Version 28-04/24

User's Manual

cAMP ELISA Kit

REF **DEIANS070**

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

For the quantitative determination of cAMP in culture supernatants, serum, and saliva from any species. Cited sample types include cell lysate, tissue, and urine.

General Description

The cyclic AMP Enzyme-Linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of extracellular cyclic AMP in plasma, saliva, serum, and culture supernatant. The optional acetylated assay format provides a significant 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 nonacetylated 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 processes.cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune and other functions. 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 metabolism, 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.

Principles of Testing

1. Standards and samples are added to wells coated with 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.

Reagents And Materials Provided

1. Assay Buffer 2, 27mL, Sodium acetate buffer containing proteins and sodium azide.

2. cyclic AMP Standard, 0.5mL, A solution of 2,000 pmol/mL cAMP.

3. Acetylation Kit, 2 vials

a. Triethylamine, 2 mL

b. Acetic Anhydride, 1 mL

4. Goat anti-Rabbit IgG Microtiter Plate, One plate of 96 wells, A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody.

5. cAMP Antibody, 5mL, A yellow solution of rabbit polyclonal antibody to cAMP.

6. cAMP Conjugate, 5mL, A blue solution of cAMP conjugated to alkaline phosphatase.

7. Wash Buffer Concentrate, 27mL, A 20× solution of tris buffered saline containing detergents.

8. pNpp Substrate, 20 mL, A solution of p-nitrophenyl phosphate.

9. Stop Solution 2, 5 mL, A solution of trisodium phosphate in water.

Materials Required But Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 1 µL and 1,000 µL.
3. Repeater pipet for dispensing 50 µL and 200 µL.
4. Disposable beakers for dilution buffer concentration.
5. Graduated cylinders.
6. Microplate shaker.
7. Lint-free paper toweling for blotting.
8. Microplate reader capable of reading at an optical density of 405nm.

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.

Specimen Collection And Preparation

Samples containing rabbit IgG will interfere with the assay. EDTA plasma may precipitate during acetylation.

Biological fluids should be diluted in Assay Buffer 2 and run directly in the assay. When using the non-acetylation protocol, a minimum 1:16 dilution is required for plasma, a 1:64 dilution for serum, a 1:16 dilution for saliva. When using the acetylation protocol, a minimum 1:64 dilution is required for both plasma and serum and a 1:2 dilution for saliva.

Culture supernatant, diluted in both assay buffer and tissue culture media, has also been validated for use in this kit. When using the non-acetylation protocol, neat culture supernatant can be used. When using the acetylation protocol, a minimum 1:4 dilution is required.

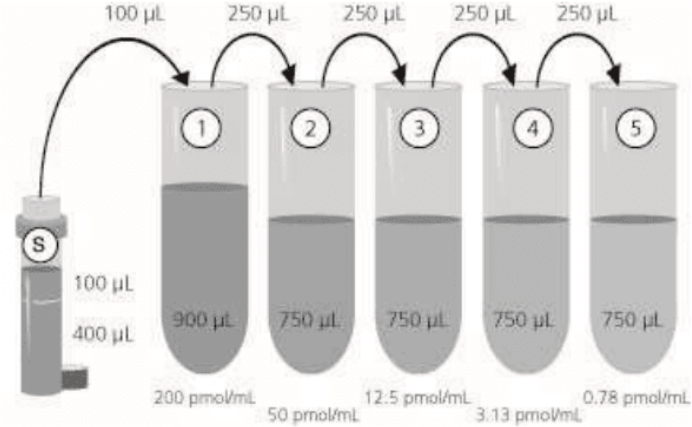
Please see Sample Recoveries section for detailed information. These are the minimum dilutions required to remove matrix interference of these samples.Please note that some samples may contain high levels of cAMP and additional dilution may be required. Samples with low levels of cAMP may be assayed in the acetylated format or the samples may be concentrated.

Reagent Preparation

1. 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.

2. cAMP Standard, non-acetylated format



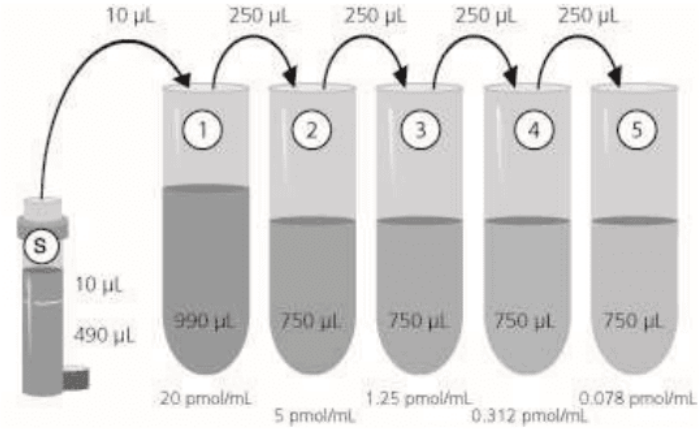
Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm × 75mm tubes #1 through #5. Pipet 900 µL of Assay Buffer 2 into tube #1. Pipet 750 µL of Assay Buffer 2 into tubes #2 through #5. Add 100 µL of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL 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.

3. Acetylation Reagent (optional)

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.

4. cAMP Standard, acetylated format (optional)



Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm × 75mm tubes #1 through #5. Pipet 990 µL of Assay Buffer 2 into tube #1. Pipet 750 µL of Assay Buffer 2 into tubes #2 through #5. Add 10 µL of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 µL of tube

#1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL 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 × 75mm tube as the B_0/NSB tube (NSB: non-specific binding, B_0: 0 pmol/mL standard). Pipet 1 mL of Assay Buffer 2 into this tube. **Add 50 µL of the Acetylating Reagent to the B_0/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.

Assay Procedure

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 B_0 wells must be section.

Acetylated standards and samples must be used within 30 minutes.

1. Pipet 100 µL of Assay Buffer 2 into the NSB (non-specific binding) and B_0 (0 pmol/mL standard) wells.
2. Add 50 µL of Assay Buffer 2 to the NSB wells.
3. Pipet 100 µL of Standards #1 through #5 to the bottom of the appropriate wells.
4. Pipet 100 µL of the samples to the bottom of the appropriate wells.
5. Pipet 50 µL of the blue conjugate into each well except the TA and Blank wells.
6. Pipet 50 µL 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 µL 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 µL of the blue conjugate to the TA wells.
10. Add 200 µL of the substrate solution into each well.
11. Incubate for 1.5 hours at room temperature with shaking.
12. Pipet 50 µL 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

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 4 parameter logistic (4PL) curve fitting program. The concentration of cAMP can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

2. Using data analysis software, plot the Average Net OD for each standard versus cAMP concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalyzed using alternative dilution(s).

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.

Typical Standard Curve

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

Non-acetylated assay format

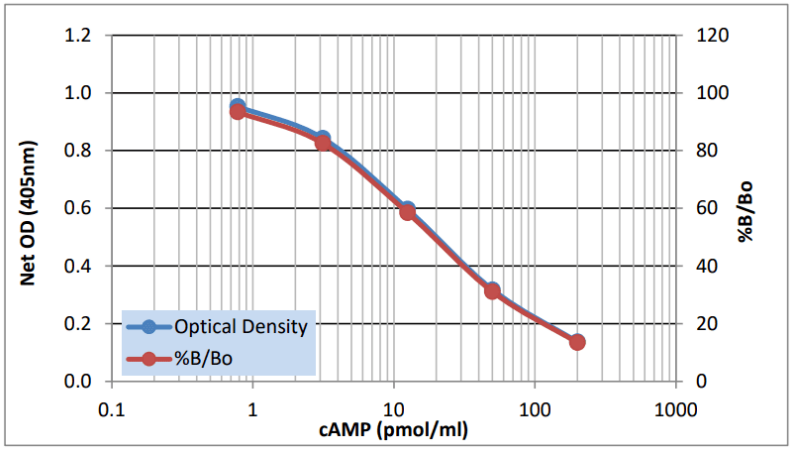
Sample	Mean OD	Net OD	%B/Bo	cAMP (pmol/mL)
TA	--	0.172	--	--
NSB	0.102	--	--	--
Bo	1.123	1.021	--	0
S1	0.239	0.137	13.44	200
S2	0.42	0.318	31.13	50
S3	0.699	0.597	58.48	12.5
S4	0.945	0.843	82.55	3.125
S5	1.055	0.953	93.42	0.781

Acetylated assay format

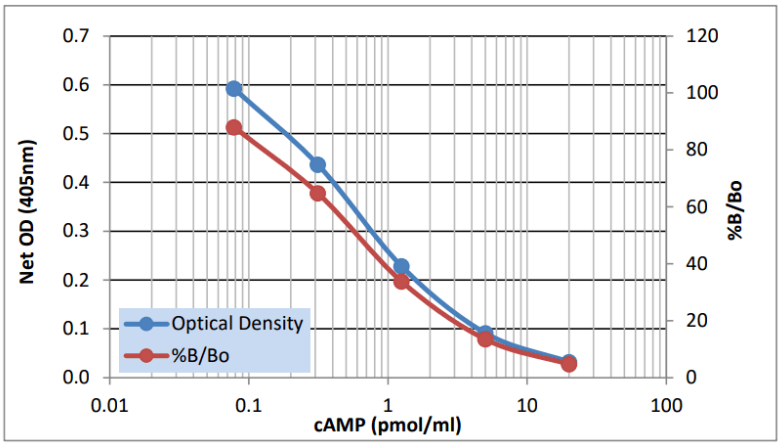
Sample	Mean OD	Net OD	%B/Bo	cAMP (pmol/mL)
TA	--	0.168	--	--
NSB	0.103	--	--	--
Bo	0.776	0.673	--	0
S1	0.135	0.032	4.74	20
S2	0.194	0.091	13.51	5
S3	0.331	0.228	33.83	1.25
S4	0.540	0.437	64.81	0.3125
S5	0.695	0.592	87.98	0.0781

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.

Non-acetylated assay format



Acetylated assay format



Precision

Intra-assay 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
20	4.19	2	4.32
5	11.94	0.5	5.42
2	13.38	0.2	5.91

Inter-assay 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
20	8.1	2	8.94
5	10.41	0.5	10.77
2	10.99	0.2	12.18

Sensitivity

The sensitivity of the assay, defined as the concentration of cAMP measured at 2 standard deviations from the mean of 18 zeros along the standard curve, was determined to be 0.49 pmol/mL in the non-acetylated assay format and 0.027 pmol/mL in the acetylated assay format.

Specificity

The specificity of the assay was determined by running serial dilutions of the analytes, including the cross-reactants, in the assay, fitting the resulting dose response curves to a 4PL curve-fit and determining the ED_{50} . The ED_{50} of each cross-reactant was then divided by the determined ED_{50} of the cAMP standard curve and multiplied by 100.

Analyte	Cross Reactivity, %
cAMP	100
AMP	<0.001
ATP	<0.001
cGMP	<0.001
GMP	<0.001
GTP	<0.001
cUMP	<0.001
CTP	<0.001

Linearity

Plasma, saliva and serum were diluted to their respective minimum recommended dilutions (MRD), spiked with cAMP and serially diluted 1:2 in Assay Buffer. Neat culture supernatant was spiked with cAMP and serially diluted in both assay buffer (AB) and tissue culture media (TCM). For the acetylated samples, plasma

and serum were diluted to their respective MRDs, spiked with cAMP, acetylated and serially diluted 1:2 in Assay Buffer. Additionally, saliva with endogenous levels of cAMP high enough to be read in the assay was diluted to its MRD and then serially diluted 1:2 in Assay Buffer. Acetylated culture supernatant was diluted 1:4, spiked with cAMP and serially diluted in both AB and TCM. All samples were run in the assay and compared to the standard curve. Results for both the non-acetylated and acetylated samples are shown in the tables below.

Dilutional Linearity, % (non-acetylated)					
Dilution	**EDTA plasma**	**Saliva**	**Serum**	**Culture supernatant (in AB)**	**Culture supernatant (in TCM)**
Neat	--	--	--	100	100
1:2	--	--	--	95.3	105
1:4	--	--	--	100	103
1:8	--	--	--	101.3	98
1:16	100	100	--	108	100
1:32	104	92	--	105	100
1:64	111	88	100	109	100
1:128	103	90	99	104	114
1:256	104	85	108	--	--
1:512	103	80	110	--	--
1:1024	104	86	120	--	--
1:2048	113	139	125	--	--
1:4096	--	--	145	--	--
1:8192	--	--	162	--	--

Dilutional Linearity, % (acetylated)					
Dilution	EDTA plasma	Saliva (non-spiked)	Serum	Culture supernatant (in AB)	Culture supernatant (in TCM)
Neat	--	--	--	--	--
1:2	--	100	--	--	--
1:4	--	75	--	100	100
1:8	--	63	--	89	100
1:16	--	83	--	97	98
1:32	--	92	--	94	87
1:64	100	144	100	10	96
1:128	110	194	96	128	96
1:256	136	--	140	--	--
1:512	172	--	164	--	--
1:1024	122	--	138	--	--
1:2048	122	--	153	--	--
1:4096	167	--	139	--	--
1:8192	--	--	--	--	--

Recovery

cAMP was spiked at three concentrations into human plasma, saliva and serum at their respective MRDs. For the acetylated samples, cAMP was spiked at the same three concentrations into human plasma, saliva and serum at their respective MRDs, acetylated and run in the assay. Matrix background was subtracted and the recovery was compared to the recovery of cAMP spiked into Assay Buffer. The average percent recovery for each matrix at the minimum recommended dilution is indicated below. Results for both non-acetylated and acetylated samples are shown in the tables below.

Sample (non-acetylated)	Spike Concentration, pmol/mL	% Recovery	Minimum Recommended Dilution
EDTA plasma	200	93	1:16
	50	152	
	20	136	
Saliva	200	115	1:16
	50	113	
	20	114	
Serum	200	123	1:64
	50	161	
	20	115	

Sample (acetylated)	Spike Concentration, pmol/mL	% Recovery	Minimum Recommended Dilution
EDTA plasma	20	85	1:64
	5	76	
	2	116	
Saliva	20	85	1:2
	5	112	
	2	193	
Serum	20	90	1:64
	5	85	
	2	80	