NEOGEN®
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ISO 9001:2000

Cyclic AMP ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #403110

For Research Use Only

Storage Conditions:
Lyophilized conjugate: -20°C or less
Do not freeze reconstituted conjugate
All other kit components: 4°C

DESCRIPTION

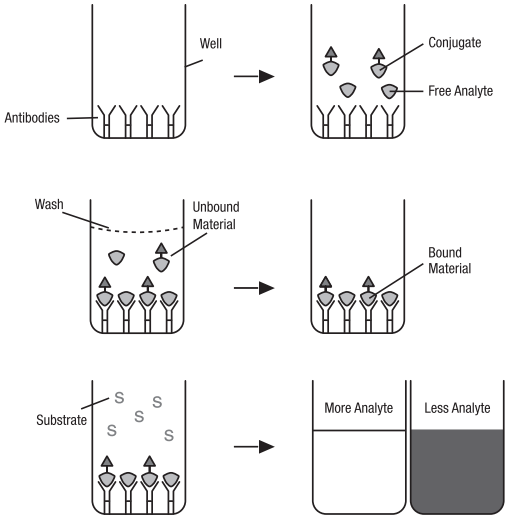
Adenosine 3',5'-cyclic monophosphate (cyclic AMP, cAMP) is one of the most important secondary messengers involved in signal transduction, gene regulation, steroid hormone biosynthesis and intracellular signaling. Its regulatory role extends to the nervous system, cell growth and differentiation, immune mechanisms and general metabolism. Cyclic AMP production is stimulated by compounds (hormones, drugs, neuromodulators) binding to target cells through cell membrane receptors. These binding events activate G-proteins which in turn activate the enzyme adenylate cyclase. Adenylate cyclase converts ATP to cyclic AMP. Cyclic AMP-dependent protein kinases can then be activated resulting in phosphorylation of substrate proteins. Cyclic AMP levels may aid in understanding the mechanisms of action of a variety of hormones and biologically active substances.

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked ImmunoSorbent Assay) for the quantitative analysis of Cyclic AMP levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and the Cyclic AMP in the sample for a limited number of binding sites.

First, the sample or standard solution is added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed to remove all unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring absorbance readings with a microplate reader with a 650 nm filter and then comparing the readings of the samples against the readings of the standards. The extent of color development is inversely proportional to the amount of Cyclic AMP in the sample or standard. For example, the absence of Cyclic AMP in the sample will result in a bright blue color, where as the presence of Cyclic AMP will result in decreased or no color development.

PRINCIPLE OF ASSAY (continued)



MATERIALS PROVIDED

1. **EIA BUFFER:** 30 mL. Provided to dilute enzyme conjugate.
2. **WASH BUFFER (10X)**: 20 mL. Dilute 10-fold with deionized or ultra pure water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the wells after the one hour incubation.
3. **SUBSTRATE:** 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after the wash step. Keep substrate refrigerated. **LIGHT SENSITIVE**.
4. **CYCLIC AMP ENZYME CONJUGATE:** Two vials of lyophilized Cyclic AMP horseradish peroxidase conjugate. Lyophilized vials should be stored at -20°C. Reconstitution with 75µL of deionized water results in a 50:1 concentrate. Store at 4°C after reconstitution. Blue capped vial.
5. **CYCLIC AMP STANDARD:** 100 µL. Cyclic AMP standard provided at the concentration of 1 µg/mL. Green capped vial.
6. **CYCLIC AMP ANTIBODY COATED PLATE:** A 96 well Costar® microplate with anti-Cyclic AMP goat antibody precoated on each well. The plate is ready for use as is. **DO NOT WASH.**

MATERIALS NEEDED BUT NOT PROVIDED

1. 300 mL deionized water to dilute wash buffer and to reconstitute lyophilized conjugate.
2. Precision pipettes that range from 10 µL-1000 µL and disposable tips.

NOTE: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.

3. Clean test tubes used to dilute the standards and conjugate.
4. Graduated cylinders to dilute and mix wash buffer.
5. Microplate reader with 650 nm filter.
6. Plastic film or plate cover to cover plate during incubation.
7. Acetic Anhydride
8. Triethylamine
9. 10mM PBS buffer, pH 7.5
10. Vortex

OPTIONAL MATERIALS:

11. 1N HCl or Neogen's Red Stop Solution
12. Microplate shaker

    If performing an extraction on samples, the following will be required:
13. 0.4 N $HClO_4$
14. 1N KOH
15. Dowex 1-x2 column (0.5x4 cm, chloride form)
16. 0.5 N HCl
17. Centrifuge
18. Freeze Dryer

WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. **DO NOT** pipette reagents by mouth.
4. Always pour substrate out of the bottle into a clean test tube - **DO NOT** pipette out of the bottle. If the pipette tip is unclean, this could result in contamination of the substrate.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be refrigerated at all times when not in use. Lyophilized conjugate should be stored at -20°C until reconstitution.
10. Ensure that the conjugate is completely reconstituted before use. Each vial, when reconstituted, provides sufficient reagent to assay 64 wells (8 strips). If more than 64 wells are to be run, reconstitute both vials and pool the reconstituted conjugate before diluting.

PROCEDURAL NOTES

1. It is not necessary to allow reagents to warm to room temperature before use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep zip-lock pouch sealed when not in use to maintain a dry environment.
3. Always use new pipette tips to pipette buffer, enzyme conjugate, standards and sample.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well, or any of the reagents already in the well. This can result in cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run samples alongside a standard curve. If testing a sample that is not extracted, standards should be diluted in the same type of medium being tested. This medium should be known to be negative.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. Before opening lyophilized conjugate vial, examine the vial to ensure that lyophilized materials have not been trapped in the cap. If material is in the cap, gently tap the upright vial to dislodge the trapped material.

PROCEDURAL NOTES (CON'T)

10. To reconstitute the lyophilized conjugate, pipette 75µL of deionized water into vial. Rehydrate the conjugate by gently rotating the vial. Do not vortex or shake the contents. Avoid excess foaming. After the solid material has gone into solution, allow the conjugate to incubate at room temperature for 45 minutes before dilution. Write the date of reconstitution on the label. Concentrated,reconstituted conjugate has a shelf life of 3 weeks when stored at 4°C.
11. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
12. Before taking an absorbance reading, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.

SAMPLE PREPARATION

This assay is non-species specific. Usually, urine and tissue culture supernatant can be assayed directly by diluting them with 10mM PBS buffer, pH 7.5. Plasma and most other mediums will need to be extracted.

EXTRACTION OF CYCLIC AMP FROM TISSUE OR CELLS

1. Tissue (100-150 mg) or cells (1-10x10^6 cells) are homogenized in 1 mL of 0.4N $HClO_4$ and then centrifuged at 20,000 xg for 10 minutes.
2. The supernatant is neutralized with 1N KOH to pH 7.0-7.5 and then applied to a Dowex 1-x2 column (0.5x4cm, Chloride form).
3. The column is washed with 5 mL of H_2O.
4. Cyclic AMP is eluted with 3 mL of 0.5N HCl.
5. This fraction is lyophilized and then dissolved in a suitable volume of standard buffer (10mM PBS buffer, pH 7.5).
6. An aliquot of each fraction is acetylated and assayed according to the following procedure.

ACETYLATION OF STANDARDS AND SAMPLES

Note: To increase the sensitivity of the assay, samples and standards must be acetylated following the procedure listed below. The samples and standards should be acetylated and assayed at the same time.

1. Use 10mM PBS, pH 7.5, to dilute all standards.
   (See Scheme I)
2. After making the standards, place clean test tubes in front of each standard and sample. Label them accordingly.
3. Now make a 2:5 Acetic Anhydride (AA), Triethylamine (TEA) mixture. Both are light sensitive. Pipette 750 µL of TEA into a small test tube. Then take 300 µL of Acetic Anhydride and pipette it in the same test tube. **VORTEX IMMEDIATELY!!** TEA must be placed in the test tube first. Vortex for 10 seconds.
4. Take the AA/TEA mixture and pipette 10 µL into all empty test tubes for standards and samples.
5. Starting with the "0" standard (10mM PBS), pipette 300 µL into the first test tube with 10 µL of AA/TEA mixture. **VORTEX IMMEDIATELY!!** Vortex for 5 seconds.
6. Repeat this procedure for all 8 standards for each sample.
7. Standards and samples are ready to be assayed immediately following acetylation. Vortex each sample and standard again for 3 - 5 seconds just before dispensing into wells.

TEST PROCEDURES

1. Prepare standards as follows:

Standard	*Preparation*
A	stock solution 1 µg/mL (Provided in green capped vial.)
B	take 20 µL of A, add to 980 µL of standard buffer and mix=20 ng/mL
C	take 200 µL of B, add to 1.8 mL of standard buffer and mix=2 ng/mL
D	take 200 µL of C, add to 1.8 mL of standard buffer and mix=0.2 ng/mL

NOTE: Standard Buffer is 10mM PBS buffer, pH 7.5

Continue standard preparation following Scheme I.

SCHEME I

Standards	*ng/mL*	*Standard buffer (µL added)*	*B standard µL*	*C standard µL*	*D standard µL*
S_0	0.00	as is	-	-	-
S_1	0.02	900	-	-	100
S_2	0.05	750	-	-	250
S_3	0.1	500	-	-	500
S_4	0.2	-	-	-	as is
S_5	0.4	800	-	200	-
S_6	1	500	-	500	-
S_7	2	-	-	as is	-

2. Determine the number of wells to be used.
3. Dilute the reconstitued Cyclic AMP enzyme conjugate. Add 1µL of enzyme conjugate into 50 µL total volume of EIA buffer for each well assayed. Mix the solution thoroughly (avoid foaming). For the entire plate, add 110 µL of the Cyclic GMP enzyme conjugate into 5.5 mL total volume of EIA Buffer. Mix the solution thoroughly (avoid foaming).

   NOTE: If more concentrated conjugate is needed than is contained in the first conj gate vial, do not use the separate contents of both vials in the same assay. Some assay variability may result. If the contents of both vials are required for an assay, pool the concentrated conjugates. Use the expiration date of the oldest reconstituted vial for the pool. Alternatively, prepare the necessary volumes of diluted conjugate and pool before using in the assay.
4. Add 50 µL of standard (S) or unknown (U) (some samples may require diluting) to the appropriate wells.

See Scheme II for suggested template design.

5. Add 50 µL of the diluted enzyme conjugate to each well. Use 8-channel pipette or 12-channel pipette for rapid addition.
6. Mix by shaking plate gently. A microplate shaker may be used.
7. Cover plate with plastic film or plate cover and incubate at room temperature for one hour.

   NOTE: Keep plate away from drafts and temperature fluctuations.

TEST PROCEDURES CON'T

8. Dilute concentrated wash buffer with deionized water (i.e. 20 mL of wash buffer plus 180 mL of deionized water). Mix thoroughly.
9. After incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
10. Wash each well with 300 µL of the diluted wash buffer. Repeat for a total of three washings. An automated plate washer can be used, however, increase wash cycles from three to five.
11. Add 150 µL of substrate to each well. Use multichannel pipette for best results. Mix by shaking plate gently.
12. Incubate at room temperature for 30 minutes.
13. Gently shake plate before taking a reading to ensure uniform color throughout each well.
14. Plate is read in a microplate reader at 650 nm. If a dual wavelength is used, set W_1 at 650 nm and W_2 at 490 nm.
15. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

NOTE: Some microplate readers can be programmed to do these subtractions automati cally when reading the plate. Consult your instrument manual.

OPTIONAL TEST PROCEDURES

16. Add 50-100 µL of 1 N HCl or Neogen's Red Stop Solution to each well to stop enzyme reaction.
17. Read plate at 450 nm, if 1 N HCl solution was used. Read plate at 650 nm, if Neogen's Red Stop Solution was used.
18. Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

NOTE: Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation approximately 10 minutes but no more than 15 minutes.

SCHEME II

	1	2	3	4	5	6	7	8	9	10	11	12
A	$^{S}0$	$^{S}0$	$^{U}1$	$^{U}1$	$^{U}9$	$^{U}9$	$^{U}17$	$^{U}17$	$^{U}25$	$^{U}25$	$^{U}33$	$^{U}33$
B	$^{S}1$	$^{S}1$	$^{U}2$	$^{U}2$	$^{U}10$	$^{U}10$	$^{U}18$	$^{U}18$	$^{U}26$	$^{U}26$	$^{U}34$	$^{U}34$
C	$^{S}2$	$^{S}2$	$^{U}3$	$^{U}3$	$^{U}11$	$^{U}11$	$^{U}19$	$^{U}19$	$^{U}27$	$^{U}27$	$^{U}35$	$^{U}35$
D	$^{S}3$	$^{S}3$	$^{U}4$	$^{U}4$	$^{U}12$	$^{U}12$	$^{U}20$	$^{U}20$	$^{U}28$	$^{U}28$	$^{U}36$	$^{U}36$
E	$^{S}4$	$^{S}4$	$^{U}5$	$^{U}5$	$^{U}13$	$^{U}13$	$^{U}21$	$^{U}21$	$^{U}29$	$^{U}29$	$^{U}37$	$^{U}37$
F	$^{S}5$	$^{S}5$	$^{U}6$	$^{U}6$	$^{U}14$	$^{U}14$	$^{U}22$	$^{U}22$	$^{U}30$	$^{U}30$	$^{U}38$	$^{U}38$
G	$^{S}6$	$^{S}6$	$^{U}7$	$^{U}7$	$^{U}15$	$^{U}15$	$^{U}23$	$^{U}23$	$^{U}31$	$^{U}31$	$^{U}39$	$^{U}39$
H	$^{S}7$	$^{S}7$	$^{U}8$	$^{U}8$	$^{U}16$	$^{U}16$	$^{U}24$	$^{U}24$	$^{U}32$	$^{U}32$	$^{U}40$	$^{U}40$

CALCULATIONS

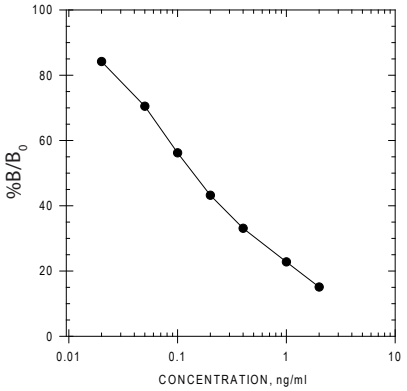
1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S_0 values is now your B_0 value. (S_1 now becomes B_1, etc.)
3. Next, find the percent of maximal binding (%B/B_0 value).To do this, divide the averages of each standard absorbance value (now known as B_1 through B_7) by the B_0 absorbance value and multiply by 100 to achieve percentages.
4. Graph your standard curve by plotting the %B/B_0 for each standard concentration on the ordinate (y) axis against concentration on the abscissa (x) axis. Draw a curve by using a curve-fitting routine

(i.e. 4-parameter or linear regression).

5. Divide the averages of each sample absorbance value by the B_0 value and multiply by 100 to achieve percentages.
6. Using the standard curve, the concentration of each sample can be determined by comparing the $\%B/B_0$ of each sample to the corresponding concentration of Cyclic AMP standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE

Cyclic AMP in 10 mM PBS Buffer



TYPICAL DATA

NOTE: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the $\%B/B_0$ should remain comparable. (Measurable wavelength: 650nm.)

Standard	*Standard Concentration (ng/mL)*	*Optical Density (Absorbance Value)*	*$\%B/B_0$*
S_0 (B_0)	0.00	1.005	100
S_1 (B_1)	0.02	0.849	85
S_2 (B_2)	0.05	0.674	67
S_3 (B_3)	0.1	0.497	49
S_4 (B_4)	0.2	0.371	37
S_5 (B_5)	0.4	0.275	27
S_6 (B_6)	1.0	0.194	19
S_7 (B_7)	2.0	0.146	14

NOTE: Standards were acetylated before testing.

CROSS REACTIVITY

Cyclic AMP	100.0%
Cyclic GMP	0.07%
Adenosine	<0.01%
Adenine	<0.01%
AMP	<0.01%
ADP	<0.01%
ATP	<0.01%
Guanine	<0.01%
GMP	<0.01%
GTP	<0.01%

NOTE: Cross reactants were acetylated.

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TECHNICAL ASSISTANCE

Technical assistance is available Monday-Friday, between 8:00 a.m. and 6:00 p.m. EST.

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