cAMP [^{125}I] RIA KIT

(REF: RK-509)

For Research Use Only. Not for use in diagnostic procedures.

The [^{125}I]cAMP RIA system (dual range) provides direct quantitative *in vitro* determination of cAMP in urine, plasma, tissues and other biological samples. cAMP may be measured in the range 25–1600 fmol per tube (8–526 pg/tube; 80–5260 pg/ml), but higher sensitivity may be obtained by acetylation of standards and unknowns prior to assay. Using this approach, standard curves ranging from 2–128 fmol/per tube (0.7–42 pg/tube; 7–420 pg/ml) are obtained. Each kit contains materials sufficient for 100 determinations permitting the construction of one standard curve and the assay of 42 unknowns in duplicate. If two curves are constructed then 34 unknowns can be measured.

Introduction

The physiological responses to many biologically important compounds are mediated through 'second messengers'. This is a term described by Sutherland for molecules which are able to transmit intracellularly, the biological effects of compounds not able to enter the target cells themselves.

cAMP was identified as playing a major role in the mode of action of adrenaline 30 years ago. In response to receptor binding, the enzyme adenylate cyclase converts ATP to cAMP, which exerts its effect by activating a protein kinase capable of phosphorylating specific substrates. Numerous hormones are known to act through this mechanism including corticotrophin (ACTH), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), calcitonin, glucagon, vasopressin and parathyroid hormone (PTH).

cAMP has now been shown to be involved in the cardiovascular and nervous systems, in immune mechanisms, cell growth and differentiation and general metabolism. There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of the physiology and pathology of many disease states.

The assay system may be used in adenylate cyclase assays which determine cAMP formation from unlabelled ATP. The method allows high sensitivity without the interference from ATP to which other adenylate cyclase assays are prone.

In recent years there has been great interest in a new generation of phosphoinositide-derived second messengers.

Receptor stimulation triggers the phospholipase C catalyzed hydrolytic cleavage of membrane phosphatidylinositol 4,5-bisphosphate to yield two second messenger molecules viz inositol 1,4,5-trisphosphate (IP3) and sn-1,2-diacylglycerol (DAG).

It is now well established that IP3 acts as a second messenger of Ca2+ mobilized hormones in a variety of cell types. DAG appears to be an essential cofactor for the enzyme protein kinase C which plays a crucial role in signal transduction.

Principle of method

The assay is based on the competition between unlabelled cAMP and a fixed quantity of 125I-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound cAMP is then reacted with the separating second antibody reagent which contains second antibody that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected either by magnetic separation or centrifugation of the separating reagent suspension and decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled cAMP in the bound fraction to be calculated. The concentration of unlabelled cAMP in the sample is then determined by interpolation from a standard curve.

Increased sensitivity may be attained by acetylation of standards and unknowns prior to assaying. This enables low cAMP concentrations to be determined in small quantities of tissue and cell cultures. Two protocols are provided depending on the degree of sensitivity required by the investigator.

Contents of the kit

1. 1 vial TRACER, lyophilized, reconstitution with 11 ml assay buffer, containing ~ 79 kBq, 2.1 µCi Adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[125I] iodotyrosine methyl ester in 0.05 M acetate buffer containing preservative after reconstitution. Store at 2-8 °C.

2. 1 vial non-acetylation STANDARD (for urine, plasma and tissue determinations), lyophilized, reconstitution with 2 ml assay buffer. The final solution contains cAMP at a concentration of 32 pmol/ml in 0.05 M acetate buffer containing preservative. Store at 2-8 °C.

3. 1 vial acetylation STANDARD (for tissue determination), lyophilized, reconstitution with 2 ml assay buffer. The final solution contains cAMP at a concentration of 2.56 pmol/ml in 0.05 M acetate buffer containing preservative. Store at 2-8°C.

4. 1 vial ANTISERUM, lyophilized, reconstitution with 11 ml assay buffer. The final solution contains rabbit anti-succinyl cAMP serum in 0.05 M acetate buffer containing 0.5% bovine serum albumin and preservative. Store at 2-8 °C.

5. 1 vial ASSAY BUFFER concentrate (10 ml), dilution to 500 ml. The diluted buffer contains 0.05 M acetate buffer, pH5.8, with preservative. Store at 2-8 °C.

6. 1 vial SEPARATING SECOND ANTIBODY REAGENT (58 ml), ready for use, containing donkey anti-rabbit serum coated on to magnetizable polymer particles with sodium azide, colour coded, blue-green. Store at 2-8 °C.

7. 1 vial ACETIC ANHYDRIDE (1 ml), ready for use. Caution: flammable, corrosive, causes burns. Store at 2-8 °C.

8. 1 vial TRIETHYLAMINE (2 ml), ready for use. Caution: flammable, harmful vapour. Store at 2-8 °C.

Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (25µl, 100µl, 500µl, 2.0 ml and 11 ml); disposable polypropylene or polystyrene tubes (12 x 75 mm) for assay procedure; disposable polypropylene or glass tubes (12 x 75 mm) for acetylation procedure; refrigerator; glass measuring cylinder (500 ml); test tube rack; vortex mixer; plastic foil; separators, comprising magnetic base and assay rack - these are for use in the magnetic separation protocol; adsorbent tissue; gamma counter.

Note: For the centrifugal protocol, the following additional equipment will be required:

Decantation racks; refrigerated centrifuge capable of 2000 xg.

Specimen collection, storage

Numerous procedures have been described for the extraction of cAMP from biological samples. These include acidic extraction procedures using trichloroacetic acid, perchloric acid, dilute hydrochloric acid and extraction with aqueous ethanol.

Some investigators also recommend the use of ion exchange chromatography following one of these extraction techniques.

Representative procedures are described below for the extraction of cAMP from tissues and cell cultures. However, it remains the responsibility of the investigator to validate the chosen extraction procedure.

Urine samples

Random, timed or 24-hour urine collections may be analyzed. If 24-hour samples are collected, it may be necessary to include a bacteriostat (2 ml 6 M hydrochloric acid per 100 ml urine is sufficient for this purpose). Samples analyzed within 24 hours of collection may be stored at 2–8°C until assayed. If analysis is not performed within 24 hours, all samples should be stored at -15°C to -30°C.

If urine contains particulate matter this should be removed by centrifugation prior to assay.

It is not necessary to extract or deproteinize urine before analysis. Urine should be diluted 1:1000 with assay buffer and assayed without acetylation.

Plasma samples

Measurements should be made in plasma not serum.

Blood should be collected into tubes containing 7.5 mM EDTA. Blood should be immediately centrifuged to remove cells and the plasma stored at -15°C to -30°C prior to analysis. If blood samples cannot be rapidly processed they should be stored in ice until it is possible to centrifuge.

It is not necessary to extract or deproteinize plasma samples before analysis. Plasma cAMP may be determined without acetylation.

Tissue samples

Tissue sections must be rapidly frozen immediately after collection so as to prevent alterations to cAMP and associated enzymes before analysis. This is usually achieved by immersion of the fresh tissue in liquid nitrogen at -196°C.

Methods of freezing biological samples for cyclic nucleotide assays are reviewed by Mayer et al.

Samples should be stored at -15°C to -30°C until the assay is conducted.

Liquid phase extraction method

1. Homogenize frozen tissue in cold 6% trichloroacetic acid at 2–8°C to give a 10% (w/v) homogenate.
2. Centrifuge at 2000 xg for 15 minutes at 2–8°C.
3. Recover the supernatant and discard the pellet.
4. Wash the supernatant 4 times with 5 volumes of water saturated diethyl ether. The upper ether layer should be discarded after each wash.
5. The aqueous extract remaining should be lyophilized or dried under a stream of nitrogen at 60°C.
6. Dissolve the dried extract in a suitable volume of assay buffer prior to analysis.

Solid phase extraction method

Note: Maximum recovery of cyclic AMP is obtained using columns containing anion exchange silica sorbents, for example Amprep SAX. These columns provide a rapid sample clean-up and effectively reduce sample handling compared with solvent extraction methods.
It remains the responsibility of the investigators to validate the chosen extraction procedure for their own application.

- Amprep extraction of cyclic AMP:

1. Column conditioning
1.1. Rinse an Amprep SAX 500 mg minicolumn (code RPN 1918) with 2 ml methanol.
1.2. Rinse the column with 2 ml distilled water.
Note: Do not allow the sorbent in the column to dry. The flow rate should not exceed 5 ml/minute.
2. Sample treatment
2.1. Homogenize 1 g (wet weight) tissue in 10 ml Hank's balanced salt solution (without calcium and magnesium) containing 5mM EDTA.
2.2. Centrifuge the homogenate for 10 minutes at 1000 xg at 4°C.
2.3. Dilute homogenate supernatant 1:10 with Hank's and apply 1 ml directly to the conditioned SAX column. Alternatively, mix 1 ml of supernatant with 1 ml undiluted acetonitrile. Vortex mix for 20 seconds, centrifuge for 10 minutes at 1500 xg at 4°C. Apply 1 ml of supernatant to the column.
3. Interference removal
3.1. Wash the column with 3 ml methanol.
4. Analyte elution
Pass 3 ml acidified methanol through the column and collect the eluate. Prepare the acidified methanol by diluting concentrated HCl to 0.1 M with absolute methanol.
The sample can be dried under nitrogen and reconstituted in assay buffer and assayed directly.
Note: If lyophilization is the preferred method of drying samples. 0.1M HCl diluted in distilled water rather than methanol can be used to elute the analyte.

Cell culture

Methods of freezing biological samples for cyclic nucleotide assays are reviewed by Mayer et al.
Samples should be stored at -15°C to -30°C until the assay is conducted.

Liquid phase extraction method

1. Add ice-cold ethanol to cell suspensions to give a final suspension volume of 65% ethanol. Allow to settle.
2. Draw off the supernatant into test tubes.
3. Wash the precipitate remaining with ice cold 65% ethanol and add the washings to the appropriate tubes.
4. Centrifuge the extracts at 2000 xg for 15 minutes at 4°C and transfer the supernatant to fresh tubes.
5. Evaporate the combined extracts under a stream of nitrogen at 60°C or in a vacuum oven.
6. Dissolve the dried extracts in a suitable volume of assay buffer prior to analysis.

Solid phase extraction method

Amprep extraction of cyclic AMP:
1. Column conditioning
1.1. Rinse an Amprep SAX 500 mg minicolumn with 2 ml methanol.
1.2. Rinse the column with 2 ml distilled water.
Note: Do not allow the sorbent in the column to dry. The flow rate should not exceed 5 ml/minute.
2. Sample treatment
2.1. Apply directly to the column.
3. Interference removal
3.1. Wash the column with 3 ml methanol.
4. Analyte elution
Pass 3 ml acidified methanol through the column and collect the eluate. Prepare the acidified methanol by diluting concentrated HCl to 0.1 M with absolute methanol.
The sample can be dried under nitrogen and reconstituted in assay buffer and assayed directly.
Note: If lyophilization is the preferred method of drying samples. 0.1 HCl diluted in distilled water rather than methanol can be used to elute the analyte.

Preparation of reagents, storage

Storage: *see Contents of the kit*. At these temperatures each reagent is stable until expiry date. The actual expiry date is given on the package label and on the quality certificate. Reconstituted components should be stored at 2–8°C and may be re-used within 14 days of dilution.

Preparation: Equilibrate all reagents and samples to room temperature prior to use.

Assay buffer: Transfer the contents of the bottle to a 500 ml graduated cylinder by repeated washing with distilled water. Adjust the final volume to 500 ml with distilled water and mix thoroughly. Assay buffer is used to reconstitute all other components.

The other components (except ready for use components): Carefully add the required volume of assay buffer and replace stopper. Mix the contents of the bottles by inversion and swirling, taking care to avoid foaming.

Non-acetylation assay

(for the measurement of urine, plasma and tissue samples in the range 25–1600 fmol/tube).

Preparation of working standards

1. Label 7 polypropylene or polystyrene tubes, 25 fmol, 50 fmol, 100 fmol, 200 fmol, 400 fmol, 800 fmol, and 1600 fmol.
2. Pipette 500 µl assay buffer into all the tubes.
3. Into the 1600 fmol tube pipette 500 µl of stock non-acetylation standard (32 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 1600 fmol to the 800 fmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 100 µl aliquots from each serial dilution give rise to 7 standard levels of cAMP ranging from 25–1600 fmol.

Assay procedure

(For a quick guide , refer to Table 1.)

1. Prepare assay buffer and standards as described in the previous section.
2. Equilibrate all reagents to room temperature.
3. Label 12x75 mm disposable tubes in duplicate for total count (TC), non-specific binding (NSB), zero standard (Bo), standards and samples.
4. Pipette **100 µl** of assay buffer into the zero standard tubes (Bo).
5. Starting with the most dilute, pipette **100 µl** of each standard into the appropriately labelled tubes.
6. Pipette **100 µl** of each unknown sample into the appropriately labelled tubes. Urine should be diluted 1:1000 and plasma 1:20 with assay buffer. A 1:1000 dilution of urine may be achieved by diluting 100 µl of sample to 2.0 ml with assay buffer (1:20 dilution). Take 100 µl of this solution (1:20) and dilute to 5.0 ml with buffer. A 1:20 solution of plasma may be achieved by diluting 100 µl of sample to 2.0 ml with assay buffer. (Other biological samples should be pretreated according to the instructions in the previous section.)
7. Pipette **100 µl** of antiserum into all tubes except the TC.
8. Vortex mix all tubes thoroughly. Cover the tubes, for example with plastic film, and incubate for 1 hour at 2–8°C.
9. Pipette **100 µ**l of tracer into all tubes. The TC tubes should be stoppered and put aside for counting.
10. Vortex mix all tubes thoroughly. Cover the tubes, for example with plastic film, and incubate for 3 hours at 2–8°C.
11. Gently shake and swirl the bottle containing separating reagent (blue-green) to ensure a homogeneous suspension. Then add **500 µl** into each tube except the TC. The TC tubes should be stoppered and put aside for counting.
12. Vortex mix all tubes thoroughly and incubate for 10 minutes at room temperature (15–30°C).
13. Separate the antibody bound fraction by using either magnetic separation or centrifugation as described below.

Magnetic separation
Attach the rack on to the separating reagent base and ensure that all the tubes are in contact with the base plate. Leave for 15 minutes. After separation do not remove the rack from the separator base. Pour off and discard the supernatant liquid. Keeping the separator inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

Centrifugation
Centrifuge all tubes for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks then pour off and discard the supernatant liquid. Keeping the tubes inverted, place them on a pad of absorbent tissues and allow to drain for 5 minutes.

14. On completion of either magnetic or centrifugal separation, firmly blot the rims of the inverted tubes on the tissue pad to remove any adhering droplets of liquid. Do not re-invert the tubes once they have been turned upright.

15. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Table 1. Non-acetylation assay protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	Bo	Stan - dard	Sam- ple
Buffer	**-**	**100**	**-**	**-**
Stan- dard	-	**-**	**100**	**-**
Sample	-	**-**	**-**	**100**
Anti- serum	**-**	**100**	**100**	**100**
Vortex mix, cover tubes and incubate for 1 hour at 2-8 °C.				
Tracer	**100**	**100**	**100**	**100**
Vortex mix and incubate for 3 hours at 2-8 °C.				
Separa- ting reagent	-	**500**	**500**	**500**
Vortex mix. Incubate for 10 minutes at room temperature (15-30 °C).				
Separate either using magnetic separator for 15 minutes or by centrifugation for 10 minutes at >1500 xg .				
Decant tubes and blot on filter paper.				
Count radioactivity (60 sec/tube).				
Calculate the results.				

Acetylation assay

(for tissue and cell culture measurements in the range 2–128 fmol/tube).

Preparation of working standards

1. Label 7 polypropylene or glass tubes, 2 fmol, 4 fmol, 8 fmol, 16 fmol, 32 fmol, 64 fmol and 128 fmol.
2. Pipette 500 µl of assay buffer into all the tubes.
3. Pipette 500 µl of stock acetylation standard (2.56 pmol/ml) into the 128 fmol tube and mix thoroughly.
4. Transfer 500 µl from the 128 fmol tube to the 64 fmol tube, and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. Remove 500 µl from the 2 fmol standard and discard. All tubes should now contain 500µl.
7. 100 µl aliquots from each serial dilution will give rise to 7 standard levels of cAMP ranging from 2–128 fmol.

Note: These tubes will be referred to as acetylation tubes containing working standards in the assay protocol section. Working standards should be freshly prepared before each assay and not re-used.

Within day assay protocol

Note: Steps 7–11 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–8. Polystyrene or polypropylene tubes may be used in steps 9–18.

(For a quick guide , refer to Table 1.)

1. Prepare assay buffer and standards ranging from 2–128 fmol/100 µl as described in the previous section.

2. Equilibrate all reagents to room temperature and mix before use.

3. Label polypropylene or glass tubes (12 x 75 mm) in for the zero standard tube and unknowns. These will subsequently be known as acetylation tubes.

4. Label polypropylene or polystyrene tubes (12 x75 mm) in duplicate for total counts (TC), zero standard tubes (Bo), each standard dilution and unknowns. These will subsequently be known as assay tubes.

5. Prepare the acetylation reagent by mixing 1 volume of acetic anhydride with 2 volumes of triethylamine in a glass vessel. Mix well. (Sufficient reagent for 50 acetylations may be obtained by mixing 0.5 ml acetic anhydride with 1.0 ml triethylamine).

6. Pipette **500 µl** of assay buffer into the zero standard acetylation tube.

7. Pipette **500 µl** of each unknown (see sample preparation section) into the appropriately labelled acetylation tubes. Tubes containing 500 µl of each working standard should already have been prepared (see reagent preparation section).

8. Carefully add **25 µl** of the acetylation reagent to all acetylation tubes containing standards and unknowns. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylation reagents.

9. Pipette duplicate **100 µl** aliquots from all acetylation tubes into the corresponding polystyrene or polypropylene assay tubes.

10. Pipette **100 µl** of antiserum into all tubes except the TC.

11. Pipette **100 µl** of [125I]cAMP into all tubes including TC tubes. The TC tubes should be stoppered and put aside for counting.

12. Vortex mix all tubes thoroughly. Cover tubes with plastic film, and incubate for 4 hours at 2–8°C.

13. Gently shake and swirl the bottle containing separating reagent (blue-green) to ensure a homogeneous suspension. Then add **500 µl** to each tube except the TC.

14. Vortex mix all tubes thoroughly and incubate for 10 minutes at room temperature (15–30°C).

15. Separate the antibody bound fraction by using either magnetic separation or centrifugation, as described below.

Magnetic separation
Attach the rack on to the separating reagent base and ensure that all the tubes are in contact with the base plate. Leave for 15 minutes. After separation do not remove the rack from the separator base. Pour off and discard the supernatant liquid. Keeping the separator inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes.

Centrifugation
Centrifuge all tubes for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks then pour off and discard the supernatant liquids. Keeping the tubes inverted, place them on a pad of absorbent tissues and allow to drain for 5 minutes.

16. On completion of either magnetic or centrifugal separation, firmly blot the rims of the inverted tubes on the tissue pad to remove any adhering droplets of liquid. Do not re-invert the tubes once they have been turned upright.

17. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Table 2. Within day protocol, Pipetting Guide (all volumes are in microlitres)

Tubes	TC	Bo	Stan - dard	Sam- ple
Buffer	**-**	**100**	**-**	**-**
Stan- dard	-	**-**	**100**	**-**
Sample	-	**-**	**-**	**100**
Anti- serum	**-**	**100**	**100**	**100**
Tracer	**100**	**100**	**100**	**100**
Vortex mix and incubate for 4 hours at 2-8 °C.				
Separa- ting reagent	-	**500**	**500**	**500**
Vortex mix. Incubate for 10 minutes at room temperature (15-30 °C).				
Separate either using magnetic separator for 15 minutes or by centrifugation for 10 minutes at >1500 xg .				
Decant tubes and blot on filter paper.				
Count radioactivity (60 sec/tube).				
Calculate the results.				

Note: For the measurement of cAMP in tissues and cell suspensions in the range 2–128 fmol/tube, the buffer, standard and samples are acetylated prior to assay.

Overnight assay protocol

Note: Steps 7–11 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–8. Polystyrene or polypropylene tubes may be used in steps 9–18.

(For a quick guide , refer to Table 1.)

Day 1

1. Prepare assay buffer and standards ranging from 2–128 fmol/100 µl as described in the previous section.
2. Equilibrate all reagents to room temperature and mix before use.
3. Label polypropylene or glass tubes (12 x 75 mm) in for the zero standard tube and unknowns. These will subsequently be known as acetylation tubes.
4. Label polypropylene or polystyrene tubes (12 x 75 mm) in duplicate for total counts (TC), zero standard tubes (Bo), each standard dilution and unknowns. These will subsequently be known as assay tubes.
5. Prepare the acetylation reagent by mixing 1 volume of acetic anhydride with 2 volumes of triethylamine in a glass vessel. Mix well. (Sufficient reagent for 50 acetylations may be obtained by mixing 0.5 ml acetic anhydride with 1.0 ml triethylamine).
6. Pipette **500 µl** of assay buffer into the zero standard acetylation tube.
7. Pipette **500 µl** of each unknown (see sample preparation section) into the appropriately labelled acetylation tubes. Tubes containing **500 µl** of each working standard should already have been prepared (see reagent preparation section).
8. Carefully add **25 µl** of the acetylation reagent to all acetylation tubes containing standards and unknowns. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylation reagents.
9. Pipette duplicate **100 µl** aliquots from all acetylation tubes into the corresponding polystyrene or polypropylene assay tubes.
10. Pipette **100 µl** of antiserum into all tubes except the TC.
11. Pipette **100 µl** of [125I]cAMP into all tubes including TC tubes. The TC tubes should be stoppered and put aside for counting
12. Vortex mix all tubes thoroughly. Cover the tubes, for example with plastic film, and incubate for between 15 and 18 hours at 2–8°C.

Day 2

13. Gently shake and swirl the bottle containing seperating reagent (blue-green) to ensure a homogeneous suspension. Then add **500 µl** to each tube except the TC.
14. Vortex mix all tubes thoroughly and incubate for 10 minutes at room temperature (15–30°C).
15. Separate the antibody bound fraction by using either magnetic separation or centrifugation, as described below.
Magnetic separation
Attach the rack on to the separating reagent base and ensure that all the tubes are in contact with the base plate. Leave for 15 minutes. After separation do not remove the rack from the separator base. Pour off and discard the supernatant liquid. Keeping the separator inverted, place the tubes on a pad of absorbent tissues and allow to drain for 5 minutes. alculation of results

Centrifugation
Centrifuge all tubes for 10 minutes at 1500 xg or greater. After centrifugation, place the tubes carefully into suitable decantation racks then pour off and discard the supernatant liquids. Keeping the tubes inverted, place them on a pad of absorbent tissues and allow to drain for 5 minutes.
16. On completion of either magnetic or centrifugal separation, firmly blot the rims of the inverted tubes on the tissue pad to remove any adhering droplets of liquid. Do not re-invert the tubes once they have been turned upright.
17. Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

Table 3. Overnight protocol, Pipetting Guide
(all volumes are in microlitres)

Tubes	TC	Bo	Stan - dard	Sam-ple
Buffer	**-**	**100**	**-**	**-**
Stan-dard	-	**-**	**100**	**-**
Sample	-	**-**	**-**	**100**
Anti-serum	**-**	**100**	**100**	**100**
Tracer	**100**	**100**	**100**	**100**
Vortex mix and incubate for 15 to 18 hours at 2-8 °C.				
Separa-ting reagent	-	**500**	**500**	**500**
Vortex mix. Incubate for 10 minutes at room temperature (15-30 °C).				
Separate either using magnetic separator for 15 minutes or by centrifugation for 10 minutes at >1500 xg .				
Decant tubes and blot on filter paper.				
Count radioactivity (60 sec/tube).				
Calculate the results.				

Calculation of results

The calculation is illustrated using representative data.The assay data collected should be similar to the data shown in tables 4 and 5.

1. Calculate the average counts per minute (cpm) for each set of replicate tubes.

2. Calculate the percent Bo/TC using the following equation:

$$\text{Bo/TC(\%)} = \frac{\text{Bo (cpm)}}{\text{TC (cpm)}} \times 100$$

If the counter background is high, it should be subtracted from all the counts.

3. Calculate the percent bound for each standard and sample using the following equation:

$$\text{B/Bo(\%)} = \frac{\text{Standard or sample (cpm)}}{\text{Bo (cpm)}} \times 100$$

A standard curve may be generated by plotting the percent B/Bo as a function of the log cAMP concentration.

Plot B/Bo(%) (y axis) against the fmol standard per tube (x axis). The fmol per tube value of the samples can be read directly from the graph. NSB is not normally determined, and is given for information only.

Table 4. Typical assay data for non-acetilation protocol

Tube	Avarage counts/ minute (cpm)	B/TC (%)	B/Bo (%)	Conc. fmol/tube
TC	31735	-	-	-
NSB	240	0.76	-	-
Bo	13836	43.6	100	-
S1	11925	-	86.2	25
S2	10515	-	76	50
S3	8764	-	63.3	100
S4	6939	-	50.1	200
S5	5188	-	37.5	400
S6	3844	-	27.8	800
S7	2683	-	19.3	1600

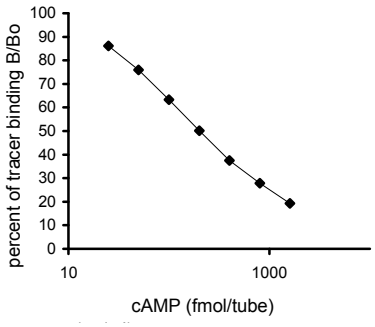


Figure 1: A typical standard curve (non-acetylation assay) (*Do not use to calculate unknown samples!)*

Table 5. Typical assay data for acetylation protocol (within day)

Tube	Conc. (fmol/ tube)	Avarage counts/ minute (cpm)	B/TC (%)	B/Bo (%)
TC	-	25422	-	-
NSB	-	342	1.3	-
Bo	-	10792	42.52	100
S1	2	9698	-	89.9
S2	4	9238	-	85.6
S3	8	7882	-	73.0
S4	16	6282	-	58.2
S5	32	4544	-	42.1
S6	64	3136	-	29.1
S7	128	2066	-	19.1

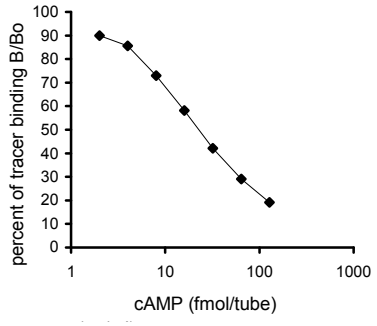


Figure 1: A typical standard curve (acetylation assay, within day protocol)
(*Do not use to calculate unknown samples!)*

Characterization of assay

Magnetic and centrifugal separation methods yield identical assay performance and results.

Stability

The components of this radioimmunoassay system will have a shelf life of at least 4 weeks from the date of despatch.
Upon arrival, all components should be stored at 2–8°C where they are stable until the expiry date printed on the end pack label.

Non-specific binding

The non-specific binding (NSB) defined as the proportion of tracer bound to the antibody in the presence of a 100-fold excess of the highest concentration cAMP standard was determined to be 2.3% (n=27) for both non-acetylation and acetylation assays. The NSB was independent of tracer batch and did not change over a 14 week storage period.

Sensitivity

The sensitivity, defined as the amount of cAMP needed to reduce the zero dose binding by two standard deviations, was 13.5 fmol for nonacetylation assays and 1.0 fmol for measurements after acetylation. For the non-acetylation assay this is equivalent to 0.14 nmol/ml for urine and 2.7 pmol/ml for plasma.

Specificity

Cross-reactivity

The antiserum cross-reactivity with related and other important compounds was determined by the 50% displacement technique. Values for both acetylation and non-acetylation assay systems are shown below:

Compound	(%) Cross-reactivity (acetylation)	(%) Cross-reactivity (non-acetylation)
cAMP	100	100
cIMP	0.01	0.01
cGMP	0.0003	0.006
cCMP	0.04	0.0007
cTMP	0.002	0.0001
AMP	0.001	0.00015
ADP	0.0002	0.00036
ATP	0.00002	0.0004
EDTA	0.0000001	<0.000004
Theophylline	0.000002	0.0002
Isobutyl methylxanthine	0.000008	0.0001

Parallelism

Urine or plasma specimens were serially diluted in assay buffer and the values measured as pmol/assay tube or fmol/assay tube are reported below. Multiplication of the assay value by the dilution factor, and correction for the sample volume assayed, yields the corrected value per ml of urine or plasma. Satisfactory agreement between results at dilutions of 1:500 to 1:8000 for urine or 1:10 to 1:640 for plasma (using the appropriate protocol) is observed. Samples were assayed without extraction.

Urine (non-acetylation)

Dilution	Sample 1		Sample 2		Sample 3	
	fmol /tube	nmol/ml	fmol /tube	nmol/ml	fmol /tube	nmol/ml
1:500	866	4.3	335	1.7	875	4.4
1:1000	420	4.3	150	1.5	441	4.4
1:2000	239	4.3	77	1.5	205	4.1
1:4000	103	4.3	37	1.5	109	4.3

Plasma (non-acetylation)

Dilution	Sample 1		Sample 2		Sample 3	
	fmol /tube	pmol/ml	fmol /tube	pmol/ml	fmol /tube	pmol/ml
1:10	227	22	475	47	269	27
1:15	167	25	252	38	175	26
1:20	150	30	176	35	118	23
1:30	118	35	105	31	71	21
1:40	80	32	58	23	44	17

Plasma (acetylation)

Dilution	Sample 2	
	fmol/tube	pmol/ml
1:50	44.1	22.1
1:150	14.8	22.2
1:450	4.11	8.5
Dilution	Sample 10	
	fmol/tube	pmol/ml
1:60	34.2	20.5
1:120	15.1	18.1
1:240	8.0	19.2

Plasma interference

Plasma matrix effects in the assay are most easily demonstrated at zero analyte concentration. The values obtained from measuring cAMP depleted plasma (obtained by leaving a plasma specimen for 24 hours at 20°C without phosphodiesterase inhibitor) were less than the sensitivity of the assay. Plasma matrix effects are, therefore extremely small.

Comparison

1. Comparison of levels of cAMP in urine determined using the magnetic separation assay with a commercial radioimmunoassay using a conventional precipitation method:

A good correlation (r=0.96, p<0.001) was demonstrated between levels of cAMP measured by magnetic separation (3.9 ± 2.3 nmol/ml) (mean ± standard deviation) and those by the commercial method (3.9 ± 2.2 nmol/ml). The commercial reference method utilized a polyethylene glycol assisted second antibody separation technique.

2. Comparison of levels of cAMP in plasma determined using the magnetic separation assay without acetylation with a commercial radioimmunoassay using a conventional precipitation method after acetylation:

A good correlation (r=0.96, p<0.001) was obtained between levels of cAMP measured by magnetic separation (22.4± 6.2 pmol/ml) and those by the commercial method (24.5 ± 5.3 pmol/ml). The commercial reference method utilized a polyethylene glycol assisted second antibody separation technique. Plasma levels were measured after acetylation.

Recovery

This was determined by adding cAMP to urine and cAMP depleted plasma and re-assaying.

Urine (non-acetylation)

Expected (nmol/ml)	Observed (nmol/ml)	Recovery %
2.79	2.85	102.2
3.91	3.84	98.2
5.70	5-59	98.1
11.0	11.5	105
16.9	18.0	106

Mean recovery = observed = 99.3%
expected

Plasma (non-acetylation)

	Expected	Observed	Recovery (%)
Sample1	33.3	35.8	107
Sample1	46.6	48.7	104
Sample1	105	120	114
Sample2	48.3	49.1	102
Sample2	61.4	69.5	113
Sample2	100	116	116

Mean recovery = observed = 101%
expected

Plasma (acetylation)

	Expected	Observed	Recovery (%)
Sample1	65	65.4	101
Sample1	109	105	96
Sample1	143	143	100
Sample2	70.8	66	93
Sample2	113	113	100
Sample2	145	149	103

Reproducibility

Between-batch reproducibility was further assessed from repeated analysis of the same samples in successive assays. These are typical of the reproducibility between batches and operators.

Non-acetylation

	Mean (fmol/tube)	SD	CV (%)	n
Urine control1	429	46	10.8	26
Urine control2	99	13	12.7	27
Plasma control1	371	36	9.7	27
Plasma control2	103	13	12.6	25
Plasma control3	57	8	13.6	24

Acetylation

	Mean (fmol/tube)	SD	CV (%)	n
Plasma control1	48.5	3.3	6.8	27
Plasma control2	24.9	1.3	5.4	26
Plasma control3	5.5	0.4	7.0	26

Additional information

Components from various lots or from kits of different manufacturers should not be mixed or interchanged.
Do not use lipaemic, haemolyzed or turbid specimens. Avoid repeated freezing and thawing of specimens.
Renal function and diseases which alter PTH concentrations can influence cAMP concentration in urine and plasma. Other factors that have been reported to alter plasma cAMP concentrations and urinary excretion are pregnancy, certain drugs (for example adrenalin) and exercise.

Precautions

Radioactivity
This product contains radioactive material. It is the responsibility of the user to ensure that local regulations or code of practice related to the handling of radioactive materials are satisfied.

Chemical hazard
Components contain sodium azide as an antimicrobial agent. Dispose of waste by flushing with copious amount of water to avoid build-up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 58 mg.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

Safety data sheets

1. Product name:

Sodium azide
CAS No. 26628-22-8
R: 22-32 Toxic if swallowed. Contact with acids liberates very toxic gas.
S: (1/2)-28-45 (Keep locked up and out of the reach of children). After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
Composition:
Sodium azide solution.
Hazards identification:
Toxic if swallowed, inhaled, or absorbed through skin. May cause eye and skin irritation.
First aid measures:
In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.
Fire fighting measures:
Dry chemical powder. Do not use water.
Accidental release:
Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Mop up spill area, place waste in a bag and hold for waste disposal. Wash spill site area after material pick-up is complete.
Handling and storage:
Wear suitable protective clothing including overalls, safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Formula weight: 65.01. Density: 1.850.
Stability and reactivity:
Avoid contact with metals and acid chlorides. This yields a very toxic gas.
Toxicological information:
LD50: 27 mg/kg oral, rat
LD50: 20 mg/kg skin, rabbit
Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Note: Inorganic azides will react with lead and copper plumbing fixtures to give explosive residues. Disposal of significant quantities of azides via such plumbing is not recommended.
Transport information :
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.

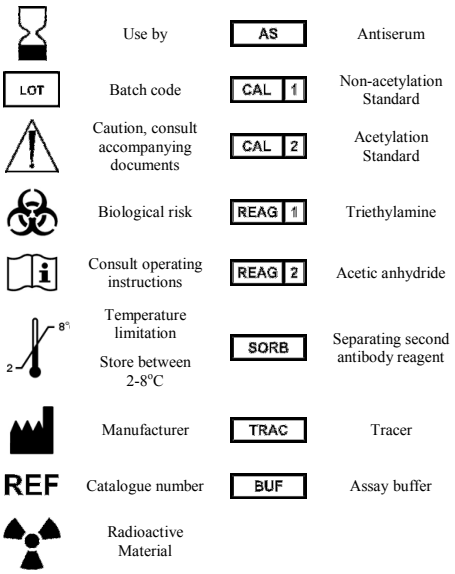
2. Product name:

Triethylamine
CAS No. 121-44-8
R: 11-36-37 Highly flammable. Irritating to eyes. Irritating to respiratory system.
S: (2-)16-26-29 (Keep out of reach of children). Keep away from sources of ignition-no smoking. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
Composition:
Colourless liquid.
Hazards identification:
Highly flammable. Irritating to eyes. Irritating to the respiratory system. Harmful it swallowed, inhaled or absorved through the skin. Causes burns.
First aid measures:
In case of concact, immediately flush eyes or skin with copiousamounts of water. Remove contaminated clothing and shoes. If inhaled remove to fresh air. In severe cases seek medical attention.
Fire fighting measures:
Carbon dioxide, dry chemical powder or polymer foam.
Accidental release:
Shut of all sources ignition. Wear suitable laboratory protective equiqment; lab coats, gloves and safety glasses. Cover with dry lime, sand or soda ash. Place in covered containers using non sparking tools and transport outdoors.
Handling and storage:
Wear suitable protective clothing including laboratory overalls,safetyglasses and gloves. Do not get in eyes, on skin, or on clothing. Do not breathe the vapour. Wash thoroughly after handling. Keep tightly closed. Keep away from heat, sparks and open flame.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Melting point: -7 °C. Boiling point: 88.8°C. Vapour pressure: 54.0 mm (20 °C). Lower explosion limit: 1.2. Upper explosion limit: 8.0. Flashpoint: 20°F. Formula weight: 101.19. Density: 0.726. Vapour density: 3.5.
Stability and reactivity:
Incompatible with acids and oxidizing agents. Therral decomposition may produce carbon monoxide, carbon dioxide and nitrogen oxides.
Toxicological information:
Skin-rabbit: 10mg/24h open mld. Skin-rabbit: 365mg open mld. Eye-rabbit: 250µg open sev. Eye-rabbit:50ppm/30d-I sev.
LD50: 460 mg/kg oral, rat
LD50: 570 mg/kg skin, rabbit
LD50: 6 g/m3/2 h inh., mus

Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Transport information :
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.

3. Product name:

Acetic anhydride
CAS No. 108-24-7
R: 10-34 Flammable. Causes burns.
S: (1/2-)26-45 (Keep locked up and out of of the reach of children). In case of contact with eyes, rinse immediately with plenty of water of seek medicial advice. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
Composition:
Acetic anhydride concentrate.
Hazards identification:
Flammable. Causes burns. Harmful it swallowed, inhaled or absorved through the skin.Matrial is destructive to the tissues of the mucous membranes, upper respiratory tract, eyes and skin.
First aid measures:
In case of concact, immediately flush eyes or skin with copious of water. Remove articles of contaminated clothing and shoes. Ensure adequate flushing of contaminated eyes by separating the eyelids with fingers. If inhaled remove to fresh air. In severe cases seek medical attention.
Fire fighting measures:
Carbon dioxide, dry chemical powder or polymer foam.
Accidental release:
Wear suitable laboratory protective equiqment; lab coats, gloves and safety glasses. Cover with activated carbon absorbent. Take up and place in a closed container. Transport outdoors. Ventilate area and wash spill after material pickup is complete.
Handling and storage:
Wear suitable protective clothing including laboratory overalls ,safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Do not breathe the vapour. Wash thoroughly after handling. Keep tightly closed. Keep away from heat, sparks and open flame.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Melting point: -73°C. Boiling point: 138-140°C. Vapour pressure: 4.6 mm (20 °C). Lower explosion limit: 2.8. Upper explosion limit: 10.3. Flashpoint: 130°F. Formula weight: 102.09. Density: 1.080. Vapour density: 3.52. Auto ignition temperature: 629°F.
Stability and reactivity:
Acids, bases, moisture, alcohols, oxidizing agents, reducing agents and finely powdered metals.
Toxicological information:
Skin-rabbit: 10 mg/24 h open mld. Skin-rabbit: 540 mg open mld. Eye-rabit: 250 microgramm open sev.
Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of they should pass through a chemical waste route.
Transport information :
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.



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