PerkinElmer™ Life Sciences, Inc.

cAMP [^{125}I] FLASHPLATE® ASSAY (ADENOSINE 3', 5'-CYCLIC MONOPHOSPHATE) CATALOG NUMBER SMP001A

For Laboratory Use

CAUTION: A research chemical for research purposes only.

I. PROPRIETARY NAME

cAMP [^{125}I] FlashPlate® Assay, Catalog Number SMP001A.

II. INTENDED USE

This product is designed to measure levels of cAMP (adenosine 3', 5' - cyclic monophosphate) in biological fluids.

III. BACKGROUND INFORMATION

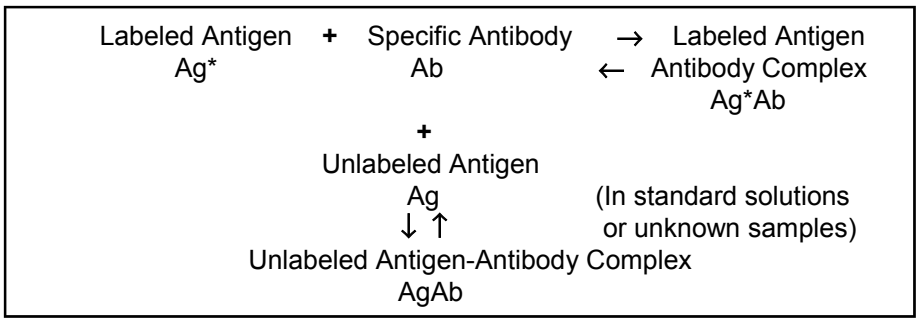
Adenosine 3', 5' cyclic monophosphate (cAMP) plays a critical role in the transmission of hormonal signals by functioning as a "second messenger"[1,2]. The binding of a hormone to its receptor can either enhance or inhibit the rate at which cAMP is produced. This is accomplished by altering the enzymatic 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 hormones[4].

The [^{125}I]-cAMP FlashPlate Assay is performed using cAMP standards and samples. Cyclic AMP can be measured in the range of 1.0. to 50 pmol/mL. The assay is accurate over a wide range of values and has a high degree of specificity.

IV. PRINCIPLES OF THE PROCEDURE

The basic principle of FlashPlate Assay 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[5].

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 [^{125}I]-cAMP FlashPlate Assay, the antibody is affixed to scintillant coated microplate wells and the counting of the bound fraction is dependent upon the distance of the material to the walls of the wells. Separation of the bound from the free antigen is not necessary to quantitate the bound tracer.

After incubation the antigen-antibody complex is counted and the data are used to construct a standard curve from which the values of the unknowns may be obtained by interpolation. Aspiration of the wells is recommended if the plate is going to be kept at room temperature for greater than 30 minutes. Aspiration prevents re-equilibration of the plates to the "new" ambient temperature. Also, the need for quench correcting due to color quenching by certain samples is eliminated.

V. REAGENT AND EQUIPMENT

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. Any reagent remaining 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 lyophilized standard is supplied. Reconstitute the contents with exactly 2.0 mL of distilled water. The reconstituted solution will contain: cAMP at a concentration of 5,000 pmol/mL, sodium acetate buffer, 0.1% sodium azide and an inert ingredient. The cAMP Standard has been calibrated spectrophotometrically using the molar absorption coefficient, $= 14.6 \times 10^3 1\ mol^{-1}\ cm^{-1}$ at 259 nm, pH 6.9. The reconstituted standard is stable for at least two months when stored at 2°-8°C. Refer to vial label for expiration date of lyophilized reagent.

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

Two vials of concentrated tracer are supplied. Each vial contains less than 370KBq (5µCi) on calibration date in 2.5 mL of a 1:1 n-propanol:water solution. Use one vial at a time as directed. Add 12.5 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 next 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 there from, 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 decontaminate. 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.

D. cAMP Carrier Serum

Five 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 wells). 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.

E. cAMP FlashPlate

Five microplates coated with solid scintillant to which anti-cyclic AMP antisera has been bound are supplied. Each plate is packaged in a sealed pouch and should not be opened until ready to use. The plate may be stored for at least two (2) months at 4°C.

F. TopSeal - A Plate Cover

Ten plate covers are provided with each kit.

VI. PRECAUTIONS

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

B. As with all immunoassay 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. The values obtained are then multiplied by the appropriate dilution factor.

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

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

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

G. WARNING: This product contains a chemical known to the state of California to cause cancer. (Note: ^{125}I Tracer)

VII. SAMPLE COLLECTION, PROCESSING AND STORAGE

A. 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.

B. 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 <u>each</u> sample extract approximately 4,000 cpm of ^{3}H-cyclic AMP marker (available from PerkinElmer) 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 as determined by the FlashPlate Assay.

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 FlashPlate 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.

7. Proceed with FlashPlate Assay.

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.

VIII. ASSAY PROCEDURE

A. 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. 96-Well Microplate Scintillation Counter

3. Laboratory vortex mixer

4. Test tube rack

5 Distilled water

6. Test tubes - 12 x 75 mm - glass and polypropylene

7. Radioactive waste container

8. 2°-8°C refrigerator or equivalent

B. Procedure

1. Suggested protocol for the preparation of standards. Mix 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	50
1.0	A	1.0	B	25
1.0	B	1.5	C	10
1.0	C	1.0	D	5
1.0	D	1.0	E	2.5
1.0	E	1.5	F	1.0

*5,000 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 wells (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 sufficient for 18 wells and a TC tube). Any remaining volume is to be discarded appropriately. Do not store and reuse this solution.

4. Add 100 µL of Assay Buffer to wells A1, A2.

   Add 100 µL of diluted standards to appropriate wells. (1.0 standard to wells B1, B2, etc). See Table 1.

5. Add 100 µL of diluted sample to appropriate wells (Sample 1, 100 µL to wells A3, A4, etc.). See Table 1.

6. Add 100 µL of working tracer to all wells that have been set up for assay and also to two test tubes (12 x 75 mm) if total counts are desired.

   All participating wells should have 200 µL of liquid.

Table I

	1	2	3	4	5	6
A B C D E F G H	0 1.0 2.5 5.0 10.0 25.0 50.0 Sample 1		Sample 2 etc.			

7. Place a plate cover on plate and incubate overnight (18-24 hrs.) at 2°-8°C.

8. After incubation, count on a 96-well microplate scintillation counter.

NOTE:

1) If FlashPlate cannot be counted within 30 minutes after removal from 4°C, aspiration of the wells is recommended to prevent re-equilibration of the assay at the "new" temperature.

2) The assay may be incubated at room temperature overnight. A drop in the B_o and shift in the standard curve will take place (Table II; b). The addition of a 100 pmol/mL standard point is recommended.

Room temperature incubation eliminates the need for aspiration of the contents of each well prior to counting. However, if color quench is a concern, wells should be aspirated.

IX. CALCULATIONS

Sample values are determined by interpolation from standard curves corrected for appropriate dilutions. The range of standard concentrations on a non-acetylated standard curve is from 0-50 pmol/mL.

A. Average the counts for each set of duplicates.

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

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

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

D. 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., 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.

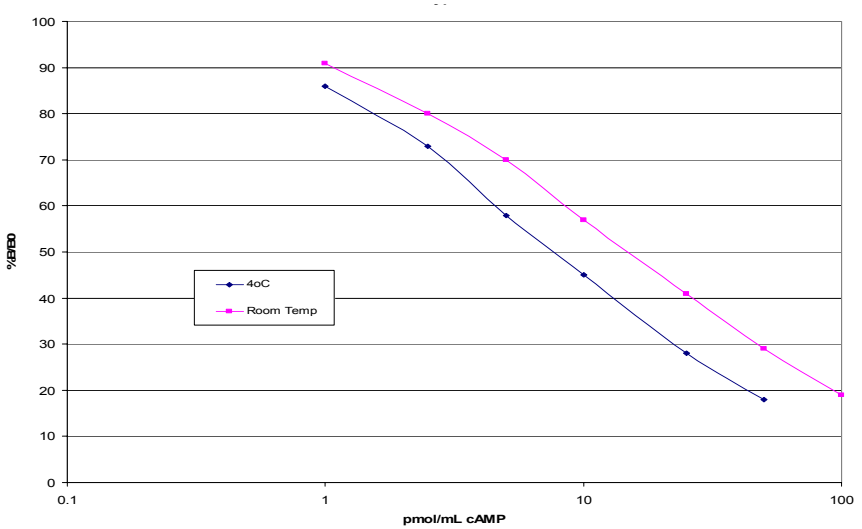
Table II
(a) Typical Data for cAMP Assay 4°C

	Well	cpm	Average	Norm. % Bound
"0" Standard	A1/A2	4869/4965	4917	-
1.0 pmol/mL	B1/B2	4177/4249	4213	86
2.5	C1/C2	3572/3668	3620	73
5.0	D1/D2	2852/2907	2879	58
10.0	E1/E2	2226/2214	2226	45
25.0	F1/F2	1405/1401	1403	28
50.0	G1/G2	855/891	873	18

(b) Typical Data for Assay RT

	Well	cpm	Average	Norm. %
"0" Standard	A1/A2	3631/3545	3588	-
1.0 pmol/mL	B1/B2	3200/3330	3265	91
2.5	C1/C2	2756/2984	2870	80
5.0	D1/D2	2463/2559	2511	70
10.0	E1/E2	1823/2267	2045	57
25.0	F1/F2	1296/1646	1471	41
50.0	G1/G2	993/1087	1040	29
100.0	H1/H2	636/728	682	19

Figure 1
cAMP Typical Standard Curves



X. PERFORMANCE CHARACTERISTICS

A. Reproducibility

Within plate precision was determined by assaying multiple standard curves on a plate. Between plate precision was determined by averaging duplicate standard curves from several plates.

4°C Overnight Incubation

Within Plate Variation				Between Plate Variation			
Standard	n	Mean ± 1 S.D. cpm	Coeff. of Var. (%)	Standard	n	Mean ± 1 S.D. cpm	Coeff. of Var. (%)
0 pmol/mL	12	2745 ± 216	7.8	0 pmol/mL	6	6314 ± 209	3.3
1	12	2374 ± 94	3.9	1	6	5277 ± 250	4.7
2.5	12	1903 ± 89	4.7	2.5	6	4435 ± 248	5.6
5	12	1555 ± 78	5.0	5	6	3659 ± 165	4.5
10	12	1187 ± 49	4.1	10	6	2763 ± 117	4.2
25	12	751 ± 48	6.4	25	6	1731 ± 76	4.4
50	12	528 ± 41	7.7	50	6	1093 ± 58	5.3

B. Sensitivity

The mean and standard deviation were determined for 16 measurements of the "zero standard" binding (B_o) in the 2°-8°C assay. The sensitivity of this assay, defined as the cAMP concentration corresponding to the mean B_o cpm minus twice the standard deviation, is < 0.4 pmol/mL.

C. Specificity

The following compounds have been checked for cross-reactivity with percentages calculated at the 50% B/B_o (B_{50}) point.

Compound	% Cross-reactivity
cGMP	0.01
GMP	< 0.01
ATP	< 0.01
ADP	< 0.01
AMP	< 0.01

XI. REFERENCES

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5. Yallow, R. S. and Berson, S. A. in *Principles of Competitive Protein Binding Assays,* Eds. Odell, W. D. and Daughaday, W. H. J. B. Lippincott Co., Philadelphia, 1971, Ch. 1.

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PC 2262-1102