Immuno Biological Laboratories,Inc.
8201Central Avenue, NE, Suite P Minneapolis, MN 55432 www.IBL-America.com (T) 1-763-780-2955 (F)1-763-780-2988

INSTRUCTION MANUAL
CYCLIC AMP RIA KIT

Catalog No. IB78169

Description

This cyclic AMP (cAMP) radioimmunoassay system provides a simple, sensitive method for measuring cAMP in biological samples at a range of 1 to 200 pmol/ml, (0.005-20 pmol/tube). The method is based on the competitive binding of cAMP in the sample and a radioiodinated derivative of cAMP [I^{125}] S-cAMP, for a highly specific antibody. The amount of labeled cAMP found in the complex decreases with increasing concentration of unlabeled cAMP in the sample. Separation of antibody bound cAMP from free cAMP is achieved through a precipitating antibody incorporated in the reagent system. Determination of the unknown is made by the comparison with a standard curve constructed in the same fashion.

Sufficient reagents are included to do 200 tubes including standards.

Introduction

Cyclic AMP was discovered by Sutherland and others in the 1950's[1,2,3]. It has since been well established that cAMP 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 resulting increases in intracellular cAMP modifies a variety of biological responses.

Summary of Test

This cyclic AMP Radioimmunoassay Kit employs a preconjugated double antibody separation system in an acetate buffer. Components of the system have been combined to minimize pipeting steps (3) and incubation (1). Standards and unknowns are combined with tracer solution and antibody. Solutions are incubated 18-20 hours at 4°C. One ml of acetate buffer is added, the tubes centrifuged and the visible pellets separated from supernatant. Radioactivity in the precipitate is counted and unknowns are determined from a standard curve.

References

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Precautions

Radioactive Materials - Use, Handling, Storage and Disposal of Wastes.

This radioactive material may be received, possessed and used only by research laboratories for in vitro laboratory tests not involving external or internal administration to humans or animals. Its receipt, acquisition, possession, use and transfer are subject to regulation and a general license of the U.S. Nuclear Regulatory Commission or of a state with which the Commission has entered into an agreement for the exercise of a regulatory authority.

Safety Precautions

1. Do not drink or smoke in areas where radioactive materials are used.
2. Do not pipet radioactive solutions by mouth.
3. Wash hands following use of radioactive materials.
4. Radioactive materials should be used only in designated areas.
5. All radioactive materials should be disposed of according to regulations of the agencies with jurisdiction over the laboratory.
6. Spills should be handled in accordance with established procedures.
7. Store radioactive compounds in clearly marked containers in predesignated areas.

Preparation of Samples

Several techniques are available for the extraction and/or partial purification from various tissues, fluids such as plasma[4], cell extracts[5], and tissue culture medium[6][7]. Since endogenous phosphodiesterases are likely contaminants of most biological samples, a denaturing, deproteinizing treatment is commonly employed. These include treatment with trichloroacetic acid[8], ethanol, [9] perchloric acid[10], followed by some chromatography such as ion-exchange or alumina.

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. The combined supernatants are extracted 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 3 minutes. Cool and lyophilize. Reconstitute with acetate buffer, filter or centrifuge to clarify.

2. Extracellular cAMP. Tissue culture media needs to be diluted at least 5 fold with acetate buffer.

B. Urine

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

C. Tissue Extraction[11]

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.

[H^3] cAMP, **Demeditec Cat. No. DEBT339**, should be used to determine recoveries. Be sure to account for the added mass of cAMP if this is done.

D. Plasma Acetylation[11]

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

Procedure:

1. Add 950 µl acetate buffer to 50ul of specimen.
2. Use 100 µl per tube. Continue with acetylation protocol.

Reagents: Description & Preparation

1. Tracer Diluent. **Catalog No. DEBT332**. One vial. Contains sodium acetate buffer (pH 6.2), normal rabbit IgG, phosphodiesterase inhibitors and sodium azide.
   Store at 4°C. Stable for 2 months.

2. cAMP Tracer Concentrate. **Catalog No. DEBT331R**. Two vials. Each contains approximately 1.5uCi (at calibration date) [I^{125}] succinyl cAMP-tyrosine methyl ester (sc-cAMP-TME-[I^{125}]) diluted in 50% n-propanol-water. Add 10.5ml of Tracer Diluent, stopper, mix by inversion. Store at 4°C. Stable 2 months from production date.

3. Sodium Acetate Concentrate. **Catalog No. DEBT333**. One vial. Dilute contents of vial to 500ml with distilled or deionized water for working buffer. Final solution 0.05M sodium acetate (pH 6.2). Store at 4°C. Stable for at least 4 weeks.

4. Cyclic AMP Standard. **Catalog No. DEBT334**. One vial. Contains 5000 picomoles, lyophilized. Add 5ml of working buffer with a volumetric pipet, stopper and mix thoroughly.
   Stable 4 weeks at 4°C.

5. Cyclic AMP Antiserum. **Catalog No. DEBT330P.** Two vials containing specific cAMP antiserum for 100 tubes. Also contains goat anti-rabbit IgG. Store at 4°C. Stable for 6 months.

   Note: Cyclic AMP Antiserum is in the form of a fine suspension, which may settle on prolonged standing.
   Always mix the solution, by inversion several times, prior to addition of the antiserum. Mixing is recommended after every 50 additions.

6. Nonspecific Binding Reagent. **Catalog No. DEBT335**. One vial. Contains 1ml, sufficient for 5 standard curves. Prepared as cAMP antiserum, but without specific antiserum.
   Stable 2 months at 4°C.

7. Acetic Anhydride. **Catalog No. DEBT336**. One vial. Contains 1ml. Moisture sensitive. Do not open cool vials or use wet pipets.
   CAUTION: Corrosive liquid - Lacrymator Combustible Liquid.

8. Triethylamine. **Catalog No. DEBT337**. One vial. Contains 2 ml. Must be kept dry. Do not open cool vials. **Caution:** Flammable. Toxic, do not breath vapors.

Specificity: Expressed as pmoles cAMP at 50% Bmax/pmoles test ligand at 50% Bmax.

c-AMP	1
AMP	550,000
ATP	200,000
c-GMP	31,000
ADP	100,000

Protocol for Non-Acetylated Samples

Working cAMP Standards should be made up from the stock solution just prior to the assay. Prepare new standards each day.

Sample protocol for working standards. (Vortex after each addition).

	pmol/ml	pmol/tube
A. 0.2 ml stock standard + 0.8 ml working buffer	200	20
B. 0.1 ml stock standard + 0.9 ml working buffer	100	10
C. 0.1 ml solution A + 0.9 ml working buffer	20	2
D. 0.1 ml solution B + 1.9 ml working buffer	5	0.5
E. 0.1 ml solution C + 0.9 ml working buffer	2	0.2
F. 0.1 ml solution C + 1.9 ml working buffer	1	0.1

Procedure: Allow solutions, except for acetate buffer, to warm to room temperature prior to setting up the assay.

1. Number 18 12x75 mm glass borosilicate tubes for the standard curve plus at least 2 tubes for each unknown or control.

2. Add 100 µl working buffer to tubes 3-6. These are the nonspecific binding (3,4) and zero standard or Bo (5,6) tubes. Tubes 1,2 are for total counts.

3. Add 100 µl working standards, starting with the lowest concentration, in duplicate, to tubes 7-18, as indicated in the summary on page 6.

4. Add 100 µl unknown samples or controls in duplicate to appropriate numbered tubes. If a volume of sample less than 100ul is used, add working buffer to make up the difference. See sample preparation.

5. Add 100 µl working tracer solution to all tubes.

6. Mix the working antibody solution by gentle inversion 2 or 3 times. (**Note:** Always mix the antibody immediately before pipeting). Pipet 100ul to tubes 5-18 and all additional sample tubes.

7. Mix NSB reagent by gentle inversion and add 100 µl to tubes 3 and 4.

8. Mix by gently shaking the test tube rack 30-60 seconds. ***DO NOT VORTEX*** Cover and incubate at 4°C, 18-20 hours.

9. Put aside tubes 1,2 for counting. Add 1ml of working buffer to the remaining tubes. Vortex. Centrifuge at 2000xg for 20 min. at 4°C. (Fixed angle rotors are not recommended).

10. Separate supernatants from pellets by aspiration or decantation. If the latter technique is used, after decanting the supernatant to a waste container, place the inverted tubes in a test tube rack with a paper towel 1-2 minutes. Blot remaining liquid from the rim and set upright.

11. Count ALL tubes for at least one minute in a gamma spectrometer.

Summary of Non-Acetylated Protocol

Tube No.	Description	Tracer	Buffer	Antibody	NSB Reagent	Unknowns & Standards
1,2	Total Counts	100 µl				
3,4	NSB tubes	100 µl	100 µl		100 µl	
5,6	Bo-zero	100 µl	100 µl	100 µl		
7,8	0.1pmol	100 µl		100 µl		100 µl
9,10	0.2pmol	100 µl		100 µl		100 µl
11,12	0.5pmol	100 µl		100 µl		100 µl
13,14	2pmol	100 µl		100 µl		100 µl
15,16	10pmol	100 µl		100 µl		100 µl
17,18	20pmol	100 µl		100 µl		100 µl
19,20 etc.	Unknowns	100 µl		100 µl		100 µl

Protocol for Acetylated Samples

Prepare working standards in the range of 0.01 to 5 pmol/ml. Start by making a 100pmol/ml solution. Prepare 5 standards as shown below. Additional standards may be added if desired.

A. 100 µl of 100pmoles/ml solution	+ 1.9 ml working buffer	5 pmol/ml
B. 400 µl solution A	+ 0.6 ml working buffer	2 pmol/ml
C. 100 µl solution A	+ 0.9 ml working buffer	0.5 pmol/ml
D. 100 µl solution B	+ 1.9 ml working buffer	0.1 pmol/ml
E. 100 µl solution C	+ 0.9 ml working buffer	0.05 pmol/ml

Prepare fresh solutions each day.

Procedure:

1. Set up total counts, nonspecific binding tubes, zero standard, standards and unknowns as described for steps 1-4 on Non-Acetylated section, using the above standards.

2. Prepare a fresh solution of 1 part acetic anhydride, 2 parts triethylamine immediately preceding addition. Add 5ul of this solution to all tubes except 1 and 2. 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. Proceed with steps of the Non-Acetylated section, starting at Step Number 5.

Calculations:

1. Determine mean values for each set of points.

2. Obtain mean net counts by subtracting the averaged nonspecific binding (tubes 3,4) from the means of all standards and unknowns.

3. Calculate relative bindings (%Bo). Divide net mean counts of each standard and unknown by net mean counts of the zero standard (tube 5,6), multiply by 100.

4. Construct a standard curve by plotting %Bo (from step 3) versus pmol cAMP/tube. Four cycle semi-logarithmic paper (smooth curve) or four cycle logarithmic-logistic paper (straight line) may be used.

5. Determine pmol/tube for each unknown. Multiply values by 10 and by dilution factor if appropriate to obtain pmol cAMP/ml.

Example Data

UNACETYLATED

Tube No.	Descrip.	cpm	Avg. Net cpm	B/Bo x100
1	TC	25336		
2	TC	26435	25480	
3	NSB	392		
4	NSB	421	406	
5	Bo	15344		
6	Bo	15212	14872	100
7	0.05pmol	14280		
8	0.05pmol	12847	13454	91.0
9	0.2 "	11923		
10	0.2 "	12350	11735	79.4
11	0.5 "	9675		
12	0.5 "	9627	9245	62.5
13	2 "	6375		
14	2 "	6122	5842	39.5
15	5 "	3515		
16	5 "	3249	2976	20.1
17	20 "	1678		
18	20 "	1514	1190	8.0

ACETYLATED

Tube No.	Descrip.	cpm	Avg. Net cpm	B/Bo x100
1	TC	26457		
2	TC	25798	26128	
3	NSB	435		
4	NSB	500	468	
5	Bo	13245		
6	Bo	12775	12542	100
7	0.005pmol	12521		
8	0.005pmol	11796	11691	93.2
9	0.02 "	10427		
10	0.02 "	10146	9819	78.2
11	0.05 "	7672		
12	0.05 "	7582	7220	57.6
13	0.2 "	4173		
14	0.2 "	4646	4002	31.9
15	0.5 "	1926		
16	0.5 "	2154	1572	12.5

DO NOT USE ABOVE FOR ACTUAL DETERMINATIONS