GE Healthcare
Life Sciences

Amersham cAMP Biotrak Enzymeimmunoassay (EIA) System

Product booklet

Codes: RPN225
RPN2251
RPN2255

Page finder

1. Legal

http//www.gelifesciences.com

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.

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 material safety data sheet(s) and/or safety statement(s) for specific advice.

Note: The assay protocol may require the use of Sulfuric acid.

Warning: Sulfuric acid is corrosive.

Please follow the manufacturer's safety data sheet relating to the safe handling and use of this material.

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

RPN225

The pack contains the following assay components, sufficient material for 96 wells. All components for this kit should be stored at 2–8°C.

Microplate: The plate contains 12 x 8 well strips coated with donkey anti-rabbit IgG, ready for use.

Assay buffer: Assay buffer concentrate. On dilution this bottle contains 0.05 M Sodium Acetate buffer, pH 6.0 containing 0.002%(w/v) Bovine Serum Albumin and 0.01%(w/v) preservative.

Standard (for non-acetylation assay): cAMP standard for non-acetylation assays in the range 25–6400 fmol/well, lyophilised. On reconstitution this bottle contains 64 pmol cAMP/ml.

Standard (for acetylation assay): cAMP standard 10.24 pmol, for acetylation assays in the range 2–128 fmol/well, lyophilised. On reconstitution this bottle contains 2.56 pmol cAMP/ml.

Antibody: Rabbit anti-cAMP, lyophilised.

Peroxidase conjugate: cAMP-Horseradish Peroxidase, lyophilised.

Wash buffer concentrate: Wash buffer concentrate. On dilution the reagent contains 0.01 M Phosphate buffer, pH 7.5 containing 0.05% Tween™ 20.

TMB substrate: Enzyme substrate containing 3,3',5,5' -Tetramethylbenzidine (TMB)/< 1% Hydrogen Peroxide. Ready for use.

Acetic Anhydride: Ready for use. Caution: flammable, corrosive, causes burns.

Triethylamine: Ready for use. Caution: flammable, harmful vapour.

Lysis reagent 1: Dodecyltrimethylammonium Bromide, 2 g, solid. Caution: harmful

Lysis reagent 2: 5 g, Solid.

RPN2251
The pack contains the following assay components, sufficient material for 96 wells. All components for this kit should be stored at 2–8°C.

Microplate: The plate contains 12 x 8 well strips coated with donkey anti-rabbit IgG, ready for use.

Assay buffer: Assay buffer concentrate. On dilution this bottle contains 0.05 M Sodium Acetate buffer,
pH 6.0 containing 0.002%(w/v) Bovine Serum Albumin and 0.01%(w/v) preservative.

Standard (for non-acetylation assay): cAMP standard for non-acetylation assays in the range 25–6400 fmol/well, lyophilised. On reconstitution this bottle contains 64 pmol cAMP/ml.

Antibody: Rabbit anti-cAMP, lyophilised.

Peroxidase conjugate: cAMP-Horseradish Peroxidase, lyophilised.

Wash buffer concentrate: Wash buffer concentrate. On dilution the reagent contains 0.01 M Phosphate buffer, pH 7.5 containing 0.05% Tween™ 20.

TMB substrate: Enzyme substrate containing 3,3',5,5' -Tetramethylbenzidine (TMB)/ Hydrogen Peroxide. Ready for use.

Lysis reagent 1: Dodecyltrimethylammonium Bromide, 2 g, solid. Caution: harmful

Lysis reagent 2: 5 g, Solid.

RPN2255
The pack contains the following assay components, sufficient material for 480 wells. All components for this kit should be stored at 2–8°C.

Microplate x5: The plate contains 12 x 8 well strips coated with donkey anti-rabbit IgG, ready for use.

Assay buffer: Assay buffer concentrate. On dilution this bottle contains 0.05 M Sodium Acetate buffer,
pH 6.0 containing 0.002%(w/v) Bovine Serum Albumin and 0.01%(w/v) preservative.

Standard (for non-acetylation assay) x5: cAMP standard for non-acetylation assays in the range 25–6400 fmol/well, lyophilised. On reconstitution this bottle contains 64 pmol cAMP/ml.

Antibody: Rabbit anti-cAMP, lyophilised.

Peroxidase conjugate: cAMP-Horseradish Peroxidase, lyophilised.

Wash buffer concentrate: Wash buffer concentrate. On dilution the reagent contains 0.01 M Phosphate buffer, pH 7.5 containing 0.05% Tween™ 20.

TMB substrate: Enzyme substrate containing 3,3',5,5' -Tetramethylbenzidine (TMB)/ Hydrogen Peroxide. Ready for use.

3.1. Other materials required

The following materials and equipment are required but not supplied:

- Pipettes or pipetting equipment with disposable polypropylene tips (50 µl, 100 µl, 500 µl, 1 ml and 5 ml)
- Disposable polypropylene test tubes
- Vortex mixer
- Refrigerator
- Glass measuring cylinders (50 ml, 100 ml, 500 ml)
- Distilled or deionised water
- Spectrophotometric plate reader capable of measuring at 450 nm
- 1.0 M Sulphuric acid
- Microplate shaker
- Magnetic stirrer and stirrer bars
- 0.4% Trypan blue solution
- Centrifuge and microplate holders for centrifuge (if using suspension cells).
- Automatic plate washer (optional)

4. Critical parameters

Important

The following points are critical:

When carrying out RPN225, RPN2251 and RPN2255 assays, please take particular note of the following instructions regarding critical steps:

- It is essential to read the complete instruction booklet before starting work.
- This is a delayed addition assay. Do not empty and wash the plate before adding the Peroxidase conjugate.
- Allow samples and all reagents to reach room temperature prior to performing the assay.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents.
- Avoid handling the tops of the wells both before and after filling.
- Keep the wells covered with lids except when adding reagents and reading.
- Standards and samples should be assayed in duplicate.
- Run a separate standard curve for each microplate.
- Carry out a microscope evaluation, with 0.4% Trypan blue, before and after lysing cells.
- **Thoroughly** wash the plate before adding the substrate. You can do this either manually or automatically provided that the following points are noted:

Manual plate washing

- Use a wash bottle.
- Completely fill each well with wash buffer.

- Completely empty each well between washes.
- After the final wash it is **essential** that all wells are emptied. Tap the plate briskly on a pad of tissues to effect this.

Automatic plate washing

- Ensure that all wells are filled and emptied **completely** with each cycle.
- Uneven washing will cause poor results. If you doubt the effectiveness of your instrument, wash manually as described above.
- There is no difference in results when using either automated or hand washing procedures, if the instrument is carefully maintained.
- To aid efficient washing in automated plate washers, the strips should be levelled with the edge of the microplate lid before each washing stage.

5. Description

The cAMP Biotrak™ competitive enzymeimmunoassay system from GE Healthcare is specifically designed for research purposes. RPN225 and RPN2251 includes protocols using novel lysis reagents in order to facilitate simple and rapid extraction of cAMP from cell cultures. These components avoid the requirement for traditional, time-consuming extraction procedures and obviate the need for removal of extraction reagents prior to measurement. It combines the use of a Peroxidase-labelled cAMP conjugate, a specific antiserum which can be immobilised on to pre-coated microplates, and a one-pot stabilised substrate solution.

Each pack of RPN225 and RPN2251 contains sufficient material for 96 wells. Each pack of RPN2255 contains sufficient material for 480 wells. Each plate allows the construction of one standard curve and the measurement of at least 36 unknowns in duplicate. The procedure may be carried out in one of four ways depending on the kit used. Please see section on protocol selection (see pages 14–15).

- Elimination of inconvenient, time-consuming extraction procedures
- Flexible method-choice of assay protocols (see pages 14–15)
- Rapid assay protocol
- Dual range 25–6400 fmol/well (non-acetylation protocol) 2–128 fmol/well (acetylation protocol) with RPN225
- Non-radioactive
- Specific for cAMP
- Precise and accurate measurement
- Ready to use substrate
- Colour coded reagents

Lysis reagent 1 hydrolyses cell membranes to release intra-cellular cAMP. Lysis reagent 2 sequesters the key component in lysis reagent 1 and ensures cAMP is free for subsequent analysis. The detergent/sequestrant complex does not interfere with antigen: antibody binding. Lysis reagent 1 is simply added to cultured cells, followed by a 5–10 minute incubation before assay (figure 1). The antiserum is reconstituted with lysis reagent 2. The assay is based on competition between unlabelled cAMP and a fixed quantity of Peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody (figure 2).

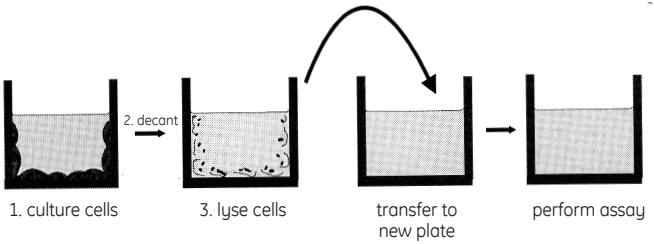


Fig 1. Cell lysis-protocol 3 intracellular method

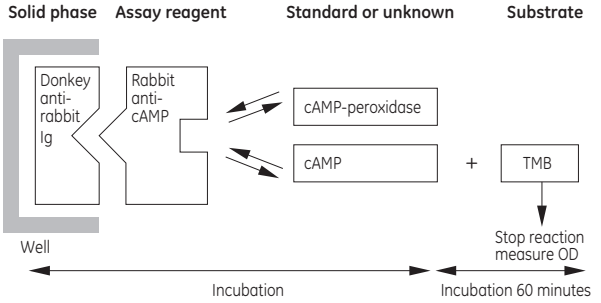


Fig 2. EIA principle

6. Protocol Selection

RPN225 suitable for use with protocols one, two, three or four.
RPN2251 suitable for use with protocols one, three or four.
RPN2255 suitable for use with protocol one.

Protocol 1. The normal non-acetylation assay (range 25–6400 fmol/well) is used for the measurement of cAMP in Urine and tissue extracts and from cell cultures prepared with traditional sample extraction methods such as acid, solvent and solid-phase methods.

Protocol 2. The acetylation assay (range 2–128 fmol/well) is used for the measurement cAMP in plasma, or in tissue extracts where higher sensitivity is required.

Protocol 3. This method describes a non-acetylation assay using the novel lysis reagents enabling the simple, direct measurement of cAMP in cultured cells where cells are grown in flasks, vessels or on plates. Cells are lysed for 10 minutes (with the reagents provided in the kit), and an aliquot is transferred to a second plate for assay. cAMP is measured in the range 25–6400 fmol/well.

Protocol 4. This method also uses the non-acetylation assay with the lysis reagents. Here the combined amount of intracellular and cell supernatant cAMP is measured. This fraction is referred to as 'total' cellular cAMP and has the additional benefit of not requiring decantation of the cell culture supernatant. cAMP is measured in the range 25–6400 fmol/well.

Curve range	Urine samples, No sample extraction needed	Plasma and tissue samples, Sample extraction required	Plasma and tissues samples where higher sensitivity is needed. Sample extraction required.	Cell cultures Using traditional extraction methods	Cell cultures Intracellular cAMP measurement. Lysis reagents and protocols provided.	Cell cultures Total cellular cAMP measurement. Lysis reagents and protocols provided.
25–6400 fmol/well	Protocol 1 (See page 16)	Protocol 1 (See page 16)		Protocol 1 (See page 16)	Protocol 3 (See page 36)	Protocol 4 (See page 45)
2–128 fmol/well			Protocol 2 (See page 28)			

Table 1. Summary of protocols

7. Protocols

7.1. Non–Acetylation EIA Procedure

(Curve range 25–6400 fmol/well, for measurement of cAMP in Urine and tissue samples and extraction of cAMP from cell cultures using traditional methods)

7.1.1. Specimen collection, sample preparation and purification

- 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 (25–28).
- Some investigators recommend the use of ion exchange chromatography following an extraction technique (5). However, it remains the responsibility of the investigator to validate the chosen extraction procedure.
- Representative procedures are described below for the extraction of cAMP from Urine and tissues. This information is provided for guidance only.

Urine

Random, timed or 24-hour Urine collections may be analysed. 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 analysed 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 deproteinise Urine before analysis. Urine should be diluted 1:1000 with assay buffer.

Tissue

Tissue sections must be rapidly frozen immediately after collection so as to prevent alteration to the 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* (24).

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

Liquid phase extraction method

1. Homogenise frozen tissue in cold 6% (w/v) Trichloroacetic acid at 2–8°C to give a 10% (w/v) homogenate.
2. Centrifuge at 2000 x g for 15 minutes at 4°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 lyophilised 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

GE Healthcare has developed a simple protocol for the extraction 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 silca sorbents, for example Amprep SAX, code RPN1918 (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.

Representative procedures are described below for the extraction of cAMP from biological samples using Amprep minicolumns. However, it remains the responsibility of the investigators to validate the chosen extraction procedure for their own application.

Amprep extraction of cyclic AMP

a. Column conditioning

1. Rinse an Amprep SAX 500 mg minicolumn (code RPN1918) with 2 ml Methanol.
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.

b. Sample treatment

1. Homogenise 1 g (wet weight) tissue in 10 mls Hank's balanced Salt solution (without Calcium and Magnesium) containing 5mM EDTA.
2. Centrifuge the homogenate for 10 minutes at 1000 x g at 4°C.
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 x g at 4°C. Apply 1 ml of supernatant to the column.

c. Interference removal

1. Wash the column with 3 ml Methanol.

d. Analyte elution

1. 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.

2. The eluate can be dried under Nitrogen and reconstituted in assay buffer then assayed directly.

Note: If lyophilisation 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.

Cell culture

(See alternative procedures – protocol 3 or 4 using proprietary lysis reagents)

1. Add ice-cold Ethanol to cell suspension to give a final suspension volume of 65% (v/v) Ethanol. Allow to settle.
2. Draw off the supernatant into test tubes.
3. Wash the remaining precipitate with ice cold 65% (v/v) Ethanol and add the washings to the appropriate tubes.
4. Centrifuge the extracts at 2000 x g for 15 minutes at 4°C and transfer the supernatant to fresh tubes.
5. Dry 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.

7.1.2. EIA Procedure

7.1.2.1. Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature prior to use. Either distilled or deionised water may be used for reagent preparation. The microplate and enzyme substrate are supplied ready for use when equilibrated to room temperature.

Assay buffer

For RPN225 and RPN2251

1. Transfer the contents of the bottle to a 500 ml graduated cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted buffer contains 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

For RPN2255

1. Transfer the contents of the bottle to a suitable vessel by repeated washing with distilled water.
2. Adjust the final volume to 2500 ml with distilled water and mix thoroughly.

Standard (for non-acetylation assay)

1. Carefully add 2.0 ml of diluted assay buffer and replace the stopper.
2. Mix the contents of the bottle until completely dissolved. The final solution should contain cAMP at a concentration of 64 pmol/ml in 0.05 M Acetate buffer containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Antiserum

For RPN225 and RPN2251

1. Carefully add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix the contents of the bottle by inversion and swirling until a 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 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

For RPN2255

1. Reconstitiute with 55 ml of assay buffer and gently mix until a complete solution is obtained..

cAMP Peroxidase conjugate

For RPN225 and RPN2251

1. Carefully add 11 ml diluted assay buffer and replace the stopper.
2. Mix until the contents are completely dissolved. The solution will contain cAMP-horseradish Peroxidase in 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

For RPN2255

1. Reconstitiute with 55 ml of assay buffer and gently mix until the contents are completely dissolved.

Wash buffer

For RPN225 and RPN2251

1. Transfer the contents of the bottle to a 500 ml graduated cylinder by repeated washings with distilled water
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted wash buffer contains 0.01 M Phosphate buffer pH 7.5 containing 0.05% (v/v) Tween 20.

For RPN2255

1. Transfer the contents of the bottle to a suitable vessel by repeated washing with distilled water.

7.1.2.2. Preparation of working standards

Note: It is important to use a clean pipette tip for each dilution. Standards should be used within 1 hour of preparation.

1. Label 8 polypropylene or polystyrene tubes (12 x 75 mm), 25 fmol, 50 fmol, 100 fmol, 200 fmol, 400 fmol, 800 fmol, 1600 fmol and 3200 fmol.

2. Pipette 500 µl diluted assay buffer into these tubes.
3. Into the 3200 fmol tube pipette 500 µl of stock non-acetylation standard (64 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 3200 fmol tube to the 1600 fmol tube and vortex mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes and vortex after each dilution.
6. 100 µl aliquots from each of the serial dilutions will give rise to 8 standard levels of cAMP from 25–3200 fmol.

Note: One hundred microlitres (100 µl) of the reconstituted stock standard provided, serves as the top standard (6400 fmol/well).

7.1.2.3. Assay method

Note: It is important that all reagents are equilibrated to room temperature before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare assay components and standards ranging from 25–6400 fmol as described in the previous sections.
2. Equilibrate reagents to room temperature and mix before use.
3. Set up the microplate with sufficient wells for running of all blanks, standards and samples. Recommended positioning of blank (B), non-specific binding (NSB) wells, standard (0–6400 fmol) and sample (S) is shown in figure 3.
4. Pipette 200 µl of diluted assay buffer into the non-specific binding (NSB) wells.
5. Pipette 100 µl of diluted assay buffer into the zero standard wells (0).

6. Pipette 100 µl of each standard into the appropriate wells, using a clean pipette tip for each standard.
7. Pipette 100 µl of unknown sample into the appropriate wells. Urine should be diluted 1:1000 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. (Other biological samples should be pretreated according to the instructions in the previous section.)
8. Pipette 100 µl of antiserum into all wells except the blank and NSB wells.
9. Cover the plate with the lid provided and gently mix and incubate at 3–5°C for exactly 2 hours.
10. Carefully pipette 50 µl cAMP–peroxidase conjugate into all wells except the blank.
11. Cover the plate, gently mix and incubate at 3–5 °C for exactly 60 minutes.
12. Aspirate and wash all wells four times with 400 µl wash buffer. Blot the plate on tissue paper ensuring any residual volume is removed during the blotting procedure.
13. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 60 minutes at room temperature (15-30°C). A blue colour will develop which can be read at 630 nm. However, we recommend halting the reaction at this point by pipetting 100 µl of 1.0 M Sulphuric acid into each well, mixing and determining the optical density in a plate reader at 450 nm. The optical density determination should be carried out within 30 minutes of the addition of the 1.0 M Sulphuric acid.

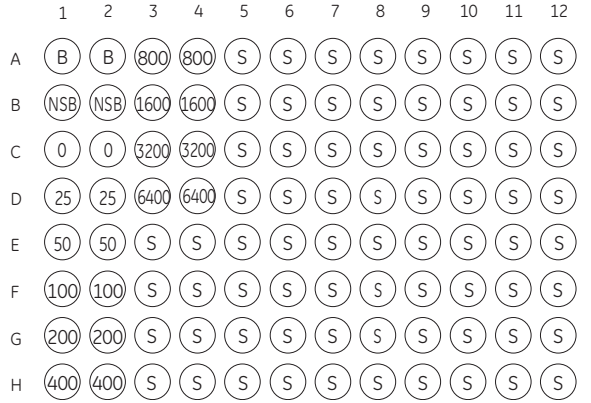


Fig 3. Recommended positioning of standard (25–6400 fmol/well) and sample (S) wells.

* Since the non–specific binding in the assay is so low, typically less than 1% it is possible to omit the substrate blank determination, enabling the assay of an extra unknown sample. If this option is taken, the non–specific wells should be used to blank the plate reader.

** Reaction can be read at 630 nm before acidification but halting reaction prior to end point determination is recommended.

7.1.3. Data Processing

7.1.3.1. Calculation of results

The calculation is illustrated using representative data and is the same for all protocols.

The assay data should be similar to that shown in table 2.

1. Calculate the average optical density (OD) for each set of replicate wells.
2. Calculate the percent bound for each standard and sample using the following relationship:

$$\%B/B_0 = \frac{(\text{standard or sample OD-NSB OD})}{(\text{zero standard OD-NSB OD})} \times 100$$

A standard curve may be generated by plotting the percent B/B_0 as a function of the log cAMP concentration. Plot $\%B/B_0$ (y axis) against fmol cAMP standard per well (*x* axis). The curve shape should be similar to figure 4, if plotted on semi–log paper. The fmol/well value of samples can be read directly from the graph. Figure 4 shows a standard curve generated from the data in table 2.

7.1.3.2. Typical assay data

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD-NSB	%B/B_0
Blank	0.046 0.045		
NSB	0.103 0.105		
0	1.612 1.637	1.521	
25	1.463 1.512	1.384	91
50	1.423 1.488	1.352	89
100	1.301 1.322	1.208	79
200	1.097 1.143	1.016	67
400	1.064 1.118	0.987	65
800	0.848 0.855	0.748	49
1600	0.651 0.645	0.544	36
3200	0.451 0.452	0.348	23
6400	0.299 0.310	0.305	20

Table 2. Typical assay data, protocol 1 (non-acetylation procedure)

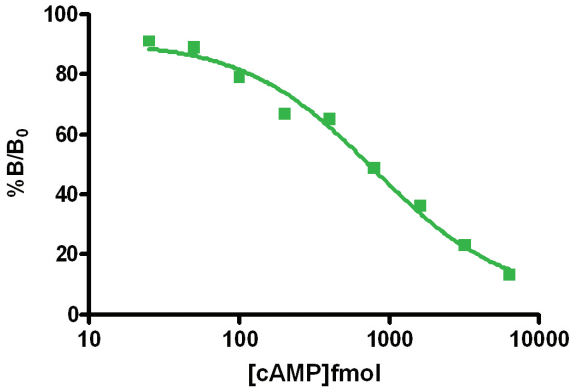


Fig 4. Typical standard curve for protocol 1 (non-acetylation procedure)

7.2. Acetylation EIA Procedure

(Curve range 2–128 fmol/well, for measurement of cAMP in plasma and tissue samples)

7.2.1. Specimen collection, sample preparation and purification

- Representative procedures are described on pages 15–18 for the extraction of cAMP from tissues. Procedures for the measurement of cAMP from plasma samples are described below. This information is provided for guidance only.

Tissue

Tissue sections must be rapidly frozen immediately after collection so as to prevent alteration to the cAMP and associated enzymes before analysis. This is usually achieved by immersion of the fresh tissue in liquid Nitrogen at -196°C. See pages 15–18 for more information on preparation of tissue samples.

Methods of freezing biological samples for cyclic nucleotide assays are reviewed by Mayer *et al*(24).

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

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 deproteinise plasma samples before analysis. Plasma should be diluted 1:100 with assay buffer.

7.2.2. EIA Procedure

7.2.2.1. Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature prior to use. Either distilled or deionised water may be used for reagent preparation. The microplate, Acetic Anhydride, Triethylamine and enzyme substrate are supplied ready for use when equilibrated to room temperature.

Assay buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted buffer contains 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Standard (for acetylation assay)

1. Carefully add 4.0 ml diluted assay buffer and replace the stopper.
2. Mix 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 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Antiserum

1. Carefully add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix the contents of the bottle by inversion and swirling until a 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 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

cAMP Peroxidase conjugate

1. Carefully add 11 ml diluted assay buffer and replace the stopper.

2. Mix until the contents are completely dissolved. The solution will contain cAMP-horseradish Peroxidase in 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Wash buffer

1. Transfer the contents of the bottle to a 500 ml graduated cylinder by repeated washings with distilled water.
2. Adjust the volume to 500 ml with distilled water and mix thoroughly. The diluted wash buffer contains 0.01 M Phosphate buffer pH 7.5 containing 0.05%(v/v) Tween 20.

Reconstituted reagents should be stored at 2–8°C and re-used within 2 weeks.

7.2.2.2. Preparation of working standards

Note: It is important to use a clean pipette tip for each dilution. Standards should be used within 1 hour of preparation.

1. Label 7 polypropylene or glass tubes (12 x 75 mm) 2 fmol, 4 fmol, 8 fmol, 16 fmol, 32 fmol, 64 fmol and 128 fmol.
2. Pipette 1 ml diluted assay buffer into all tubes, except the 128 fmol.
3. Pipette 1 ml of stock acetylation standard (2.56 pmol/ml) into the 128 fmol tube.
4. Pipette 1 ml of stock acetylation standard (2.56 pmol/ml) into the 64 fmol tube and mix thoroughly.
5. Transfer 1 ml from the 64 fmol tube to the 32 fmol tube and mix thoroughly
6. Repeat this doubling dilution successively with the remaining tubes.
7. Remove 1 ml from the 2 fmol standard and discard. All tubes should now contain 1 ml.

8. 50 µl aliquots from each serial dilution give rise to 7 standard levels of cAMP from 2–128 fmol.

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

7.2.2.3. Assay method

Note: Steps 7–12 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–8. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare the assay buffer and working standards as described in the 'reagent preparation' section.
2. Equilibrate all reagents to room temperature and mix before use.
3. Label polypropylene or glass tubes (12 x 75 mm) for zero standard and unknowns. These will subsequently be known as acetylation tubes.
4. Set up the microplate with sufficient wells for running of all blanks, standards and samples as required. Recommended positioning of blank (B), non-specific binding (NSB), standard (0–128 fmol) and sample (S) wells is shown in figure 5.
5. Prepare the acetylation reagent by mixing 1 volume Acetic Anhydride with 2 volumes of Triethylamine in a glass vessel. Mix well (sufficient reagent for 50 acetylations may be attained by mixing 0.5 ml Acetic Anhydride with 1.0 ml Triethylamine).

6. Pipette 1 ml of diluted assay buffer into the zero standard acetylation tube.
7. Pipette 1 ml of each unknown (see sample preparation section) into the appropriately labelled acetylation tubes. Plasma should be diluted 1:100 with assay buffer. A 1:100 dilution of plasma may be achieved by diluting 100 µl of sample to 2.0 ml with assay buffer (1:20 dilution). Take 200 µl of this solution (1:20) and dilute to 1 ml. Tubes containing 1 ml of each working standard should have been prepared (see p.26).
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 acetylating reagents.
9. Pipette 100 µl of antiserum into all wells except the blank and non-specific binding (NSB) wells.
10. Pipette duplicate 50 µl aliqouts from all aceylation tubes including the zero standard into the appropriate wells.
11. Pipette 150 µl of assay buffer into the non-specific binding wells.
12. Cover the plate with the lid provided, gently mix and incubate at 3–5°C for exactly 60 minutes.
13. Pipette 100 µl of cAMP Peroxidase conjugate into all wells except the blank.
14. Cover the plate, gently mix and incubate at 3-5°C for exactly 60 minutes.
15. Aspirate and wash all wells four times with 400 µl wash buffer. Blot the plate on tissue paper ensuring any residual volume is

removed during the blotting procedure. **Thorough washing is essential for good performance.**

16. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 60 minutes at room temperature (15-30°C). A blue colour will develop which can be read at 630 nm. However, we recommend halting the reaction at this point by pipetting 100 µl of 1.0 M Sulphuric acid into each well, mixing and determining the optical density in a plate reader at 450 nm. The optical density determination should be carried out within 30 minutes of the addition of the 1.0 M Sulphuric acid.

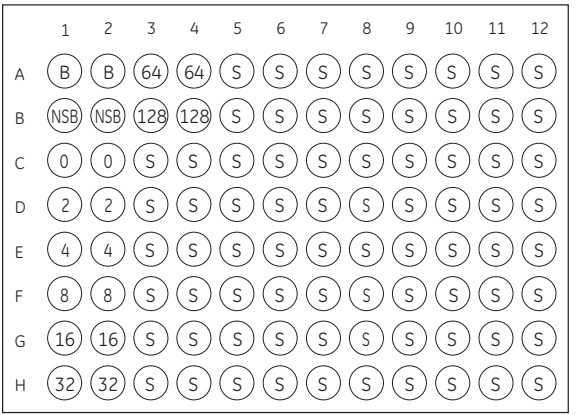


Fig 5. Recommended positioning of standard (2–128 fmol/well) and sample (S) well.

7.2.3. Data processing

7.2.3.1. Calculation of results

The method for calculating results is shown on page 25. The assay data should be similar to that shown in table 3. Figure 6 shows a standard curve generated from the data in table 3.

7.2.3.2. Typical assay data

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD-NSB	%B/B_0
Substrate blank	0.050		
	0.050		
NSB	0.078		
	0.082		
0	1.624	1.570	
	1.675		
2	1.430	1.382	88
	1.493		
4	1.296	1.253	80
	1.370		
8	1.140	1.081	69
	1.182		
16	0.954	0.888	57
	0.982		
32	0.739	0.654	42
	0.729		
64	0.532	0.449	29
	0.525		
128	0.390	0.308	20
	0.386		

Table 3. Typical assay data (acetylation procedure)

* For the calculation of $\%B/B_0$ values, non-specific binding values have been subtracted from the appropriate optical densities.

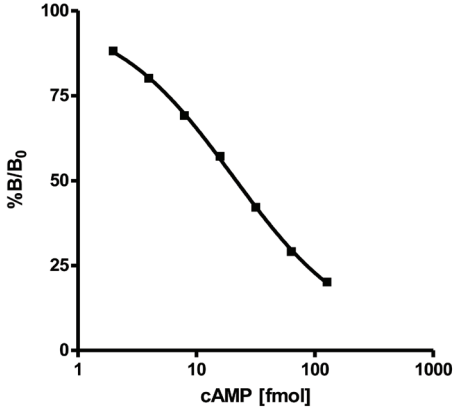


Fig 6. Typical standard curve for protocol 2 (acetylation procedure)

7.3. Intracellular cAMP measurement using the non-acetylation EIA procedure with the novel lysis reagents

(Curve range 25–6400 fmol/well, for cell culture samples)

7.3.1. EIA Procedure

7.3.1.1. Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature prior to use. Either distilled or deionised water may be used for reagent preparation. The microplate and enzyme substrate are supplied ready for use when equilibrated to room temperature.

Assay buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted buffer contains 0.05 M Acetate buffer pH 5.8 containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

NOTE: Lysis reagents 1 and 2 (solids) require 20 minutes mixing at room temperature to dissolve in assay buffer. This is readily achieved using a beaker and magnetic stirrer. Lysis reagent buffer 1A and buffer 2A may appear slightly opaque after mixing. When lysis buffers 1B and 2B are prepared these solutions should be clear. This will not affect assay performance.

Lysis reagent 1

1. Transfer the contents of the bottle (lysis reagent 1, solid) to a 100 ml graduated cylinder by repeated washing with assay buffer. Dissolve in 60 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 80 ml with assay buffer and mix thoroughly. The final solution contains a 2.5% solution of Dodecyltrimethylammonium Bromide in assay buffer. This is lysis buffer 1A. Stir continuously when used.
3. Take 10 ml of lysis buffer 1A and make up to 100 ml with assay buffer to give a final 0.25% solution of Dodecyltrimethylammonium Bromide in assay buffer. This is lysis reagent 1B. It is used for the intracellular measurement of cAMP and for the preparation of standards.

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) to a 100 ml graduated cylinder. Dissolve in 80 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.
2. Adjust the final volume to 100 ml with assay buffer and mix thoroughly. This is lysis buffer 2A.
3. Take 10 ml of lysis buffer 2A and make up to a final volume of 40 ml with assay buffer and mix thoroughly. This is lysis reagent 2B.

Standard (for non-acetylation assay)

Add 2 ml diluted lysis reagent 1B (prepared as described above) and replace the stopper. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 64 pmol/ml in lysis reagent 1B.

Antiserum

Carefully add 11.0 ml of lysis reagent 2B replace the stopper. Gently mix the contents of the bottle by inversion and swirling until a complete solution is obtained. Vigorous agitation and foaming should be avoided. The contents will contain anti-cAMP serum in lysis reagent 2B.

cAMP Peroxidase conjugate
Do not add lysis reagent 1 or 2 to the conjugate.

Carefully add 11.0 ml of diluted assay buffer and replace the stopper. Mix the contents until completely dissolved. The solution will contain cAMP-horseradish Peroxidase in 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Wash buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted wash buffer contains 0.01 M Phosphate buffer pH 7.5 containing 0.05% (w/v) Tween 20.

7.3.1.2. Cell lysis methods – intracellular cAMP measurement
(**Note:** For cell lysis method for total cellular cAMP measurement, see protocol 4). Two lysis methods are described for intracellular cAMP measurement.

Adherent cells

1. Culture cells (100 µl volumes) in standard 96-well microplates (tissue-culture grade), with cell concentrations of between 10^4–10^6 cells/ml.
2. Incubate plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over-confluent (e.g. at 10^7 cells/ml) as cells may be lost during decantation.**
3. Add 100 µl of drug, agonist etc. under study. Incubate for suitable time period.
4. Decant or aspirate excess culture media.
5. Add 200 µl/well of diluted lysis reagent 1B

6. Agitate cells after lysis reagent 1B is added. This is to facilitate cell lysis and can be achieved by shaking the plate on a microplate shaker for 10 minutes after adding the lysis reagent.
7. Carry out a microscopic evaluation using Trypan blue to check cells have lysed. Cell membranes may still be visible after cell lysis. Lysed cells are now ready for use in the enzymeimmunoassay protocol and should be processed immediately in the immunoassay (see 'assay method').

Suspension cells

Note: If suspension cells are used, special microplate adapters are needed for the centrifugation step.

1. Culture cells (100 µl) in standard 96-well microplates (tissue-culture grade) with cell concentrations of between 10^4–10^6 cells/ml.
2. Incubate plate overnight at 37°C (5% CO_2 and 95% humidity).
3. Add 100 µl of drug, agonist etc. under study. Incubate for a suitable time period.
4. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 x g for 3 minutes to form a pellet in each well. Note: the actual centrifugal speed required is dependent on the cells under study and should be validated by the investigator.
5. Gently decant or aspirate excess media and resuspend pellet in 200 µl of lysis reagent 1B.
6. Agitate cells after lysis reagent 1B is added. This is to facilitate cell lysis and can be achieved by shaking the plate on a microplate shaker for 10 minutes after adding the lysis reagent.
7. Carry out a microscopic evaluation using Trypan blue to check cells have lysed. Cell membranes may still be visible after cell lysis. Lysed cells are now ready for use in the enzymeimmunoassay protocol and should be immediately processed in the immunoassay (see 'assay method').

7.3.1.3. Preparation of working standards

Note: It is important to use a clean pipette tip for each dilution. Standards should be used within 1 hour of preparation.

1. Label 8 polypropylene or polystyrene tubes (12 x 75 mm), 25, 50, 100, 200, 400, 800, 1600 and 3200 fmol.
2. Pipette 500 µl of lysis reagent 1B into all tubes.
3. Into the 3200 fmol tube pipette 500 µl of stock non-acetylation standard (64 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 3200 fmol tube to the 1600 fmol tube and vortex mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes and vortex after each dilution.
6. 100 µl aliquots from each of the serial dilutions will give rise to 8 standard levels of cAMP from 25–3200 fmol.

Note: One hundred microlitres (100 µl) of the reconstituted stock standard provided, serves as the top standard (6400 fmol/well).

7.3.1.4. Assay method

It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare assay buffer, lysis reagents and standards ranging from 25–6400 fmol as described in the previous section.
2. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB.
3. Set up the microplate with sufficient wells to enable the running of all blanks, standards and samples as required. Recommended

positioning of substrate blank (B), non-specifi c binding (NSB), standard (0–6400 fmol) and sample (S) wells is shown in figure 2.

4. Pipette 100 µl of lysis reagent 1B and 100 µl of lysis reagent 2B into the NSB wells.
5. Pipette 100 µl of lysis reagent 1B into the zero standard (0) wells.
6. Pipette 100 µl of each standard into the appropriate wells, using a clean pipette for each standard.
7. Pipette 100 µl of unknown sample into the appropriate wells. (See previous section for sample preparation).
8. Pipette 100 µl of antiserum into all wells except the blank and NSB wells.
9. Cover the plate with the lid provided, gently mix and incubate at 3–5°C for exactly 2 hours.
10. Carefully pipette 50 µl cAMP-peroxidase conjugate into all wells except the blank.
11. Cover the plate, gently mix and incubate at 3–5°C for exactly 60 minutes.
12. Aspirate and wash all wells four times with 400 µl wash buffer. Blot the plate on tissue paper ensuring any residual volume is removed during the blotting procedure. **Thorough and careful washing is essential for good performance.**
13. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 60 minutes at room temperature (15-30°C). A blue colour will develop which can be read at 630 nm. However, we recommend halting the reaction at this point by pipetting 100 µl of 1.0 M Sulphuric acid into each well, mixing and determining the optical density in a plate reader at 450 nm. The optical density determination should be carried out within 30 minutes of the addition of the 1.0 M Sulphuric acid.

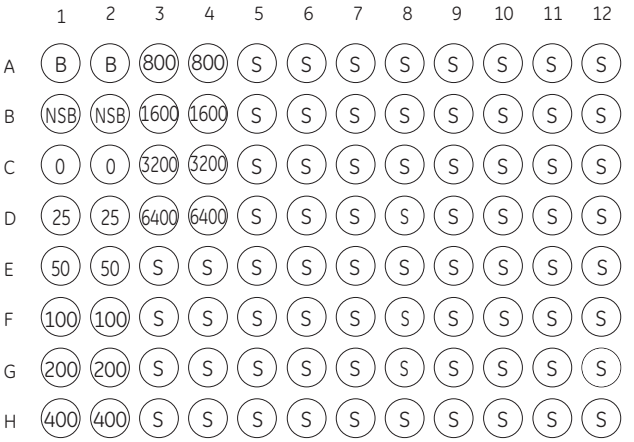


Fig 7. Recommended positioning of standard (25–6400 fmol/well) and sample (S) wells

7.3.2. Data processing

7.3.2.1. Calculation of results

The method for calculating results is shown on page 25. The assay data should be similar to that shown in table 4. Figure 8 shows a standard curve generated from the data in table 4.

7.3.2.2. Typical assay data

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD-NSB	%B/B_0
Substrate	0.045		
blank	0.043		
NSB	0.112		
	0.112		
0	1.533	1.422	
	1.534		
25	1.492	1.379	97
	1.489		
50	1.447	1.319	93
	1.415		
100	1.345	1.255	88
	1.388		
200	1.322	1.182	83
	1.266		
400	1.057	0.946	66
	1.058		
800	0.78	0.656	46
	0.759		
1600	0.594	0.473	33
	0.576		
3200	0.406	0.293	20
	0.404		
6400	0.285	0.173	12
	0.284		

Table 4. Typical assay data for the intra- and 'total' cellular cAMP assays (protocols 3 and 4).

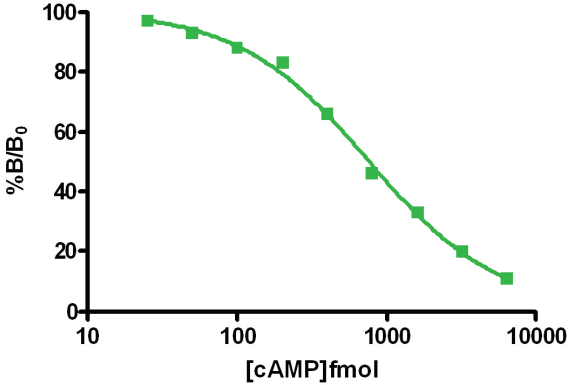


Fig 8. Typical standard curve (for intra- and total cellular cAMP protocols)

7.4. Total Cellular cAMP measurement using the non-acetylation EIA procedure with the novel lysis reagents

(Curve range 25–6400 fmol/well, for cell culture samples)

7.4.1. EIA Procedure

7.4.1.1. Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature prior to use. Either distilled or deionised water may be used for reagent preparation. The microplate and enzyme substrate are supplied ready for use when equilibrated to room temperature.

Assay buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted buffer contains 0.05 M Acetate buffer pH 5.8 containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

NOTE: lysis reagents 1 and 2 (solids) require 20 minutes mixing at room temperature to dissolve in assay buffer. This is readily achieved using a beaker and magnetic stirrer. Lysis reagent buffer 1A and buffer 2A may appear slightly opaque after mixing. When lysis buffer 1B and 2B are prepared these solutions should be clear. This will not affect assay performance.

Lysis reagent 1

1. Transfer the contents of the bottle (lysis reagent 1, solid) to a 100 ml graduated cylinder by repeated washing with assay buffer. Dissolve in 60 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 80 ml with assay buffer and mix thoroughly. The final solution contains a 2.5% solution of Dodecyltrimethylammonium Bromide in assay buffer. This is lysis buffer 1A, which is used in the cell lysis method for the 'total' cellular cAMP assay. Stir continuously when used. Stir for 30 minutes after storage.
3. Take 10 ml of lysis buffer 1A and make up to 100 ml with assay buffer to give a final 0.25% solution of Dodecyltrimethylammonium Bromide in assay buffer. This is lysis reagent 1B and is used for the preparation of working standards only.

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) to a 100 ml graduated cylinder. Dissolve in 80 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.
2. Adjust the final volume to 100 ml with assay buffer and mix thoroughly. This is lysis buffer 2A.
3. Take 10 ml of lysis buffer 2A and make up to a final volume of 40 ml with assay buffer and mix thoroughly. This is lysis reagent 2B.

Standard (for non-acetylation assay)

Add 2 ml diluted lysis reagent 1B and replace the stopper. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 64 pmol/ml in lysis reagent 1B.

Antiserum

Carefully add 11.0 ml of lysis reagent 2B and replace the stopper. Gently mix the contents of the bottle by inversion and swirling until a complete solution is obtained. Vigorous agitation and foaming should be avoided. The contents will contain anti-cAMP serum in lysis reagent 2B.

cAMP Peroxidase conjugate
Do not add lysis reagent 1 or 2 to the conjugate.

Carefully add 11.0 ml of diluted assay buffer and replace the stopper. Mix the contents until completely dissolved. The solution will contain cAMP-horseradish Peroxidase in 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Wash buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly.

Reconstituted reagents should be stored at 2–8°C and re-used within two weeks.

7.4.1.2. Cell lysis method – total cellular cAMP measurement
(**Note:** For cell lysis methods and intracellular cAMP measurement, see page 36)

The following method measures the combined amount of intracellular and cell supernatant cAMP.

Suspension and adherent cells

1. Culture adherent or suspension cells (160 µl/well) in flat-bottomed 96-well microplates (tissue-culture grade), with cell concentrations between 10^4 –10^6 cells/well.
2. Incubate the plate overnight at 37°C, (5% CO_2 and 95% humidity).
3. Add 20 µl aliquots of agonist or cell stimulant directly to the cell culture samples. Do not decant or aspirate the culture media. Incubate agonist/cell stimulant with cultures depending on required experimental conditions.

4. Add 20 µl of lysis reagent 1A (2.5% Dodecyltrimethylammonium Bromide in assay buffer). The final volume in the wells should be 200 µl, each containing 0.25% Dodecyltrimethylammonium Bromide (final concentration) which is equivalent to the lysis reagent 1B.
5. Following the addition of lysis reagent 1A, agitate cells to facilitate cell lysis. This can be readily achieved by vigorous, successive pipetting. Incubate the plate for 10 minutes at room temperature (in order to achieve cell lysis).
6. Carry out a microscopic evaluation with Trypan blue. Lysed cells are now ready for use in the assay (see 'assay protocol'). **Cell membranes may still be visible after lysis.**
7. Transfer 100 µl aliquots of cell lysate to the donkey anti-rabbit Ig coated plate for assay.

7.4.1.3. Preparation of working standards

Note: It is important to use a clean pipette tip for each dilution. Standards should be used within 1 hour of preparation.

1. Label 8 polypropylene or polystyrene tubes (12 x 75 mm), 25, 50, 100, 200, 400, 800, 1600 and 3200 fmol.
2. Pipette 500 µl lysis reagent 1B into these tubes.
3. Into the 3200 fmol tube pipette 500 µl of stock non-acetylation standard (64 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 3200 fmol tube to the 1600 fmol tube and vortex mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes and vortex after each dilution.
6. 100 µl aliquots from each of the serial dilutions will give rise to 8 standard levels of cAMP from 25–3200 fmol.

Note: One hundred microlitres (100 µl) of the reconstituted stock standard provided, serves as the top standard (6400 fmol/well).

7.4.1.4. Assay protocol

It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare assay buffer, lysis reagents and standards ranging from 25–6400 fmol as described in the previous section.
2. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB.
3. Set up the microplate with sufficient wells to enable the running of all blanks, standards and samples as required. Recommended positioning of substrate blank (B), non-specific binding (NSB), standard (0–6400 fmol) and sample (S) wells is shown in figure 9.
4. Pipette 100 µl of lysis reagent 1B and 100 µl of lysis reagent 2B into the NSB wells.
5. Pipette 100 µl of lysis reagent 1B into the zero standard (0) wells.
6. Pipette 100 µl of each standard into the appropriate wells, using a clean pipette for each standard.
7. Pipette 100 µl of unknown sample into the appropriate wells. (See previous section for sample preparation).
8. Pipette 100 µl of antiserum into all wells except the blank and NSB wells.
9. Cover the plate with the lid provided, gently mix and incubate at 3–5°C for exactly 2 hours.
10. Carefully pipette 50 µl cAMP-peroxidase conjugate into all wells except the blank.
11. Cover the plate, gently mix and incubate at 3–5°C for exactly 60 minutes.

12. Aspirate and wash all wells four times with 400 µl wash buffer. Blot the plate on tissue paper ensuring any residual volume is removed during the blotting procedure. **Thorough and careful washing is essential for good performance.**

13. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 60 minutes at room temperature (15-30°C). A blue colour will develop which can be read at 630 nm. However, we recommend halting the reaction at this point by pipetting 100 µl of 1.0 M Sulphuric acid into each well, mixing and determining the optical density in a plate reader at 450 nm. The optical density determination should be carried out within 30 minutes of the addition of the 1.0 M Sulphuric acid.

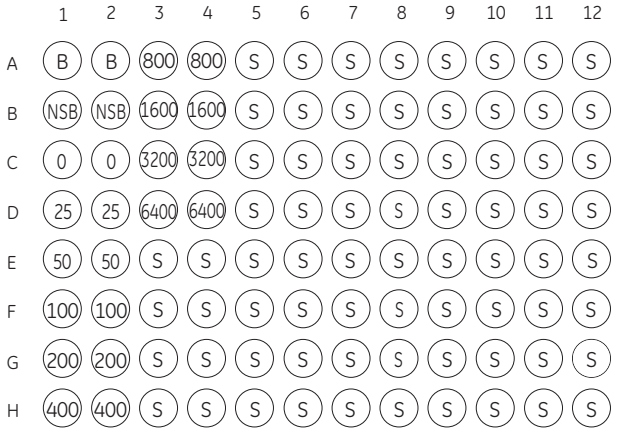


Fig 9. Recommended positioning of standard (25–6400 fmol/well) and sample (S) wells

7.4.2. Data Processing

7.4.2.1. Calculation of results

The method for calculating results is shown on page 25. The assay data should be similar to that shown in table 5. Figure 10 shows a standard curve generated from the data in table 5.

7.4.2.2. Typical assay data

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD-NSB	%B/B_0
Substrate blank	0.045 0.043		
NSB	0.112 0.112		
0	1.533 1.534	1.422	
25	1.492 1.489	1.379	97
50	1.447 1.415	1.319	93
100	1.345 1.388	1.255	88
200	1.322 1.266	1.182	83
400	1.057 1.058	0.946	66
800	0.78 0.756	0.656	46
1600	0.594 0.576	0.473	33
3200	0.406 0.404	0.293	20
6400	0.285 0.285	0.173	12

Table 5. Typical assay data for intra- and total cellular cAMP

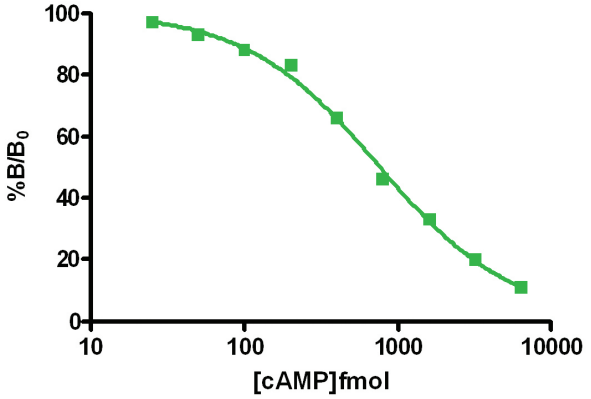


Fig 10. Typical standard curve (for intra- and total cellular cAMP protocols)

8. Additional Information

8.1. Expected values

Individual laboratories should establish their own normal ranges. GE Healthcare has achieved the following values of cAMP in normal subjects using the enzyme immunoassay system:

Urine 1.96 ± 0.94 nmol/ml (n = 18)

Plasma 13.96 ± 3.8 pmol/ml (n = 20)

8.2. Limitations of use

Do not use lipaemic, haemolysed 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.

8.3. Specificity

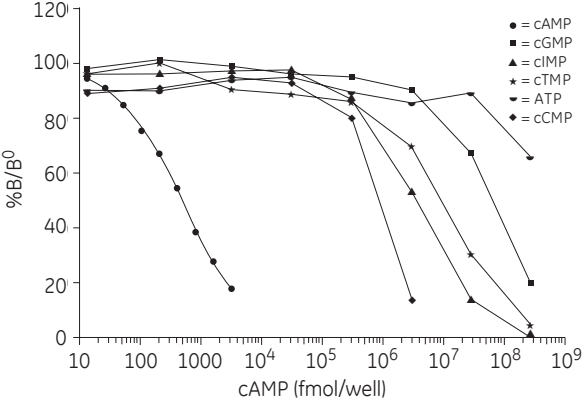
The cross-reactivity, as determined by the concentration giving 50% B/B_0, is shown in the following table and graphically in figures 11 and 12.

Compound	% Cross-reactivity (50% B/B_0 displacement)	
	Non-acetylation (protocols 1, 3, 4)	Acetylation (protocol 2)
cIMP	1	<0.005
cGMP	<0.05	<0.005
cCMP	<0.05	<0.005
cTMP	<0.05	<0.005
AMP	<0.05	<0.005
ADP	<0.05	<0.005
ATP	<0.05	<0.005
EDTA	<0.05	<0.005
Theophylline	<0.05	<0.005
cAMP	100	100

Table 6. Cross-reactivity

Fig 11.

(a) Cross–reactivity profile for protocols 1,3,4 (non-acetylation assay)



(b) Cross–reactivity profile for protocols 1,3,4 (non-acetylation assay)

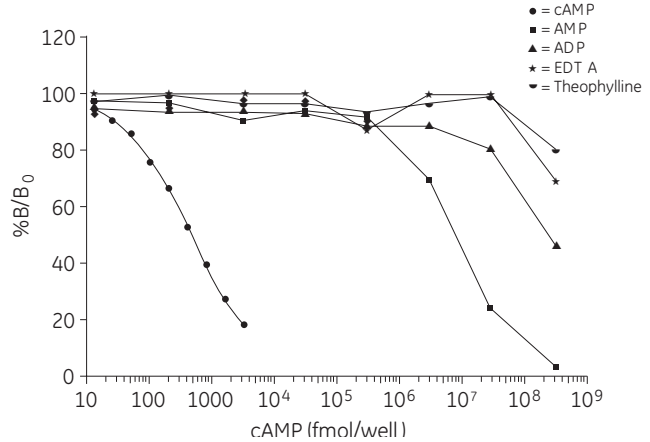
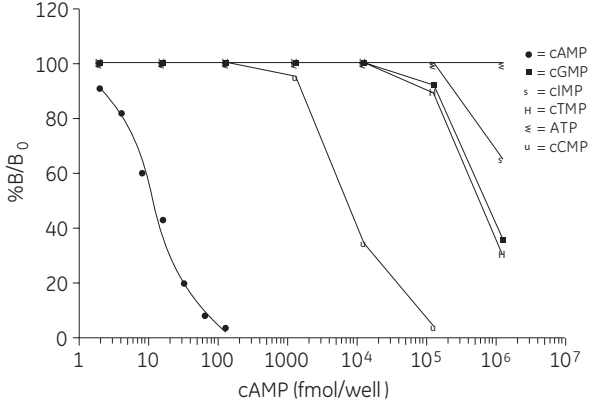
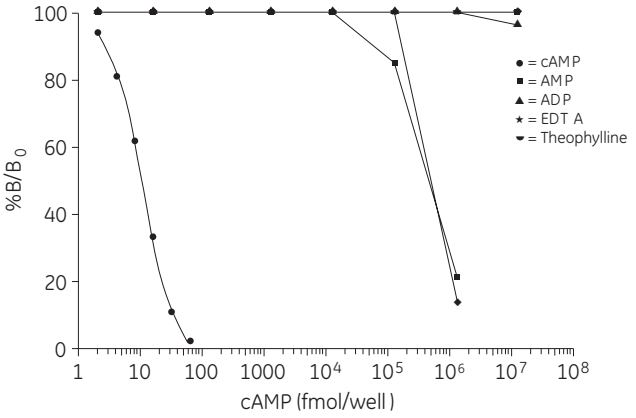


Fig 12.

(a) Cross–reactivity profile for protocol 2 (acetylation assay)



(b) Cross–reactivity profile for protocol 2 (acetylation assay)



8.4. Sensitivity

The sensitivity, defined as the amount of cAMP needed to reduce the zero dose binding by two standard deviations, was as follows:

Protocols 1, 3, 4 (non-acetylation assay)	12 fmol/well	38.4 pg/ml
Protocol 2 (acetylation assay)	2 fmol/well	14 pg/ml

8.5. Precision

Within-assay precision

The within-assay precision for duplicate determinations was calculated by measuring controls in the assay. The results are shown below:

	Protocols 1, 3, 4 non-acetylation assay (mean values as fmol/well)			**Protocol 2 acetylation assay (mean values as fmol/well)**		
Control	Mean ± SD	%CV	n	Mean ± SD	%CV	n
A	1561± 14.4	9.23	14	7.8 ± 0.5	6.3	14
B	299.0 ± 20.1	6.72	14	18.7 ± 0.8	4.3	14
C	576.4 ± 28.0	4.86	14	34.6 ± 2.3	6.5	14

Between-assay precision

The between-assay precision was assessed by repeated measurement of the same controls in successive assays. The results are shown below:

Table 7.

	Protocols 1, 3, 4 non-acetylation assay (mean values as fmol/well)			Protocol 2 acetylation assay (mean values as fmol/well)		
Control	Mean ± SD	%CV	n	Mean ± SD	%CV	n
A	11.4 ± 9.2	8	8	06.2 ± 0.73	11.7	11
B	295 ± 26.8	9.1	8	10.9 ± 0.89	08.2	11
C	739 ± 143	15	8	21.8 ± 3.79	17.4	11

Precision profile

A precision profile was generated by preparing ten replicates of each of the standards and calculating the standard deviation and percent coefficient of variation at each concentration.

Protocols 1, 3, 4 non-acetylation assay			Protocol 2 acetylation assay		
Standard	Standard deviation	%CV	Standard	Standard deviation	%CV
12.5	1.2	9.7	2	0.4	19.6
25	2.4	10.8	4	0.4	8.9
50	3.4	6.8	8	0.4	4.7
100	9.6	9.3	16	0.8	4.8
200	10.7	5.3	32	0.7	2.2
400	38.9	10.4	64	1.7	2.6
800	42.8	5.3	128	2.3	1.8
1600	65.9	3.9			
3200	106	3.3			

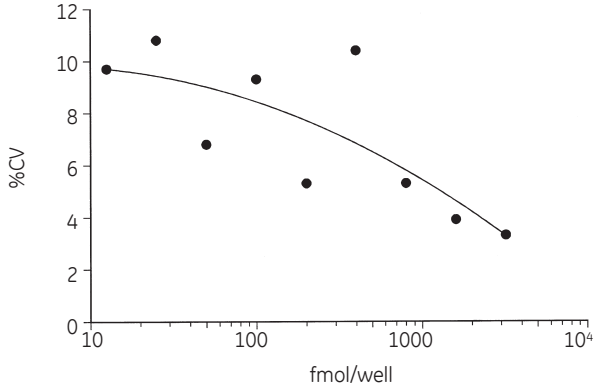


Fig 13a. Precision profile protocols 1, 3, 4,

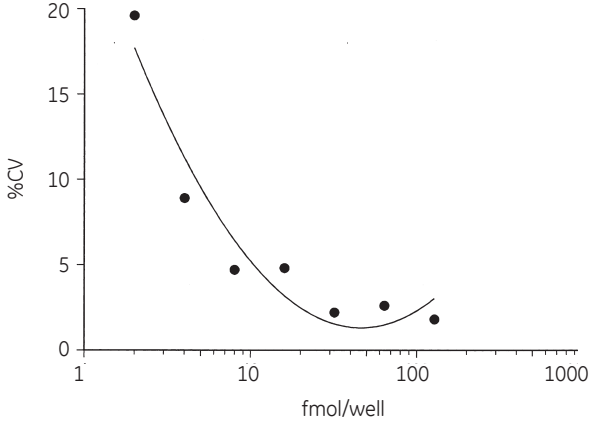


Fig 13b. Precision profile protocol 2

9. Troubleshooting guide

Problems	Remedies
1. Low optical densities	**1.** Check plate reader wavelength. **2.** Check incubation time and temperature. **3.** Check that reagents are equilibrated to room temperature before use. **4.** Check that the kit reagents have been stored correctly.
2. High optical densities	**1.** Ensure that every well is completely filled and emptied at every wash step. **2.** Ensure that automatic washers are functioning correctly. **3.** Check incubation times and temperatures. **4.** Ensure that plates have been blotted on tissue paper after washing.
3. Flat curves/poor reproducibility	**1.** Check pipette calibration. **2.** Check preparation of working standards. **3.** Ensure troughs used with multichannel pipettes are separate and dedicated to individual components. **4.** Ensure that washing procedures have been carried out correctly.

10. Background and references

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 specfic substrates. Numerous hormones are known to act through this mechanism including Corticotrophin (ATCH), luteinising 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 system (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 catalysed Hydrolytic cleavage of membrane Phosphatidylinositol

4,5-biphosphate 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 Ca^{2+}mobilised hormones in a variety of cell types (21). DAG appears to be an essential co-factor for the enzyme protein kinase C which plays a crucial role in signal transduction (17,22).

Levels of IP3 and DAG can be determined using GE Healthcare assay system TRK1000.

1. Sutherland, E.W., *et al., Circulation.* **37**, 279, (1968).

2. Rall, T.W., *et al., J.Biol. Chem.* **224**, 463, (1957).

3. Cook, W.H., *et al., Am. Chem*. Soc. **79**, 3607, (1957).

4. Suthgerland, E.W. and Rall, T.W., *J. Am. Chem.* Soc. **79**, 3608, (1957).

5. Lipkin, D., *et al., Am. Chem. Soc.* **81**, 6198, (1959).

6. Hamet, P. *et al, Advances in Cyclic Nucleotide Research.* **12**, 11, Hamet, P. and Sands, H., eds, Raven Press, New York, (1980).

7. Drummond, G., *Advances in Nucleotide Research.* **15**, 373, Greenard, P. and Robinson, G.A., eds, Raven Press, New York, (1983).

8. Plaut, M. *et al., Advances in Nucleotide Research.* **12**, 161, Hamet, P. and Sands, H., eds, Raven Press, New York, (1980).

9. Boyton, A.L. and Whitefield, J.F., *Advances in Cyclic Nucleotide Research.* **15**, 193, Greenard, P. and Robinson, G.A., eds, Raven Press, New York, (1983).

10. Exton, J.H., *Advances in Cyclic Nucleotide Research.* **12**, 319, Hamet, P. and Sands, H., eds, Raven Press, New York, (1980).

11. Albano, J.D.M. *et al., Analyt. Biochem.* **60**, 130, (1974).

12. Albano, J.D.M. *et al., Analyt. Biochem. Soc. Trans.* **1**, 477, (1973).

13. Thomas, J.A. and Singhal, R.L., *Biochem. Pharmacol.* **22**, 507, (1973).

14. Volker, T.T., *et al., Analyt.Biochem.* **144**, 347, (1985).

15. Berridge, M.J., *Biochem. J.* **220**, 345-360, (1984).

16. Berridge, M.J. and Irvine, *R.F., Nature*. **312**, 315-321, (1984).

17. Nishizuka, Y., *Nature.* **308**, 693-698, (1984).

18. Majerus, P.W. *et al., Science.* **234**, 1519-1526, (1986).

19. Michell, R.H., *Phosphoinositides and Receptor Mechanisms*, Putney, J. W. Jnr., Alan R., eds. Liss Inc., New York. 1-24, (1986).

20. Berridge, M.J., *Phosphoinositides and Receptor Mechanisms.* 24-45, Putney, J. W. Jnr., Alan R., eds, Liss Inc., New York, (1986).

21. Michell, R.H., *Bicochem. Biophys. Acta*. **415**, 81-147, (1975).

22. Nishizuka, Y., *Science*. **223**, 305-312, (1986).

23. Bos, E.S. *et al, J. Immunoassay* **2**, 187-204, (1981).

24. Mayer, S.E.., *et al., Methods in Enzymology*. **38**, 3. Hardman, J.C. and O'Malley, B.W., eds, Academic Press, New York, (1974).

25. Goldburg, N.D. and O'Toole, A.G., *Methods of Biological Analysis*.
20, 1-39. Glick, D., ed Interscience Publishers, John Wiley and Sons Inc., London, (1971).

26. Harper, J.F.H. and Brooker, G., J. *Cyclic Nucleotide Res.* **1**, 207, (1975).

27. Steiner, A.L., *Methods of Hormone Radioimmunoassay.* 3, Jaffe, B.M. and Behrman, H.R., eds, Academic Press, New York, (1979).

28. Rosenburg, N., *et al. FEBS Letts*. **137**, 105, (1982).

11. Related products

Biotrak signal transduction assay range

Cyclic GMP	EIA	RPN226
Amprep SAX 500 mg (pack of 50)		RPN1918
Amprep SAX 100 mg (pack of 100)		RPN1908

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