GE Healthcare

Amersham cAMP [^{125}I] Biotrak Assay System (dual range) with Magnetic Separation for all Biological Samples

100 tubes

Product Booklet

Code: RPA509

Page finder

1. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited.
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.* Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: Radioactive material.

Instructions relating to the handling, use, storage and disposal of radioactive materials.

1. Upon receipt, vials or ampoules containing radioactive material should be checked for contamination. All radioactive materials should be stored in specially designated areas and suitable shielding should be used where appropriate. Access to these areas should be restricted to authorized personnel only.
2. Radioactive material should be used by responsible persons only in authorized areas. Care should be taken to prevent ingestion or contact with skin or clothing. Protective clothing, such as laboratory overalls, safety glasses and gloves should be worn whenever radioactive materials are handled. Where this is appropriate, the operator should wear personal dosimeters to measure radiation dose to the body and fingers.
3. No smoking, drinking or eating should be allowed in areas where radioactive materials are used. Avoid actions that could lead to the ingestion of radioactive materials, such as the pipetting of radioactive solutions by mouth.
4. Vials containing radioactive materials should not be touched by hand; wear suitable protective gloves as normal practice. Use forceps when handling vials containing 'hard' beta emitters such as

phosphorus-32 or gamma emitting labelled compounds. Ampoules likely to contain volatile radioactive compounds should be opened only in a well ventilated fume cabinet.

5. Work should be carried out on a surface covered with absorbent material or in enamel trays of sufficient capacity to contain any spillage. Working areas should be monitored regularly.
6. Any spills of radioactive material should be cleaned immediately and all contaminated materials should be decontaminated or disposed of as radioactive waste via an authorized route. Contaminated surfaces should be washed with a suitable detergent to remove traces of radioactivity.
7. After use, all unused radioactive materials should be stored in specifically designated areas. Any radioactive product not required or any materials that have come into contact with radioactivity should be disposed of as radioactive waste via an authorized route.
8. Hands should be washed after using radioactive materials. Hands and clothing should be monitored before leaving the designated area, using appropriate instruments to ensure that no contamination has occurred. If radioactive contamination is detected, hands should be washed again and rechecked. Any contamination persisting on hands and clothing should be reported to the responsible person so that suitable remedial actions can be taken.
9. Certain national/international organizations and agencies consider it appropriate to have additional controls during pregnancy. Users should check local regulations.

Most countries have legislation governing the handling, use, storage, disposal and transportation of radioactive materials. The instructions set out above complement local regulations or codes of practice. Such regulations may require that a person be nominated to oversee radiological protection. Users of radioactive products must make themselves aware of and observe the local regulations or codes of practice which relate to such matters.

Warning: Contains sodium azide, triethylamine and acetic anhydride. See safety data sheets supplied.

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 sheets for specific advice).

2.2. Storage

Store at 2–8°C.

2.3. Expiry

The expiry date is stated on the package and will be at least 4 weeks from the date of despatch.

3. Components of the assay system

The pack contains the following assay components.

Standard (for urine, plasma and tissue determinations)
cAMP standard 64 pmol, for non-acetylation assays in the range 25–1600 fmol/tube. Lyophilized. On reconstitution this bottle contains 32 pmol cAMP/ml.

Standard (for tissue determination)
cAMP standard 5.12 pmol, for acetylation assays in the range 2–128 fmol/tube, lyophilized. On reconstitution this bottle contains 2.56 pmol cAMP/ml.

Tracer
Adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I] iodotyrosine methyl ester ~59 kBq, 1.6 µCi, lyophilized.

Antiserum
Rabbit anti-succinyl cAMP serum, lyophilized.

Amerlex-M second antibody reagent
Donkey anti-rabbit serum coated on to magnetizable polymer particles containing sodium azide. Colour coded blue-green, 55 ml, ready for use.

Buffer
Assay buffer concentrate 10 ml. On dilution this bottle contains a 0.05 M acetate buffer wth preservative.

Acetic anhydride
1 ml ready for use. Caution: flammable, corrosive, causes burns.

Triethylamine
2 ml ready for use. Caution: flammable, harmful vapour.
See safety data sheet supplied.

4. Critical parameters

- Use a sample preparation procedure appropriate for the type of specimen under test.
- All reagents should be allowed to equilibrate to room temperature before use.
- When using the acetylation protocol, the acetylation step should be performed as quickly as possible.

5. Additional materials and equipment required

The following materials and equipment are required:

Pipettes or pipetting equipment with disposable tips (25 µl, 100 µl, 500 µl, 2.0 ml and 11.0 ml)

Vortex mixer

Refrigerator

Disposable polypropylene or glass tubes (12x75 mm) for acetylation procedure

Disposable polystyrene or polypropylene tubes (12x75 mm) for assay procedure

Glass measuring cylinder (500 ml)

Distilled or deionized water

Gamma scintillation counter

Amerlex-M separators, comprising magnetic base and assay rack, are available from Johnson & Johnson. These are for use in the magnetic protocol.

Additional packs of 4 assay racks are also available from Johnson & Johnson.

Disposable polypropylene tubes are supplied by Sarstedt International Rommelsdore, 5223 Numbrecht, West Germany

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

Decantation racks

Refrigerated centrifuge capable of 2000 xg

6. Description

- Simple, non-centrifugal separation
- Precise and accurate measurements
- Choice of assay range: includes ultra-high sensitivity option (2–128 fmol/tube; 7–420 pg/ml)
- Plasma and urine determinations without extraction or acetylation
- HPLC purified high specific activity iodinated tracer

The Biotrak™ cAMP[^{125}I] assay system from GE Healthcare provides a simple, reliable and precise quantitative determination of cAMP in urine, plasma, tissues and other biological samples. The system utilizes a high specific activity [^{125}I]2′-0-succinyl-cAMP tyrosine methyl ester tracer, together with a highly specific and sensitive antiserum. Separation of the antibody bound from free fraction is achieved with a second antibody Amerlex™-M preparation, thus allowing a simple magnetic separation.

The assay is based on the competition between unlabelled cAMP and a fixed quantity of ^{125}I-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 (figure 1).

The antibody bound cAMP is then reacted with the Amerlex-M 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 Amerlex-M 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.

Non-acetylation protocol

This enables measurement of cAMP in urine, plasma, tissues and cell cultures in the range 25–1600 fmol/tube.

Acetylation protocol

This enables measurement of low levels of cAMP in small quantities of biological materials in the range 2-128 fmol/tube.

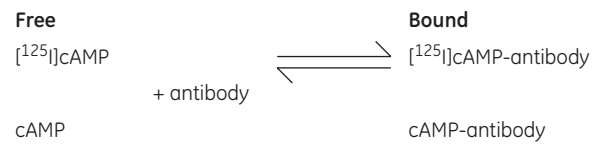

Figure 1.

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. The sensitivity of the non-aceylation protocol is 44 pg/ml and the acetylation protocol, 3 pg/ml. Each pack contains sufficient material for 100 assay tubes and includes reagents for the acetylation reaction. If one standard curve is constructed, 42 unknowns can be measured in duplicate. If two curves are constructed then 34 unknowns can be measured.

7. Specimen collection and sample preparation

- 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(24-27).
- Some investigators also recommend the use of ion exchange chromatography(25) 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

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

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

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* (23).

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: To eliminate lengthy sample extractions GE Healthcare have developed some novel lysis reagents. These reagents are provided in the cAMP EIA (RPN 225) and the cAMP SPA kits (RPA 538, RPA 542 and RPA 559). Simply add lysis reagent to the cells, incubate for 5 minutes and assay.

GE Healthcare has developed a simple protocol for the extraction and purification of cAMP from biological samples by ion-exchange chromatography using disposable Amprep™ minicolumns.

Maximum recovery of cyclic AMP is obtained using columns containing anion exchange silica sorbents, for example Amprep SAX, code RPN 1918 (500 mg) which are available from GE Healthcare. These columns provide a rapid sample clean-up and effectively reduce sample handling compared with solvent extraction methods.

Representative procedures are described below for the extraction of cAMP from biological samples. Both Amprep and liquid phase extraction methods are described. However 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* (23).

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.

The above protocol was kindly provided by Dr B L Brown, University of Sheffield.

Solid phase extraction method
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. 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 M HCl diluted in distilled water rather than methanol can be used to elute the analyte.

8. Assay procedure

8.1. Reagent preparation

Note: All reagents should be allowed to equilibrate to room temperature. Either distilled or deionized water may be used for reagent preparation, acetic anhydride and triethylamine are supplied ready for use. Reconstituted components should be stored at 2–8°C and may be re-used within 14 days of dilution.

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. The diluted buffer contains 0.05 M acetate buffer pH5.8, with preservative.

Tracer
Carefully add 11.0 ml diluted buffer and replace the stopper. Mix the contents of the bottle by inversion and swirling.
The solution will contain adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I] iodotyrosine methyl ester in 0.05 M acetate buffer containing preservative.

Antiserum
Carefully add 11.0 ml assay buffer and replace the stopper. Gently mix the contents of the bottle by inversion and swirling until complete solution is obtained. Vigorous agitation and foaming should be avoided. The contents will contain anti-cAMP serum in 0.05 M acetate buffer containing 0.5% bovine serum albumin and preservative.

Standard (for non-acetylation assay)
Carefully add 2.0 ml assay buffer and replace the stopper. Mix the contents of the bottle until completely dissolved. The final solution should contain cAMP at a concentration of 32 pmol/ml in 0.05 M acetate buffer containing preservative.

Standard (for acetylation assay)
Carefully add 2.0 ml assay buffer and replace the stopper. Mix the bottle until the contents are completely dissolved. The final solution should contain cAMP at a concentration of 2.56 pmol/ml in 0.05 M acetate buffer containing preservative.

8.2. 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 protocol

1. Prepare assay buffer and standards ranging from 25–1600 fmol as described in the previous section.
2. Equilibrate all reagents to room temperature and mix before use.
3. Label polypropylene or polystyrene tubes in duplicate for total counts (TC), zero standard tubes (B_0), standards and samples.
4. Pipette 100 µl of assay buffer into the zero standard tubes (B_0).

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 µl of [^{125}I]cAMP into all tubes.

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 Amerlex-M second antibody 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 Amerlex-M separator 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.

8.3. 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 500 µl.

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 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.

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 (1 2 ×75 mm) in duplicate for total counts (TC), zero standard tubes (B_0), 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 [^{125}I]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 Amerlex-M second antibody 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 Amerlex-M separator 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 1. Within day acetylation protocol (all volumes are in microlitres)

	Total counts (TC)	**Zero standard (B_0)**	**Standards**	**Samples**
Buffer*	-	100	-	-
Standard*	-	-	100	-
Samples*	-	-	-	100
Antiserum	-	100	100	100
Tracer	100	100	100	100
Vortex mix, cover tubes and incubate at 2–8°C for 4 hours.				
Amerlex-M second antibody	-	500	500	500
Vortex mix. Incubate for 10 minutes at room temperature. Separate **either** using Amerlex-M separator for 15 minutes **or** by centrifugation for 10 minutes at >1500 xg. Decant supernatants, drain for 5 minutes and count.				

* 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 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.

* This protocol is not subject to routine quality control

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 (B_0), 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 [^{125}I]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 Amerlex-M second antibody 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 Amerlex-M separator 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. Overnight acetylation protocol (all volumes are in microlitres)

	Total counts (TC)	**Zero standard (B_0)**	**Standards**	**Samples**
Buffer*	-	100	-	-
Standard*	-	-	100	-
Samples*	-	-	-	100
Antiserum	-	100	100	100
Tracer	100	100	100	100
Vortex mix, cover tubes and incubate for 15 to 18 hours at 2–8°C.				
Amerlex-M second antibody	-	500	500	500
Vortex mix. Incubate for 10 minutes at room temperature. Separate **either** using Amerlex-M separator for 15 minutes **or** by centrifugation for 10 minutes at >1500 xg. Decant supernatants, drain for 5 minutes and count.				

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

9. Data processing

9.1. Calculation of results

The calculation is illustrated using representative data.

The assay data collected should be similar to the data shown in tables 2 and 3.

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

2. Calculate the percent B_0/TC using the following equation:

$$\%/B_0/TC = \frac{B_0 \text{ cpm}}{\text{TC cpm}} \times 100$$

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:

$$\%B/B_0 = \frac{(\text{Standard or sample cpm}) \times 100}{B_0 \text{ cpm}}$$

A standard curve may be generated by plotting the percent B/B0 as a function of the log cAMP concentration.

Plot $\%B/B_0$ (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 (see figures 2 and 3).

NSB is not normally determined, and is given for information only.

9.2. Typical assay data

Table 2. Typical assay data: for non-acetylation protocol

Tube	Counts/ minute*	Average counts/minute	%B/TC	%B/B_0
Total count(TC)	31589 31880	31735	-	-
Non-specific binding† (NSB)	199 281	240	0.76	-
Zero standard (B_0)	13431 14240	13836	43.6	100.0
25 fmol standard	11711 12139	11925	-	86.2
50 fmol standard	10274 10755	10515	-	76.0
100 fmol standard	8586 8938	8764	-	63.3
200 fmol standard	6963 6914	6939	-	50.1
400 fmol standard	5173 5202	5188	-	37.5
800 fmol standard	3915 3773	3844	-	27.8
1600 fmol standard	2752 2613	2683	-	19.3

* Corrected for instrument blank.

† Non-specific binding is not usually determined and is given for information only.

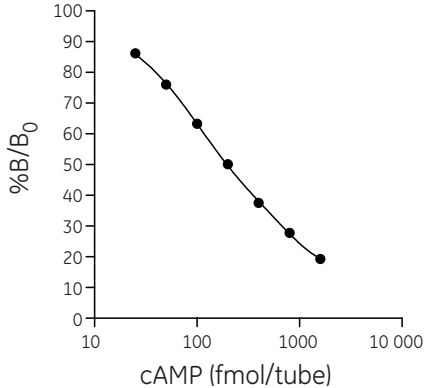

Figure 2. Typical standard curve: non-acetylation protocol

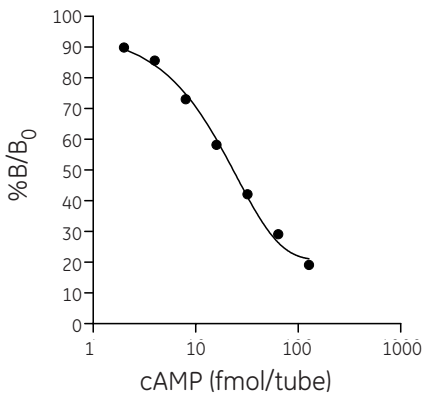

Figure 3. Typical standard curve: acetylation protocol (within day assay)

Table 3. Typical assay data: for acetylation protocol (within day)

Tube	Counts/ minute*	Average counts/minute	%B/TC	%B/B_0
Total count (TC)	25521 25322	25422	-	-
Non-specific binding (NSB)†	337 347	342	1.3	-
Zero standard (B_0)	10621 10963	10792	42.5.2	100.0
2 fmol standard	9653 9742	9698	-	89.9
4 fmol standard	9250 9226	9238	-	85.6
8 fmol standard	7832 7932	7882	-	73.0
16 fmol standard	6277 6286	6282	-	58.2
32 fmol standard	4550 4537	4544	-	42.1
64 fmol standard	3105 3167	3136	-	29.1
128 fmol standard	2037 2095	2066	-	19.1

* Corrected for instrument blank.

† Non-specific binding is not usually determined and is given for information only.

10. Additional information

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

10.1. Limitations of use

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.

10.2. 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	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	22.7	475	47.5	269	26.9
1:15	167	25.1	252	37.8	175	26.3
1:20	150	30.0	176	35.2	118	23.6
1:30	118	35.4	105	31.5	71	21.3
1:40	80	32.0	58	23.2	44	17.6

Plasma (acetylation)

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

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.

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 (see page 34). Plasma matrix effects are, therefore extremely small.

Comparison of levels of cAMP in urine determined using the Biotrak 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 (see figure 4).

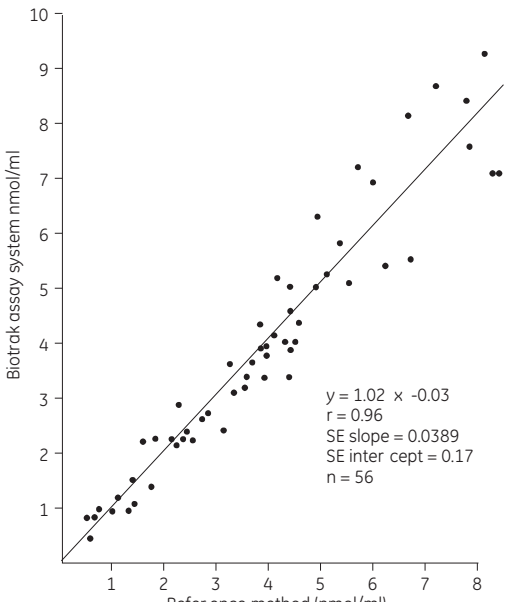

Figure 4. Comparison of urinary cAMP values obtained using the Biotrak assay system and a reference method.

Comparison of levels of cAMP in plasma determined using the Biotrak 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 (see figure 5).

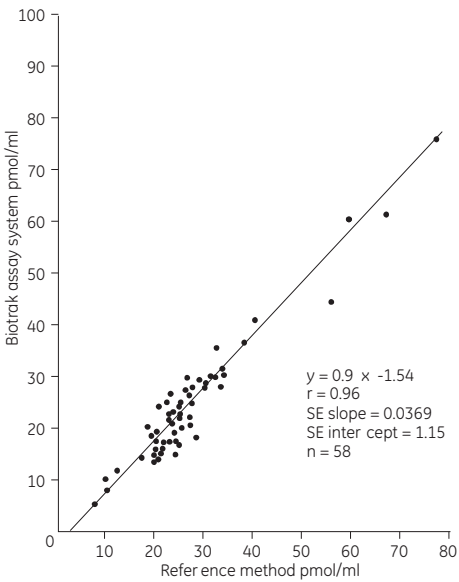

Figure 5. Comparison of plasma cAMP values obtained using the Biotrak assay system and a reference method.

10.3. 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 non-acetylation 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.

10.4. Reproducibility

The between-assay reproducibility for duplicate determinations is presented graphically as precision profile in figures 6 and 7. This was determined by pooling the standard deviation of duplicate determinations over 20 assay runs using different batches of reagents.

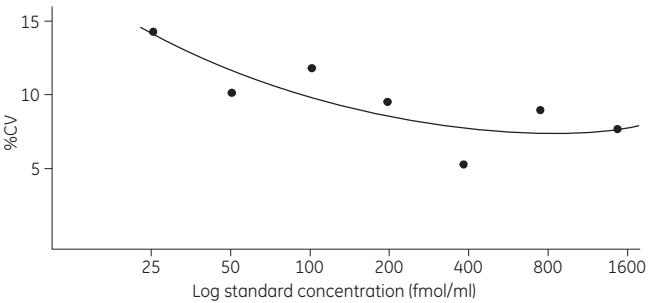

Figure 6. Precision profile - non-acetylation protocol.

Note: The line drawn represents the line of best fit for the parabola as determined by least squares.

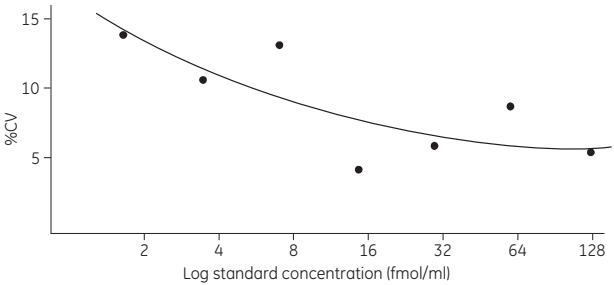

Figure 7. Precision profile - acetylation protocol.

Note: The line drawn represents the line of best fit for the parabola as determined by least squares.

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	Standard deviation	Coefficient of variation (%)	Sample number
Urine control 1	429	46	10.8	26
Urine control 2	99	13	12.7	27
Plasma control 1	371	36	9.7	27
Plasma control 2	103	13	12.6	25
Plasma control 3	57	8	13.6	24

Acetylation

	Mean fmol/tube	Standard deviation	Coefficient of variation (%)	Sample number
Control 1	48.5	3.3	6.8	27
Control 2	24.9	1.3	5.4	26
Control 3	5.5	0.4	7.0	26

10.5. 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 = $\frac{\text{observed}}{\text{expected}}$ = 99.3%

Plasma (non-acetylation)

	Expected	**Observed**	**Recovery (%)**
Sample 1	33.3	35.8	107
	46.6	48.7	104
	105	120	114
Sample 2	48.3	49.1	102
	61.4	69.5	113
	100	116	116

Mean recovery = $\frac{\text{observed}}{\text{expected}}$ = 101%

Plasma (acetylation)

	Expected	Observed	Recovery (%)
Sample 1	65	65.4	101
	109	105	96
	143	143	100
Sample 2	70.8	66	93
	113	113	100
	145	149	103

Mean recovery = $\frac{\text{observed}}{\text{expected}}$ = 105.3%

10.6. Amerlex-M accessories

Amerlex-M separator (comprising 50 tube rack and magnetic base)

Amerlex-M multivortexer

Amerlex-M racks (4 in a pack)

These products are available from Johnson & Johnson, or their assigned distributors.

11. Background

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 (1).

cAMP was identified as playing a major role in the mode of action of adrenaline 30 years ago (2–5). 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 (6) and nervous systems (7), in immune mechanisms (8), cell growth and differentiation (9) and general metabolism (10). 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 (11–14). The method allows high sensitivity without the interference from ATP to which other adenylate cyclase assays are prone (14).

In recent years there has been great interest in a new generation of phosphoinositide-derived second messengers (15–20).

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 (IP_3) and *sn*-1,2-diacylglycerol (DAG).

It is now well established that IP3 acts as a second messenger of Ca^{2+} mobilized hormones in a variety of cell types (21). DAG appears to be an essential cofactor for the enzyme protein kinase C which plays a crucial role in signal transduction (17,22).

Levels of IP_3 and DAG can be determined using GE Healthcare assay systems TRK 1000 and RPN 200.

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13. Related products

Signal transduction assay range

*Cyclic AMP, direct	EIA	RPN225
Cyclic AMP, [^{3}H]	RR	TRK432
*Cyclic AMP, [^{125}I], direct	SPA	RPA538
*Cyclic AMP, [^{125}I], direct	SPA	RPA542
Cyclic AMP screening assay	SPA	RPA556
*Cyclic AMP screening direct assay	SPA	RPA559
Cyclic GMP	EIA	RPN226
Cyclic GMP, [^{3}H]	RIA	TRK500
Cyclic GMP, [^{125}I]	RIA/AM	RPA525
Cyclic GMP, [^{125}I]	SPA	RPA540
sn-1,2-Diacylglycerol (DAG)	EA	RPN200
D-*myo*-Inositol 1,4,5-trisphosphate (IP_3), [^{3}H]	RR	TRK1000
MAP kinase	EA	RPN84
Protein kinase C	EA	RPN77

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