Cyclic AMP Competitive EIA Kit

Cat. No. 99-0050
Size: 2x 96 Tests
For Research Use Only
Lot No.

The Cyclic AMP EIA Kit is a competitive immunoassay for quantitative detection of human acetylated and non-acetylated Cyclic AMP in cell culture extract, supernatant, tissue extract, and urine. For Research Use Only.

INTRODUCTION

Cyclic AMP mediates the cellular action of numerous hormones in virtually all animals, plants and bacteria. Generally, this interaction occurs at the plasma membrane where the hormone binds the cell and activates the cAMP-generating enzyme, adenyl cyclase. The increase in intracellular cAMP modifies a variety of biological responses.

REAGENTS AND MATERIALS PROVIDED

2 x 96 well precoated plates
1vial Sodium Acetate Concentrate
2 vials Cyclic AMP-Alkaline Phosphatase Conjugate
1 vial Cyclic AMP Standard (5000 picomoles)
1 vial Cyclic AMP Antiserum (12 ml)
1 vial Acetic Anhydride* (1 ml)
1 vial Triethylamine** (2 ml)
1 vial p-Nitrophenyl Phosphate (PNPP)
1 vial Diethanolamine Buffer
1 vial 5x Wash Buffer Concentrate (100 ml)

*Corrosive liquid, lacrymator, combustible. Do not inhale or ingest. Use in a fume hood.
** Flammable, toxic. Do not breathe vapors

MATERIALS REQUIRED BUT NOT PROVIDED

5 ml and 10 ml graduated pipettes, beakers, flasks, and cylinders
10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
50 µl to 300 µl adjustable multichannel micropipette, disposable tips, and reservoir
Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

STORAGE

Store at 2° - 8°C except Cyclic AMP-Alkaline Phosphatase Conjugate, which needs to be stored at -20°C.

PRINCILPLE OF TEST

Cyclic AMP (cAMP) EIA system provides a simple, sensitive method for measuring cAMP in biological samples at a range of 0.05 to 100 pmoles/ml. The assay method is based on the competitive binding by cAMP and an alkaline phosphate derivative of cAMP for a limited amount of specific antibody. The amount of enzyme-labeled cAMP bound to antibody decreases with increasing concentration of cAMP. Separation of bound cAMP from free cAMP is achieved by a second antibody bound to the sides of 96-well (8 well strips) plates.

ASSAY RANGE

Non-Acetylated - 0.5 pmole/ml – 100 pmole/ml
Acetylated - 0.05 pmole/ml – 10 pmole/ml

PREPARATION OF REAGENTS

1. **Sodium Acetate Concentrate**: Dilute contents to 500ml with distilled or deionized water (stable at least 2 months at 4ºC).
2. **Cyclic AMP-Alkaline Phosphatase Conjugate**: For one plate reconstitute one vial with acetate buffer. For the whole kit reconstitute both vials and combine them. Reconstitute with volume given on the label. The solution is stable for 2 months at -20ºC.
3. **Cyclic AMP Standard**: Contains 5000 pmoles, lyophilized. Add exactly 5.0ml sodium acetate buffer. Mix thoroughly. Stable for 2 months at 4ºC.
4. **5x Wash Buffer Concentrate**. Contains 100ml. Dilute contents to 500ml with deionized water.

SAMPLE PREPARATION

Several techniques are available for the extraction and/or partial purification from various tissues [5], fluids such as plasma[3], cell extracts[4], and tissue culture medium[6]. Since endogenous phosphodiesterases are likely contaminants of most biological samples, a denaturing, deproteinizing treatment is commonly employed. These include treatment with trichloroacetic acid[7], ethanol[8], perchloric acid[9], followed by some chromatography such as ion-exchange or alumina. Samples should not contain metal chelators, e.g., EDTA or EGTA.

A. Tissue Culture Extracts or Media (Acetylate for all formats)

1. Intracellular cAMP
   a. Monolayers: Wash the plates 3 times with physiological saline (Ca^{++}, Mg^{++} free). Obtain a cell count. Flood the cells with 5% trichloroacetic acid. After 5 minutes, remove supernatant, wash once with water. Combine TCA supernatant with water wash and extract with ether as in C. Lyophilize if necessary.
   b. Suspensions: Wash the cells with PBS by centrifugation in polypropylene tubes, add 0.05M HCl, and place the tubes in boiling water for 3 minutes. Cool and lyophilize. Reconstitute with sodium acetate buffer, filter or centrifuge to clarify.
2. Extracellular cAMP: Tissue culture media needs to be diluted at least 5 fold with sodium acetate buffer. Store at -20ºC or lower.

B. Urine

Store at -20ºC or lower. Dilute urine 100 fold with acetate buffer just prior to assay. Use 100ul/tube. Use non-acetylated protocol.

C. Tissue Extraction[10]

Homogenize 1 part (by weight) with 9 parts 5-10% trichloroacetic acid with an instrument such as a Polytron (Brinkman Instruments). Clarify by centrifugation and extract the supernatant with five volumes of water saturated ether in a screw cap centrifuge tube (centrifugation may be used to separate the layers if necessary). Remove the ether layer and repeat extraction of aqueous layer two times. Remove residual ether from the aqueous layer. The sample may be heated up to approximately 50°C.

D. Plasma Acetylation[11]

Heparinized or citrated plasma is brought to 0.5mM IBMX, cooled in an ice bath and used for assay immediately or stored at -70°C.

Procedure:

1. Add 950ul acetate buffer to 50ul of specimen.
2. Use 210ul and continue with acetylation protocol.

PROCEDURE: For non-Acetylated Samples

1. Allow solutions to equilibrate to room temperature prior to setting up the assay.
2. Remove strips not used from the frame, store dry at 4ºC. Use 16 wells for blank, maximum binding (Bmax) and 6 standards in duplicate, and at least 2 wells for each unknown.
3. Add 150ul buffer to the blanks, 100ul buffer to the Bmax and 100ul of each standard and unknown to appropriate wells.
4. Add 50ul of diluted cAMP-Alkaline Phosphatase tracer to all wells and 50ul cAMP antiserum to all wells, except the blanks. Incubate 3 ½ hours at room temperature or 18-24 hours at 4ºC.
5. Aspirate all wells and fill with diluted Wash Buffer, aspirate completely. Repeat for a total of 5 washes (See NOTE).
6. Tap the wells gently on paper towels to remove remaining liquid. Add 200ul PNPP, 4mg/ml in Diethanolamine Buffer to all wells (cover tightly with new sealing tape). Incubate approx. 2 hr. at 37ºC. Remove sealing tape, measure absorbance at 405-410nm. However, data may be taken at any time. (Do not allow absorbance of the Bmax to exceed 1.5). Extend incubation, if necessary.

NOTE : Samples that could contain large quantities of endogenous alkaline phosphatase should be evaluated for possible interference. It may be necessary to do additional washing and/or deproteinize the sample. A “sample blank” (i.e., no specific antibody) should be done on such samples.

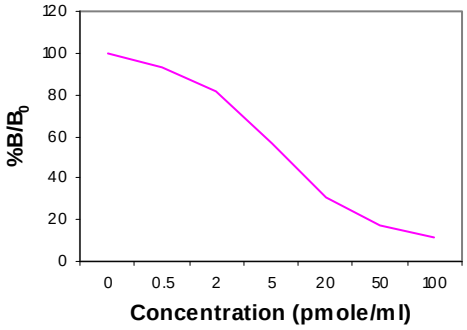
PROCEDURE: For Acetylated Samples

1. Prepare Bmax, standards (above) and unknowns as previously described. Transfer 420ul acetate buffer (blank and Bmax) and 210ul of each standard and unknown to labeled 12x75mm glass tubes.
2. Prepare a fresh solution of 1 part acetic anhydride, 2 parts triethylamine immediately preceding addition. Add 10ul of this solution to all tubes and 20ul to the blank and Bmax tube. The acetylating reagent must be added directly into test solution and immediately vortex mixed. **Note:** The reagent is very unstable. Mix only enough to last 3-5 minutes (approximately 50-60 tubes).
3. Let stand 30 for mins. Transfer 100ul (in duplicate) of each solution to a well, including blank and Bmax. Add 50ul acetate buffer to blank wells.
4. Proceed with Non-Acetylated Protocol starting at Step 3.
   Incubation with substrate should be 2 hours at 37ºC.

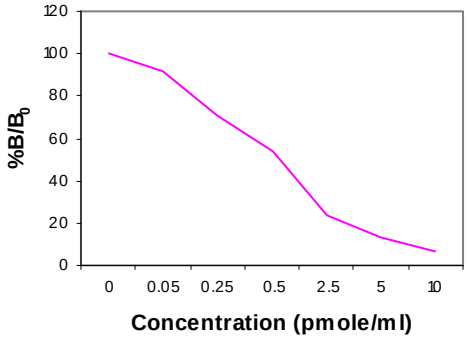
CALCULATION OF RESULTS

1. Determine mean values for each set of points.
2. Obtain mean net absorbance by subtracting the averaged nonspecific binding (blank) from the means of all standards and unknowns.
3. Calculate relative bindings (%B/Bo). Divide net mean absorbance of each standard and unknown by net mean absorbance of the zero standard (0 pmole/ml) multiply by 100.
4. Construct a standard curve by plotting % B/Bo (from step 3) versus pmole cAMP/ml. Four cycle semi-logarithmic paper (smooth curve) or four cyclic logarithmic-logistic paper (straight line) may be used.
5. Determine pmoles/ml for each unknown.

Standard Curve for Acetylated cAMP



Standard Curve for Non-Acetylated cAMP



PERFORMANCE CHARACTERISTICS

1. Specificity

Acetylated	pmole/ml @ 50% Bmax	Non-Acetylated	pmole/ml @ 50% Bmax
cAMP	1.2	cAMP	15
cGMP	11,000	cGMP	1×10^5
AMP	3.6×10^5	AMP	3×10^6
ATP	4.4×10^5	ADP	9×10^5
ADP	1.6×10^6	ATP	8×10^5

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