PerkinElmer Life and Analytical Sciences, Inc.

cAMP [^{125}I]
RADIOIMMUNOASSAY KIT
(ADENOSINE 3', 5'
CYCLIC MONOPHOSPHATE)
CATALOG NUMBER NEK033

For Laboratory Use
CAUTION: A research chemical for research purposes only.

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I. PROPRIETARY NAME

cAMP [^{125}I] RIA Kit
PerkinElmer Life Sciences, Inc. Catalog Number: NEK033, 200 Tubes

II. INTENDED USE

This kit is designed to measure cAMP levels in plasma, urine and tissues.

III. EXPLANATION OF TEST

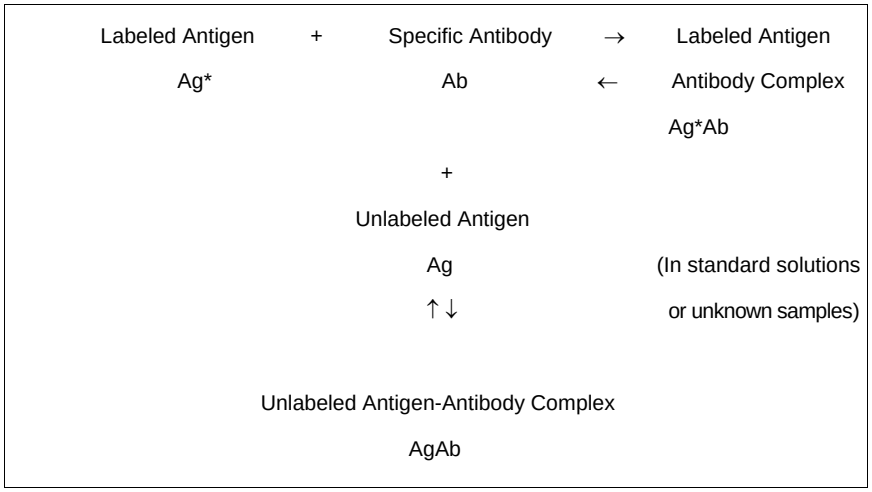
Adenosine 3', 5' cyclic monophosphate (cAMP) plays a critical role in the transmission of hormonal signals by functioning as a "second messenger"[1,2]. The binding of a hormone to its receptor can either enhance or inhibit the rate at which cAMP is produced. This is accomplished by altering the enzymatic activity of adenyl cyclase, the membrane associated enzyme which catalyzes the production of cAMP from ATP[3]. By this mechanism, intracellular levels of cAMP are altered in response to hormonal stimulation. In turn, the intracellular level of cAMP regulates the enzymatic activity of a protein kinase which phosphorylates other substances setting off a cascade of cellular events which leads to the expression of the hormone[4].

The cAMP Assay is a double-antibody RIA which utilizes a prereacted antibody complex. The assay is performed using acetylated or non-acetylated standards and samples. Acetylation of the sample allows for a more sensitive assay of cAMP[5,6]. The assay is accurate over a wide range of values and has a high degree of specificity.

IV. PRINCIPLE OF THE METHOD

The basic principle of radioimmunoassay is the competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. This interaction is illustrated in Figure 1[7].

Figure 1



When unlabeled antigen from standards or samples and a fixed amount of the labeled antigen are allowed to react with a constant and limiting amount of antibody, decreasing amounts of the labeled antigen are bound to the antibody as the amount of unlabeled antigen is increased. In the cAMP [^{125}I] Kit, separation of the bound from free antigen is achieved by using a prereacted primary antibody/secondary antibody complex. The second antibody is derived from an animal species different from that used to generate the primary antibody.

After incubation and centrifugation, the supernatant is discarded and the antigen-antibody complex is counted to quantitate the bound tracer. The data are used to construct a standard curve from which the values of the unknowns may be obtained by interpolation.

V. REAGENTS

All necessary reagents are supplied and are intended FOR LABORATORY USE. Kits are shipped at ambient temperatures and must be stored upon receipt at refrigerator temperature (2-8°C). The reagents are stable for the times indicated if the specific precautions given below are followed. Sodium azide has been added as an antibacterial agent where appropriate.

NOTE: The National Institute for Occupational Safety and Health has issued a bulletin citing the potentially explosive hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide is added at a minimal concentration, it is still recommended that copious amounts of water be used to flush the drain pipeline after disposal of these reagents in the plumbing system. Copper-free and lead-free discharge lines should be used whenever possible. Decontamination procedures should be followed prior to maintenance on drain lines which have been used for azide-containing reagents. Recommended decontamination procedures are available from RIA Technical Services.

NOTE: An excess of each reagent beyond the labeled content is added to each container in order to allow the withdrawal of the number of aliquots required to fill the stated quantity of tubes. Any reagent remaining after the stated quantity of tubes have been prepared should be discarded.

A. cAMP Sodium Acetate Buffer

One vial of concentrated buffer is supplied. Dilute to 500 mL with distilled water. The final solution will contain sodium acetate buffer, pH 6.2, and a stabilizer. The diluted buffer is stable for at least two months when stored at 2-8°C. Refer to vial label for exact expiration date of the concentrated reagent.

B. cAMP Standard

One vial of liquid standard is supplied. The solution contains: cAMP at a concentration of 50,000 pmol/mL, sodium acetate buffer, and 0.09% sodium azide. The cAMP Standard has been calibrated spectrophotometrically using the molar absorption coefficient, Œ = 14.6 x 10^3l mol^{-1} cm^{-1} at 259 nm, pH 6.9. The standard is stable for at least one year when stored at 2-8°C. Refer to vial label for expiration date of reagent.

C. cAMP Antiserum Complex

One vial of lyophilized, prereacted, first and second antibody is supplied. Reconstitute with 21 mL of distilled water. The resulting solution will contain the prereacted antibody complex, and an inert ingredient, in sodium phosphate buffer, pH 6.0. This solution may appear cloudy and will settle upon standing. Before use and during prolonged use, mix thoroughly.

The reconstituted antiserum complex is stable for at least two months when stored at 2-8°C. Refer to vial label for expiration date of lyophilized reagent.

D. cAMP [^{125}I] Tracer (Succinyl cAMP Tyrosine Methyl Ester [^{125}I])

Two vials of concentrated tracer are supplied. Each vial contains less than 74KBq (2 µCi) on calibration date in 1 mL of a 1:1 n-propanol water solution. Use one vial at a time as directed. Add 5.0 mL of distilled water to each vial as required. The concentrate and diluted tracer are stable for at least two months when stored at 2-8°C. This material is radioactive and the user should follow the precautions listed on the following page.

INSTRUCTIONS RELATING TO THE HANDLING, USE, STORAGE, AND DISPOSAL OF THIS RADIOACTIVE MATERIAL

This radioactive material may be received, acquired, possessed, and used only by research laboratories for in vitro laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations 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 regulatory authority.

1. All radioactive materials must be labeled and secured in specifically designated posted areas. Records of receipt and survey must be maintained.

2. All work with these materials must be carried out only in authorized areas.

3. Prohibit mouth pipetting of radioactive materials.

4. There must be no smoking or eating within the work area.

5. Hands must be washed after handling radioactive materials.

6. Any spilled material must be wiped up quickly and thoroughly and the contaminated substances transferred to a suitable receptacle. The surfaces involved must be washed thoroughly with an appropriate decontaminant. Monitor to ensure the area has been effectively decontaminated.

7. When use of the Tracer reagent has been completed, empty and decontaminate the vial. This radioactive material can be discarded into the sanitary sewerage system using copious amounts of water to ensure a minimal discharge concentration.

8. Prior to disposal of the empty, uncontaminated Kit and Tracer containers to unrestricted areas, remove or deface the radioactive material labels or otherwise clearly indicate that the containers no longer contain radioactive material.

E. cAMP Carrier Serum

Two vials of lyophilized carrier serum are supplied. Use one vial at a time. Reconstitute the contents of each vial with exactly 6.0 mL of distilled water. (One vial of reconstituted carrier serum is sufficient for 100 tubes.) The resulting solution will contain carrier serum, 0.1% sodium azide, a stabilizer, and an inert ingredient in sodium acetate buffer, pH 6.2. Refer to vial label for expiration date of lyophilized reagent. Store at 2-8°C.

F. cAMP Acetic Anhydride

One vial containing 1 mL is supplied. CAUTION: FLAMMABLE, CORROSIVE, LACHRYMATOR. Store tightly closed in the refrigerator. Allow vial to equilibrate to room temperature before use. Protect from moisture. This material is stable for at least two months under these conditions. Refer to vial label for expiration date.

G. cAMP Triethylamine

One vial containing 1 mL is supplied. CAUTION: FLAMMABLE, VAPOR HARMFUL. Store tightly closed in the refrigerator. Allow vial to equilibrate to room temperature before use. Refer to vial label for expiration date.

H. cAMP Precipitator

One bottle containing 100 mL is supplied ready to use and contains a precipitation enhancer and sodium azide in sodium acetate buffer. Refer to bottle label for expiration date. Store at 2-8°C. Shake well before using.

VI. SAMPLE COLLECTION, PROCESSING AND STORAGE

A. Plasma: EDTA-treated plasma should be used. Collect blood by venipuncture in a 5 or 10 mL glass blood collection tube containing EDTA (lavender top). The usual precautions for venipuncture apply. Samples should be kept on ice after drawing. Separation of cells from plasma should be carried out as soon as possible after collection in a refrigerated centrifuge (15 minutes, approximately 760 x g). Plasma may be stored at -20°C for at least four weeks. Repeated freezing and thawing should be avoided.

B. Urine: Voided urine specimens are required for the assay. Urine samples should be centrifuged to remove any particulate

matter present. Random, timed, or 24 hour urine collections can be used. For twenty-four hour specimens, it may be necessary to prevent bacterial growth by collecting urine into acid (2 mL of 6 N HCl per 100 mL urine). Urine collected utilizing antibacterial agents should be validated as a suitable specimen by the operator. Urine samples can be stored undiluted at 2-8°C for 24 hours, but should be frozen at -20°C for longer storage.

C. Tissue Samples: Precipitation of proteins from plasma or tissues has been accomplished with trichloroacetic acid (TCA), perchloric acid, or ethanol followed, in some cases, by ion exchange or alumina column chromatography. The decision as to which procedure to use depends on the nature of the sample and is left up to the individual investigator.

This protocol is applicable to tissue culture cells as well as solid tissue; however, it is the responsibility of the investigator to validate this procedure for each specific application. As part of this validation, we recommend adding phosphodiesterase to some samples and noting the loss of immunoreactivity.

1. Homogenize the frozen tissue sample at 4°C with 6% TCA to make a 1 mL 10% (w/v) homogenate. Add an equal volume of cold 10% TCA to cell culture preparations or supernatants.
2. To determine the recovery of cyclic AMP during extraction, add to each sample extract approximately 4,000 cpm of ^{3}H-cyclic AMP marker (NET275 [2,8 ^{3}H] Cyclic AMP, diluted 1:200 in 50% Ethanol / 50% H_2O solution, available from PerkinElmer Life Sciences) to the TCA extract. At a specific activity of approximately 37 Ci/mmol and at a 50% counting efficiency, this represents approximately 0.1 pmol (100 fmol) of cyclic AMP. This amount must be taken into account when calculating the cyclic AMP content of the tissue.
3. Centrifuge TCA extracts at 2,500 x g at 4°C for 15 minutes.
4. Collect the supernatant and extract 4 times with 5x volume of water-saturated ether. Discard the ether phase.
5. Place sample in a water bath at 70-80°C and evaporate to dryness under a stream of air.

6. Dissolve the residue in Assay Buffer (volume depends on the amount of c-AMP in the sample) and use 100 µL directly in the Assay or the sample may be diluted. The decision as to which procedure to use for the sample depends on expected levels of cyclic AMP in the sample.

   If necessary, the TCA extract may be purified further by ion exchange column chromatography. In this example, it is not necessary to remove the TCA with ether as the sample may be applied directly to the column.

   a. Prepare a 0.6 x 5.0 cm column of Dowex 50w - X8 (H+), 200-400 mesh in water. This is conveniently done in a disposable Pasteur capillary pipet.

   b. Prior to the sample additions to the column, it should be characterized to locate which fraction contains ^{3}H-cyclic AMP marker. Use water as the eluate.

   c. After the elution volume containing ^{3}H-cyclic AMP has been determined, allow the water to drain into the resin bed, pipet 1 mL of the TCA extract onto the column and start collecting 1 mL samples.

   d. After the TCA extract has drained into the column, add water and continue to collect the effluent.

   e. Combine the fractions previously determined to contain ^{3}H-cyclic AMP marker and continue at Step 5. We have found recoveries > 90% with this procedure.

      If an excess of cyclic AMP is suspected in the sample, dilute with Assay Buffer.

VII. NON-ACETYLATED PROCEDURE (URINE)

A. Reagents Supplied - sufficient for 200 tubes

1. 1 vial cAMP Antiserum Complex, lyophilized

2. 2 vials cAMP [^{125}I]-Tracer, 1 mL (concentrate)

3. 2 vials cAMP Carrier Serum, lyophilized

4. 1 vial cAMP Standard, liquid

5. 1 vial cAMP Buffer, 25 mL (concentrate)

6. 1 bottle cAMP Precipitator, 100 mL

B. Equipment and Reagents Required

In addition to the reagents supplied with the kit, the following materials are required:

1. Pipettors and/or pipets that can accurately and precisely deliver the required volumes.

2. Gamma scintillation counter.

3. Laboratory vortex mixer.

4. Test tube rack.

5. Distilled water.

6. Centrifuge - refrigerated (swing bucket head).

7. Test tubes - 12 x 75 mm - glass.

8. Test tube - 13 x 100 mm - glass.

9. Absorbent paper for blotting.

10. Radioactive waste container.

11. 2-8°C refrigerator or equivalent.

C. Urinary Radioimmunoassay Protocol

NOTE: A full understanding of the Protocol and Precautions is necessary for successful completion of the RIA. Read these two sections carefully before proceeding with the assay.

Seven levels of cAMP standards are recommended, each point to be run in duplicate.

1. Prepare standard solutions as follows, mixing each solution thoroughly before adding it to the next tube. Prepare the standards fresh each day.

Pipet mL cAMP Std.	From Tube	Add mL Assay Buffer	Into Labeled Tube	Concentration (pmol/mL)
0.1	*	9.9	A	500
0.2	A	1.8	B	50
1.0	B	1.0	C	25
1.0	C	1.5	D	10
1.0	D	1.0	E	5
1.0	E	1.0	F	2.5
1.0	F	1.5	G	1.0
1.0	G	1.0	H	0.5
*50,000 pmol/mL (stock standard reagent)				

2. Prepare the urine sample as follows: First, dilute the urine specimen tenfold by adding 100 µL of urine to 900 µL of Assay Buffer. Then, take 100 µL of the tenfold dilution and add it to 4.9 mL of Assay Buffer. This produces a 1/500 dilution of the sample.

3. Prepare Working Tracer Solution by adding one volume of diluted cAMP [^{125}I]-Tracer to one volume of the reconstituted cAMP Carrier Serum. Make enough of the Working Tracer Solution to run the desired number of tubes (e.g., 1.0 mL of cAMP Carrier Serum and 1.0 mL of cAMP [^{125}I]-Tracer will provide 2.0 mL of solution which is theoretically sufficient for 20 tubes). Any remaining volume is to be discarded appropriately. Do not store and reuse this solution.

4. Number a series of 20 tubes to be used for the standard curve, plus two additional tubes for each sample. The assay may be set up at room temperature.

5. Tubes 1 and 2 measure the total counts added and receive only the Working Tracer Solution.

6. Add 200 µL of Assay Buffer to tubes 3 and 4 (blank tubes).

7. Add 100 µL of Assay Buffer to tubes 5 and 6 (zero standard tubes).

8. Add 100 µL of each standard solution or sample to the appropriate tubes (see Table I).

9. Add 100 µL of the Working Tracer Solution to all tubes (see Step 3).

10. Thoroughly mix Antiserum Complex and add 100 µL to all tubes, except Total Count tubes and Blank tubes.

11. Mix all tubes, except 1 and 2, by using a vortex mixer.

12. Cover and incubate overnight (16-18 hours) at 2-8°C.

13. Set tubes 1 and 2 aside, and add 500 µL of 2-8°C cAMP Precipitator to all the other tubes. Mix well with a vortex mixer and centrifuge 2-8°C for 15 minutes at approximately 1,200 x g.

14. Decant by gently inverting all tubes once, preferably at the same time, discarding the supernatant into a radioactive waste container. Keeping the tubes inverted, place them on absorbent paper for blotting. To facilitate removal of remaining droplets, gently tap the rims of the tubes on the paper. Allow tubes to drain for 20-30 seconds.

15. Count all tubes, including Tubes 1 and 2, in a gamma counter. At the usual counting efficiency of 50-70%, a counting time of one minute should be sufficient.

16. Calculations are described in Section IX.

TABLE I - PROTOCOL FOR NON-ACETYLATED STANDARD CURVE
(All volumes are in microliters)

	Tube	Assay Buffer	Stds.	Sam-ple	Working Tracer	Antiserum Complex		Preci-pitator
Total Cts.	1, 2	-	-	-	100	-		-
Blank	3, 4	200	-	-	100	-	Mix.	500
"0" Std.	5, 6	100	-	-	100	100	Cover	500
0.5 Std.	7, 8	-	100	-	100	100	and	500
1 Std.	9, 10	-	100	-	100	100	incu-	500
2.5 Std.	11, 12	-	100	-	100	100	bate	500
5 Std.	13, 14	-	100	-	100	100	16-18	500
10 Std.	15, 16	-	100	-	100	100	hours	500
25 Std.	17, 18	-	100	-	100	100	at 2 -	500
50 Std.	19, 20	-	100	-	100	100	8°C.	500
Sample	21, 22	-	-	100	100	100		500
Centrifuge at 1,200 x g for 15 minutes at 2-8°C. Decant and blot tubes. Count pellets.								

VIII. ACETYLATED PROCEDURE (PLASMA)

A. Reagents Supplied - sufficient for 200 tubes

1. 1 vial cAMP Antiserum Complex, lyophilized
2. 2 vials cAMP [^{125}I]-Tracer, 1 mL (concentrate)
3. 2 vials cAMP Carrier Serum, lyophilized
4. 1 vial cAMP Standard, liquid
5. 1 vial cAMP Buffer, 25 mL (concentrate)
6. 1 vial cAMP Triethylamine
7. 1 vial cAMP Acetic Anhydride
8. 1 bottle cAMP Precipitator, 100 mL

B. Equipment and Reagents Required

In addition to the reagents supplied with the kit, the following materials are required:

1. Pipettors and/or pipets that can accurately and precisely deliver the required volumes.

2. Gamma scintillation counter.

3. Laboratory vortex mixer.

4. Test tube rack.

5. Distilled water.

6. Centrifuge - refrigerated (swing bucket head).

7. Test tubes - 12 x 75 mm - glass.

8. Test tube - 13 x 100 mm - glass.

9. Absorbent paper for blotting.

10. Radioactive waste container.

11. 2-8°C refrigerator or equivalent.

C. Acetylated (Plasma) Radioimmunoassay Protocol

NOTE: A full understanding of the Protocol and Precautions is necessary for successful completion of the RIA. Read these two sections carefully before proceeding with the assay.

1. Dilute the cAMP stock standard reagent (50,000 pmol/mL) fifty-fold in a 13 x 100 mm test tube by adding 100 µL of the 50,000 pmol/mL solution to 4.9 mL of Assay Buffer. The resulting solution will be 1,000 pmol/mL. Prepare a 40 pmol/mL solution by adding 0.1 mL of the 1,000 pmol/mL solution to 2.4 mL of Assay Buffer. Place 200 µL of the 40 pmol/mL solution in a marked glass tube.

2. Prepare the plasma samples for acetylation as follows: Pipet 100 µL of the plasma sample into 400 µL of Assay Buffer (1:5 dilution). Place 100 µL of the 1:5 dilution in a marked glass test tube.

3. Prepare 150 µL of acetylation reagent by mixing together in a glass test tube 100 µL of Triethyl-amine and 50 µL of Acetic Anhydride. Vortex before using. Make up a fresh solution each time.

4. Prepare 10 mL of Modified Assay Buffer by adding 50 µL of the acetylation reagent (Step 3) to 10 mL of Assay Buffer. Mix well and let incubate at room temperature for at least three minutes. Modified Assay Buffer is used for the preparation of the cAMP standards and in the "blank" and "zero standard" tubes (see Table II).

5. Acetylate the 40 pmol/mL standard prepared in Step 1 (i.e., the 200 µL aliquot) by adding to it 10 µL of the freshly prepared acetylation reagent. Allow the reaction to proceed for at least three minutes at room temperature and then add 1.8 mL of Assay Buffer. Label this 4 pmol/mL standard as tube A. The amount of acetylation reagent used is sufficient to acetylate 25,000 pmols of cAMP.

6. Acetylate the previously prepared 100 µL aliquot of diluted sample (Step 2) by adding 5 µL of acetylation reagent to each tube. Immediately vortex and let the sample incubate for at least three minutes at room temperature. Then add 900 µL of Assay Buffer to each tube. This makes a 1:50 dilution of the sample.

7. Dilute the 4 pmol/mL standard with Modified Assay Buffer (see Step 4) as shown below. The standards should be prepared fresh each day. Do not store for reuse.

Pipet mL cAMP Std.	From Tube	Add mL Modified Assay Buffer	Into Labeled Tube	Concentration (pmol/mL)
-	-	-	A	4.0
1.0	A	1.0	B	2.0
1.0	B	1.0	C	1.0
1.0	C	1.0	D	0.5
1.0	D	1.0	E	0.25
1.0	E	1.5	F	0.10
1.0	F	1.0	G	0.05

8. Prepare Working Tracer Solution by adding one volume of diluted cAMP [^{125}I]-Tracer to one volume of the reconstituted cAMP Carrier Serum. Make enough of the Working Tracer Solution to run the desired number of tubes (e.g., 1.0 mL of cAMP Carrier Serum and 1.0 mL of cAMP [^{125}I]-Tracer will provide 2.0 mL of solution which is theoretically sufficient for a maximum of 20 tubes). Any

remaining volume is to be discarded appropriately. Do not store and reuse this solution.

9. Number a series of 20 tubes to be used for the standard curve, plus two additional tubes for each sample. The assay may be set up at room temperature.

10. Tubes 1 and 2 measure the total counts added and receive only the Working Tracer Solution.

11. Add 200 µL of Modified Assay Buffer to Tubes 3 and 4 (blank tubes).

12. Add 100 µL of Modified Assay Buffer to Tubes 5 and 6 (zero standard tubes).

13. Add 100 µL of each standard solution or sample to the appropriate tubes (see Table II).

14. Add 100 µL of Working Tracer Solution to all tubes.

15. Thoroughly mix Antiserum Complex and add 100 µL to all tubes, except Total Count tubes and Blank tubes.

16. Mix all tubes, except 1 and 2, by using a vortex mixer.

17. Cover and incubate overnight (16-18 hours) at 2-8°C.

18. Set Tubes 1 and 2 aside, and add 500 µL of 2-8°C cAMP Precipitator to all the other tubes. Mix well with a vortex mixer and centrifuge at 2-8°C for 15 minutes at approximately 1,200 x g.

19. Decant by gently inverting all tubes once, preferably at the same time, discarding the supernatant into a radioactive waste container. Keeping the tubes inverted, place them on absorbent paper for blotting. To facilitate removal of remaining droplets, gently tap the rims of the tubes on the paper. Allow tubes to drain for 20-30 seconds.

20. Count all tubes, including Tubes 1 and 2, in a gamma counter. At the usual counting efficiency of 50-70%, a counting time of one minute should be sufficient. Include an instrument blank.

21. Calculations are described in Section IX.

TABLE II - PROTOCOL FOR ACETYLATED STANDARD CURVE
(All volumes are in microliters)

	Tube	Assay Buffer	Stds.	Sample	Work-ing Tracer	Antise-rum Complex		Precipi-tator
Total Cts.	1, 2	-	-	-	100	-	Mix. Cover and incu-bate 16-18 hours at 2-8°C.	-
Blank	3, 4	200	-	-	100	-		500
"0" Std.	5, 6	100	-	-	100	100		500
0.05 Std.	7, 8	-	100	-	100	100		500
0.10 Std.	9, 10	-	100	-	100	100		500
0.25 Std.	11, 12	-	100	-	100	100		500
0.5 Std.	13, 14	-	100	-	100	100		500
1.0 Std.	15, 16	-	100	-	100	100		500
2.0 Std.	17, 18	-	100	-	100	100		500
4.0 Std.	19, 20	-	100	-	100	100		500
Sample	21, 22	-	-	100	100	100		500
Centrifuge at 1,200 x g for 15 minutes at 2 - 8°C. Decant and blot tubes. Count pellets.								

IX. PROCEDURE FOR CALCULATING UNKNOWNS IN URINE AND PLASMA SAMPLES

NOTE: Urine and plasma sample values are determined in an identical manner, i.e., by interpolation from their respective standard curves. The range of standard concentrations on a non-acetylated (urine) standard curve is from 0-50 pmol/mL. The range of standard concentrations on an acetylated (plasma) standard curve is from 0-4 pmol/mL.

A. If all tubes have been counted for the same period of time, use the total accumulated counts, otherwise, correct all counts to a common count rate.

B. Average the counts for each set of duplicates.

C. Calculate the average Net Counts for all standards and samples by subtracting from each the average blank counts.

D. Express the average Net Counts for each standard and sample as a percentage of the average Net Counts for the zero standard. (This is termed "normalized" percent bound or % B/Bo.)

$$\% \text{ B/Bo} = \frac{\text{Average Net Counts of Standard or Sample}}{\text{Average Net Counts of Zero Standard}} \times 100$$

E. Using semi-logarithmic or log-logit graph paper, plot % B/Bo for each standard against the corresponding concentration of cAMP in pmol/mL.

F. Determine the concentration of cAMP in the samples by interpolation from the standard curve. Since identical volumes are used for standards and samples, and the standard curve is expressed as pmol/mL of cAMP, samples can be read as pmol/mL and then multiplied by the appropriate dilution factor (i.e., 50 for plasma samples, 500 for urine samples) to calculate the sample concentration. Any samples with concentrations above the range of the standard curve must be diluted and re-assayed. The values are then multiplied by the appropriate dilution factor. See Tables III and IV for sample calculations, and Figure 2 for typical standard curve.

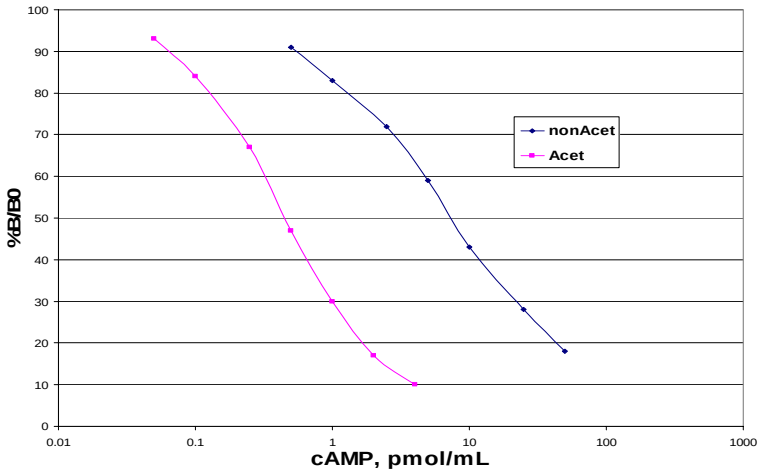
TABLE III - TYPICAL DATA FOR NON-ACETYLATED (URINE) ASSAY

	Tube Number	Counts Bound	Average Counts Bound	Net Counts Bound	Normalized % Bound
Blank	1 2	1016 950	983	-	-
"0" Standard	3 4	16892 17078	16985	16002	100
0.5 pmol/mL	5 6	15340 15662	15501	14518	91
1.0 pmol/mL	7 8	14305 14369	14337	13354	83
2.5 pmol/mL	9 10	12436 12461	12448	11466	72
5.0 pmol/mL	11 12	10385 10508	10446	9463	59
10 pmol/mL	13 14	7837 8034	7936	6952	43
25 pmol/mL	15 16	5287 5502	5394	4412	28
50 pmol/mL	17 18	3764 3844	3804	2812	18

TABLE IV - TYPICAL DATA FOR ACETYLATED (PLASMA) ASSAY

	Tube Number	Counts Bound	Average Counts Bound	Net Counts Bound	Normalized % Bound
Blank	1 2	923 1003	963	-	-
"0" Standard	3 4	15856 16227	16042	15079	100
0.05 pmol/mL	5 6	14942 15008	14975	14012	93
0.10 pmol/mL	7 8	13671 13452	13562	12598	84
0.25 pmol/mL	9 10	10839 11179	11009	10046	67
0.50 pmol/mL	11 12	8003 8050	8026	7064	47
1.0 pmol/mL	13 14	5412 5571	5492	4528	30
2.0 pmol/mL	15 16	3589 3539	3564	2601	17
4.0 pmol/mL	17 18	2493 2578	2536	1572	10

Figure 2
cAMP Typical Standard Curve



X. PRECAUTIONS

A. Incubation conditions should be standardized for proper day to day internal quality control.

B. As with all radioimmunoassay procedures, pipetting is crucial. It is essential that pipetting be accurate and reproducible.

C. Samples with concentrations above the range of the standard curve may be reassayed after dilution with Assay Buffer (urine samples) or Modified Assay Buffer (plasma samples). The values obtained are then multiplied by the appropriate dilution factor. During the preparation of plasma samples, it is important to make an initial 1:50 dilution of the acetylated sample as previously specified, and then to dilute further with Modified Assay Buffer.

D. The use of grossly hemolyzed or lipemic samples should be avoided.

E. The presence of exogenous radioactivity in clinical samples may lead to erroneous results.

F. Avoid using a centrifuge which may overheat due to prolonged use.

G. The reagents in this kit should be used as a unit. Do not mix different lots of any component within a given assay.

H. This product has not been tested for use with any methods other than those stated in this Instruction Manual.

I. The average Net Counts for the "0" Standard represents the counts bound to the antibody in the absence of added cAMP. This figure should fall between 40-60% of the average net total counts. The acetylated procedure will generally have a slightly lower Bo than the non-acetylated procedure.

XI. PERFORMANCE CHARACTERISTICS FOR THE NON-ACETYLATED (URINE) PROCEDURE

A. <u>Recovery</u>

To demonstrate the accuracy of the method, recovery studies were performed by adding known quantities of cAMP to

aliquots of Assay Buffer and three urine samples. The resulting solutions were re-assayed and the recovery of added cAMP was determined. Typical results are given below.

		+ 5 nmol/mL cAMP		+ 15 nmol/mL cAMP		+45 nmol/mL cAMP	
Sample	nmol/mL Base Value	nmol/mL Measured	% Rec	nmol/mL Measured	%Rec	nmol/mL Measured	% Rec
Assay Buffer	0	5.4	108	15.6	104	44.1	98
A	2.4	7.6	104	17.5	101	49.5	105
B	1.4	6.2	96	18.2	112	51.2	111
C	3.0	8.5	110	19.0	107	48.8	102

B. Reproducibility

Precision was determined by multiple duplicate analyses of urine pools. Typical results are given in nmol/mL.

Within Assay Variation				Between Assay Variation			
Sample	n	Mean ± 1 S.D.	Coeff. of Var. (%)	Sample	n	Mean ± 1 S.D.	Coeff. of Var. (%)
A	10	5.4 ± 0.19	3.5	A	6	9.5 ± 0.89	9.0
B	10	2.4 ± 0.07	2.9	B	6	3.4 ± 0.16	4.7
C	9	1.3 ± 0.03	2.3	C	6	7.1 ± 0.69	9.7
D	10	4.0 ± 0.07	1.8	D	6	3.2 ± 0.22	6.8
				E	6	3.8 ± 0.25	6.5
				F	6	3.5 ± 0.2	5.7
				G	6	5.0 ± 0.56	11.0
				H	6	1.9 ± 0.18	9.4

C. Linearity

To demonstrate the absence of non-specific urine interferences, five samples were assayed by the standard procedure and diluted to varying degrees with Assay Buffer. The recommended 100 µL aliquot was used for all samples. Typical results are given in nmol/mL, corrected for dilution

Sample	1:100	1:250	1:500	1:1000
A	-	6.0	5.8	5.8
B	2.5	2.5	2.4	2.5
C	-	5.8	5.8	5.2
D	1.4	1.4	1.4	1.3

D. Sensitivity

The mean and standard deviation were determined for 8 duplicate measurements of the zero standard binding. The sensitivity of the method, defined as the cAMP concentration corresponding to the mean cpm minus twice the standard deviation, is < 0.025 pmol/mL.

E. Specificity

The following compounds have been checked for cross-reactivity. The percentages are calculated at the 50% B/Bo point.

Compound	% Cross Reactivity
CGMP	~ 0.02
GMP	< 0.01
ATP	< 0.01
ADP	< 0.01
AMP	< 0.01

XII. PERFORMANCE CHARACTERISTICS FOR ACETYLATED (PLASMA) PROCEDURE

A. Recovery

To demonstrate the accuracy of the method, recovery studies were performed by adding known quantities of cAMP to aliquots of Modified Assay Buffer and three plasma samples. The resulting solutions were re-assayed and the recovery of added cAMP was determined. Typical results are given on below.

		+25 pmol/mL cAMP		+50 pmol/mL cAMP		+100 pmol/mL cAMP	
Sample	pmol/mL Base Value	pmol/mL Measured	% Rec	pmol/mL Measured	% Rec	pmol/mL Measured	% Rec
Modified Assay Buffer	0	24	96	44	88	94	94
A	10	34	94	57	94	96	86
B	7.5	50	90	54	93	100	92

B. Reproducibility

Precision was determined by multiple duplicate analyses of plasma pools. Typical results are given in pmol/mL.

Within Assay Variation				Between Assay Variation			
Sample	n	Mean ± 1 S.D.	Coeff. Of Var. (%)	Sample	n	Mean ± 1 S.D.	Coeff. of Var. (%)
A	10	16 ± 0.49	3	A	6	16 ± 0.98	6.1
B	10	6.3 ± 0.26	4.1	B	6	5.6 ± 0.51	9.1
C	10	12.8 ± 0.24	1.9	C	6	27 ± 3.2	11.8
D	10	35.9 ± 0.88	2.4	D	6	16 ± 2.4	15
E	10	24.7 ± 0.48	1.9	E	6	11.6 ± 1.6	14
				F	6	11 ± 1.5	14

C. Linearity

To demonstrate the absence of non-specific serum interferences, five plasma samples were assayed by the standard procedure and diluted to varying degrees with Modified Assay Buffer. The recommended 100 µL aliquot was used for all samples. Typical results are given in pmol/mL, corrected for dilution.

Sample	1:50	1:100	1:200
A	13	11	12
B	16	14	13
C	18	18	16
D	9	8	-
E	20	20	17

D. Sensitivity

The mean and standard deviation were determined for 8 duplicate measurements of the zero standard binding. The sensitivity of the method, defined as the cAMP concentration corresponding to the mean cpm minus twice the standard deviation, is < 0.025 pmole/mL.

E. Specificity

The following compounds have been checked for cross-reactivity. The percentages are calculated at the 50% B/Bo point.

Compound	% Cross Reactivity
CGMP	~ 0.02
GMP	< 0.01
ATP	< 0.01
ADP	< 0.01
AMP	< 0.01

XIII. CORRELATION

The cAMP concentraion of plasma, urine, and cell culture samples were measured. Summarized below is the correlation obtained comparing the present with the previous antibody.

Sample Type	N	Range (pmole/mL)	Slope	Y-intercept	Correlation Coefficient
Plasma	40	8 to 75	0.99	-0.01	0.98
Urine	16	60 to 9070	0.96	-141	0.99
Cell Culture	12	876 to 1942	1.04	-192	0.98

XIV. REFERENCES

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