

Introduction

The cyclic AMP Enzyme-Linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of extracellular cyclic AMP in saliva, serum, and culture supernatants. The optional acetylated assay format provides an approximate 10-fold increase in sensitivity and is ideal for samples with extremely low levels of cAMP. If expected levels of cAMP are unknown, the investigator may evaluate a few samples in the non-acetylated format in order to determine if higher sensitivity is required.

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important "second messengers" involved as a modulator of physiological processes⁵. cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune and other functions⁶⁻⁹. 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). Because cAMP has been shown to be involved in the cardiovascular and nervous systems, immune mechanisms, cell growth and differentiation, and general metabolism¹⁰⁻¹², there remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures. The investigation of cAMP may help to provide a clearer understanding of the physiology and pathology of many disease states.

Principle

1. Standards and samples are added to wells coated with a GxR IgG antibody. A blue solution of cAMP conjugated to alkaline phosphatase is then added, followed by a yellow solution of rabbit polyclonal antibody to cAMP.
2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the cAMP in the sample or conjugate. The plate is washed, leaving only bound cAMP.
3. pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the cAMP conjugate.
4. Stop solution is added. The yellow color is read at 405nm. The amount of signal is indirectly proportional to the amount of cAMP in the sample.

Materials Supplied

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. **Assay Buffer 2**

27 mL, Product No. 80-0055

Sodium acetate buffer containing proteins and sodium azide

2. **cyclic AMP Standard**

0.5 mL, Product No. 80-0056

A solution of 2,000 pmol/mL cAMP

3. **Acetylation Kit**

2 vials, Product No. 950-001

a. Triethylamine

2 mL, Product No. 80-0063

b. Acetic Anhydride

1 mL, Product No. 80-0064

Triethylamine and acetic anhydride are lachrymators. Caution- corrosive, flammable, and harmful vapor.

4. **Goat anti-Rabbit IgG Microtiter Plate**

One plate of 96 wells, Product No. 80-0060

A clear plate of break-apart strips coated with a Goat anti-Rabbit polyclonal antibody

5. **cAMP ELISA Antibody**

5 mL, Product No. 80-0604

A yellow solution of rabbit polyclonal antibody to cAMP

6. **cAMP ELISA Conjugate**

5 mL, Product No. 80-0053

A blue solution of cAMP conjugated to alkaline phosphatase

Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.

Activity of conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).

7. **Wash Buffer Concentrate**

27 mL, Product No. 80-1286

Tris buffered saline containing detergents

8. **pNpp Substrate**

20 mL, Product No. 80-0075

A solution of p-nitrophenyl phosphate

9. **Stop Solution**

5 mL, Product No. 80-0247

A solution of trisodium phosphate in water

Stop solution is caustic. Keep tightly capped.

10. **cAMP Assay Layout Sheet**

1 each, Product No. 30-0106

11. **Plate Sealer**

1 each, Product 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 pipet for dispensing 50 µL and 200 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. Microplate shaker
7. Lint-free paper toweling for blotting
8. Microplate reader capable of reading at 405 nm

Reagent Preparation

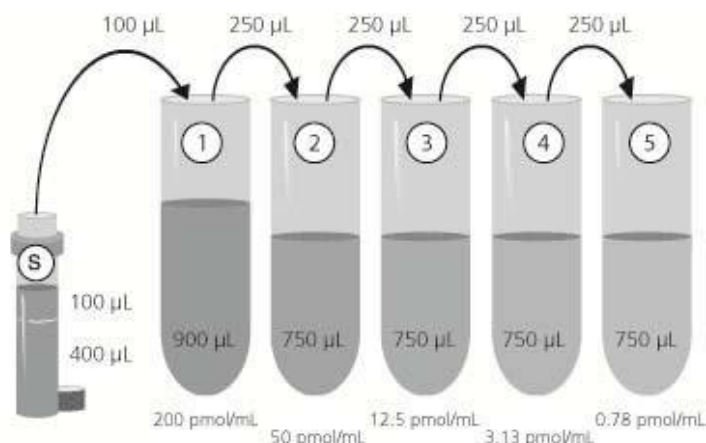
Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

Wash Buffer

Prepare the wash buffer by diluting 5 mL of the supplied Wash Buffer 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.

cAMP Standard, non-acetylated format

Two diluent options are available for the preparation of the standard curve, NSB, and Bo wells. Use Assay Buffer 2 for the standard diluent when the sample is serum or saliva. For culture supernatants, use the same non-conditioned media for the standard diluent.



Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm x 75mm tubes #1 through #5. Pipet 900 μ L of the appropriate sample diluent into tube #1. Pipet 750 μ L of the appropriate sample diluent into tubes #2 through #5. Add 100 μ L of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Add 250 μ L of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Diluted standards should be used within 60 minutes of preparation.

The concentrations of cAMP in the tubes are labeled above.

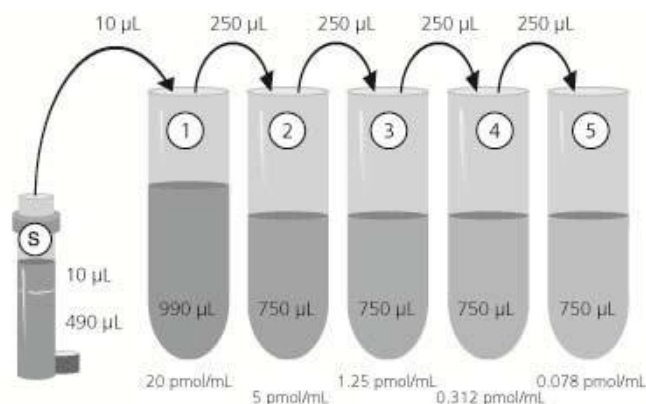
Acetylation Reagent (optional)

Triethylamine and acetic anhydride are lachrymators. Caution- corrosive, flammable, and harmful vapor.

Prepare the Acetylating Reagent by adding 0.5 mL of Acetic Anhydride to 1 mL of Triethylamine. Note that this volume is sufficient to add to 30 mL of diluted standards and samples. Use the prepared reagent within 60 minutes of preparation. Discard any unused portion of the Acetylating Reagent.

cAMP Standard, acetylated format (optional)

Two diluent options are available for the preparation of the standard curve, NSB, and Bo wells. Use Assay Buffer 2 for the standard diluent when the sample is serum or saliva. For culture supernatants, use the same non-conditioned media for the standard diluent.



Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm x 75mm tubes #1 through #5. Pipet 990 μ L of the appropriate sample diluent into tube #1. Pipet 750 μ L of the appropriate sample diluent into tubes #2 through #5. Add 10 μ L of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 μ L of tube #1 to tube #2 and vortex thoroughly. Add 250 μ L of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Acetylate all **standards and samples** by adding 10 μ L of the Acetylating Reagent for each 200 μ L of the standard or sample. Add the Acetylating Reagent directly to the diluted standard or sample and vortex immediately after the addition of the Acetylating Reagent.

Label one 12mm x 75mm tube as the Bo/NSB tube. Pipet 1 mL of the appropriate standard diluent into this tube. Add 50 μ L of the Acetylating Reagent to the Bo/NSB tube and use in Steps 2 and 3 of the Assay Procedure.

The acetylated standards should be used within 30 minutes of preparation.

The concentrations of cAMP in the tubes are labeled above.

Sample Handling

Samples must be stored frozen at or below -20°C to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.

Samples containing rabbit IgG will interfere with the assay. EDTA plasma may precipitate during acetylation. Biological fluids, such as serum and saliva, should be diluted in Assay Buffer 2 and run directly in the assay. A minimum 1:10 dilution is required for serum and a 1:4 dilution for saliva (see Sample Recoveries section). These are the minimum dilutions required to remove matrix interference of these samples.

Culture supernatants may be run directly in the assay provided the same non-conditioned media is used as the standard diluent.

Please note that some samples may contain high levels of cAMP and additional dilution may be required. Samples with low levels of cAMP may be assayed in the acetylated format or the samples may be concentrates.

Sample Recoveries

cAMP standard was spiked into the following matrices diluted with Assay Buffer 2 and measured in the kit. The results were as follows:

Sample	Non-Acetylated Format		Acetylated Format	
	% 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

Assay Procedure

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

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard and reagent.

All standards and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

Note: If the acetylated format of the assay is to be run, all standards, samples, and the diluent for the NSB and Bo wells must be acetylated as per the instructions in the Reagent Preparation section. Acetylated standards and samples must be used within 30 minutes.

1. Pipet 100 μ L of the appropriate standard diluent (Assay Buffer 2 or non-conditioned culture media) into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells.
2. Add 50 μ L of Assay Buffer 2 to the NSB wells.
3. Pipet 100 μ L of Standards #1 through #5 to the bottom of the appropriate wells.
4. Pipet 100 μ L of the samples to the bottom of the appropriate wells.
5. Pipet 50 μ L of the blue conjugate into each well except the TA and Blank wells.
6. Pipet 50 μ L of the 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.

7. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature.
8. Empty the contents of the wells and wash by adding 400 μ L of wash buffer to every well. Repeat 2 more times for a total of 3 washes. 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.
9. Pipet 5 μ L of the blue conjugate to the TA wells.
10. Add 200 μ L of the substrate solution into each well.
11. Incubate for 1 hour at room temperature without shaking.
12. Pipet 50 μ L stop solution into each well.
13. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Calculation of 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 4 parameter logistic (4PL) curve fitting program.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

To normalize for protein content, divide the resulting picomole per mL determinations (pmol/mL) by the total protein concentration (mg/mL) in each sample. This is expressed as pmol cAMP per mg of total protein.

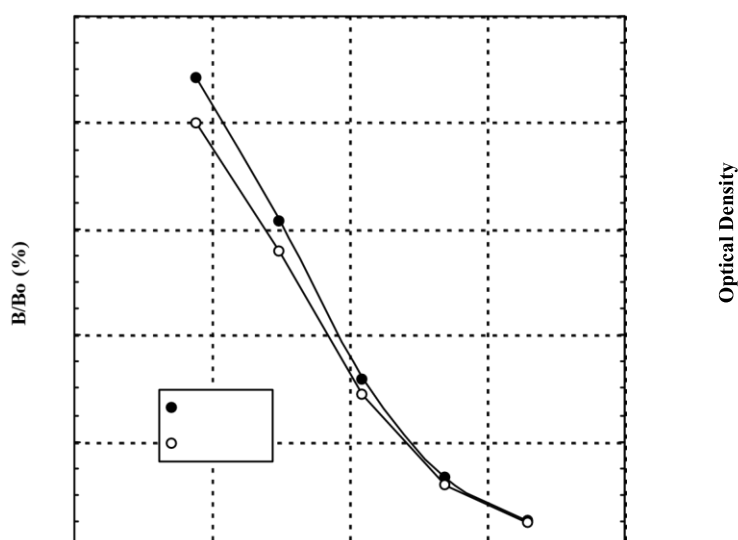
Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Typical Results

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

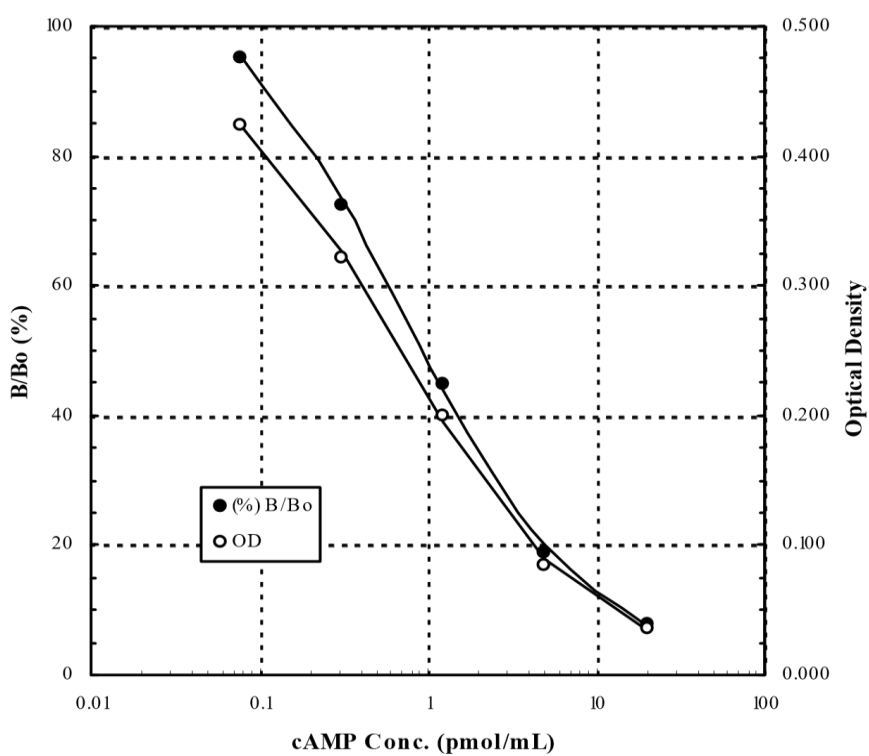
Non-acetylated assay format

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.086)	---	---
TA	0.454	---	---
NSB	0.001	0.18%	---
Bo	0.543	100%	0
S1	0.028	5.1%	200
S2	0.071	13.1%	50
S3	0.173	31.8%	12.5
S4	0.334	61.5%	3.125
S5	0.479	88.2%	0.781
Unknown 1	0.135	24.8%	18.93
Unknown 2	0.357	65.8%	2.57



Acetylated assay format

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.093)	---	---
TA	0.463	---	---
NSB	-0.003	-0.87%	---
B ₀	0.445	100%	0
S1	0.036	8.0%	20
S2	0.084	18.9%	5
S3	0.199	44.7%	1.25
S4	0.323	72.5%	0.3125
S5	0.424	95.3%	0.0781
Unknown 1	0.059	13.3%	9.20
Unknown 2	0.143	32.0%	2.27



Performance Characteristics

Specificity

The cross reactivities for a number of related compounds were determined by diluting the cross reactants in the kit assay buffer at a concentration of ten times the high standard. These samples were then measured in the assay.

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%

Sensitivity

The sensitivity of the assay, defined as the concentration of cAMP measure at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 0.30 pmol/mL in the non-acetylated assay format and 0.039 pmol/mL in the acetylated assay format.

Linearity

A buffer sample containing cAMP was serially diluted 1:2 in the kit assay buffer and measured in the assay. The results are shown in the table below.

Non-acetylated			
Dilution	Expected (pmol/mL)	Observed (pmol/mL)	% Recovery
Neat	---	49.2	---
1:2	24.6	23.1	94%
1:4	12.3	13.7	112%
1:8	6.15	6.9	112%
1:16	3.07	3.4	111%
Acetylated			
Dilution	Expected (pmol/mL)	Observed (pmol/mL)	% Recovery
Neat	---	5.42	---
1:2	2.71	2.86	106%
1:4	1.36	1.23	91%
1:8	0.68	0.51	75%
1:16	0.34	0.28	83%

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing cAMP in a single assay.

Non-acetylated Format		Acetylated Format	
pmol/mL	%CV	pmol/mL	%CV
1.18	10.5	0.40	7.40
5.96	2.50	0.90	6.80
18.6	2.90	5.58	7.70

Inter-assay precision was determined by measuring buffer controls of varying cAMP concentrations in multiple assays over several days.

Non-acetylated Format		Acetylated Format	
pmol/mL	%CV	pmol/mL	%CV
1.13	13.7	0.46	11.2
4.95	11.2	0.98	11.2
19.2	8.40	4.75	7.90

References

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