

GE Healthcare

Amersham
cAMP Biotrak SPA
Screening Assay System

5 x 96 wells

Product Booklet

Code: RPA556



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1. Legal

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Topcount is a trademark of Packard Instrument Co.

Microbeta is a trademark of Wallac Oy.

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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 regulations or codes of practice which relate to such matters.

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 statements 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.

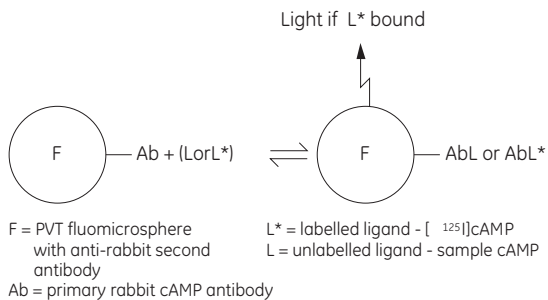


Figure 1. Assay principle

3. Components of the assay system

The pack contains the following assay components, sufficient material for 5 x 96 wells. All components for this kit should be stored at 2–8°C.

Assay buffer

Assay buffer concentrate. On dilution the contents of this bottle will produce a 0.05 M acetate buffer containing 0.01% sodium azide

Standard, 2 vials

cAMP standard 512 pmol, for assays in the range 0.2–12.8 pmol/well, lyophilized. On reconstitution this bottle contains 256 pmol/ml in 2.0 ml.

Tracer, 2 vials

Adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[¹²⁵I]iodotyrosine methyl ester, 147.5 kBq, 4 µCi, lyophilized.

Antiserum, 2 vials

Rabbit anti-succinyl cAMP serum, lyophilized.

SPA anti-rabbit reagent

Donkey anti-rabbit IgG coupled to SPA fluomicrospheres, lyophilized.

4. Description

The Biotrak™ cAMP assay system from GE Healthcare has been specifically designed for research purposes. This assay system utilizes the novel technique of scintillation proximity assay (SPA), which eliminates the need to separate antibody bound from free ligand common to heterogeneous radioimmunoassays. The SPA fluomicrospheres used in this assay are second generation polyvinyl toluene (PVT) based beads. These remain in suspension for relatively longer periods of time when compared to the first generation yttrium silicate based SPA beads. This greatly improves pipetting accuracy and facilitates complete automation of the assay.

cAMP may be routinely measured in the range
0.2–12.8 pmol/well (1.32–84.28 ng/ml).

The sensitivity of the assay system is 0.1 pmol/well (0.65 ng/ml).

Alternative protocols for increased sensitivity (assay range: 0.025–3.2 pmol/well) and improved automation are provided on pages 30–31.

Each pack contains sufficient material for 5 × 96 wells.

A separate standard curve should be constructed for each plate, allowing 39 unknowns to be measured in duplicate per plate. This would allow testing of 195 unknowns in duplicate.

- No separation step
- Automatable
- Sample collection and preparation procedures included.

5. Critical parameters

The following points are critical.

- Do not agitate the plates during the course of the assay incubation period.
- A separate standard curve must be run on each plate.
- The scintillation proximity assay fluomicrospheres are insoluble. The bottle contents should be magnetically stirred to ensure a homogeneous suspension when pipetting into the wells. This is particularly important during any automation process. The fluomicrospheres must not be allowed to settle.
- When researchers are using highly coloured samples, colour quench correction may be necessary. Please contact your local GE Healthcare representative for further information.
- Removal of organic solvents from samples must be carried out prior to assay.
- For cell culture samples better recoveries are obtained if the standard curve is formulated in the culture media.

6. Additional equipment and reagents required

- Microplate liquid scintillation counter
- 96 well microplates compatible with the scintillation counter
- Plate sealers
- Disposable polypropylene or polystyrene tubes (12 x 75 mm) for preparation of working standards
- Pipettes or pipetting equipment (50 µl, 1 ml, 2 ml, 5 ml)
- Glass measuring cylinder (2000 ml)
- Distilled or deionized water
- Test tube rack to hold tubes
- Vortex mixer
- Magnetic stirrer

Disposable polypropylene tubes are supplied by Sarstedt International Rommelsdore, 5223 Numbrecht, Germany Microplate liquid scintillation counters are available from Packard Instrument Co. Inc., Meriden, CT 06450 and Wallac Oy, Finland. Compatible 96 well microplates and plate sealers are available from a wide range of suppliers such as Dynatech Laboratories, Inc., Chantilly, Virginia 22021, Packard and Wallac.

This assay system is designed for use in a microplate format. However, assays can be carried out in miniscintillation vials. These are available from a wide range of suppliers such as Pharmacia-LKB, Packard and Beckman Instruments, for example Bio-Vial™ (13 x 55 mm). These vials are counted in traditional rack liquid scintillation counters. Do not add scintillant to the vials.

Plates used with the TopCount™ and Microbeta™ liquid scintillation counter need to be sealed with an adhesive clear plastic. These should be ordered with the plates along with a roller to seal the plates effectively.

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 (5–8). Some investigators also recommend the use of ion exchange chromatography (6) following one of these extraction techniques.

For direct measurement of cAMP use the CAMP direct SPA screening assay system, RPA559 from GE Healthcare. This kit contains novel lysis reagents which eliminate traditional time consuming extraction procedures.

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.

7.1. Tissue samples

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

Methods of freezing biological samples for cyclic nucleotide assay are reviewed by Mayer *et al* (4).

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% (w/v) trichloroacetic acid at $2-8^{\circ}\text{C}$ 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 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

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 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 methods.

The columns can be used with the Amprep Manifold-10 (RPN1930) or the Super Separator-24 (RPN1940) to facilitate the extraction of 10 or 24 samples simultaneously. Alternatively, a syringe can be used for a simple manual extraction. The syringe is easily connected to the column with the column adaptor provided in each pack of Amprep minicolumns. Further column adaptors are available from GE Healthcare (code RPN1927). Please enquire for further details of the Amprep range of products available from GE Healthcare.

Representative procedures are described overleaf for the extraction of cAMP from biological samples using Amprep minicolumns. However, it remains the responsibility of the

investigator to validate the chosen extraction procedure in his own application.

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.

Sample treatment - tissue

1. Homogenize 1 g (wet weight) tissue in 10 ml Hank's balanced salt solution (without calcium and magnesium) containing 5 mM EDTA.
2. Centrifuge the homogenate for 10 minutes at 1000 xg 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 column.

Removal of interference

1. Wash the column with 3 ml methanol.

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 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.

7.2. Cell culture samples

Methods of freezing biological samples for cyclic nucleotide assay are reviewed by Mayer *et al* (4). Samples should be stored at -15°C to -30°C until the assay is conducted.

Liquid phase extraction method

Cell suspension

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 a test tube.
3. Wash the remaining precipitate with ice cold 65% (v/v) ethanol and add the washings to the appropriate tube.
4. Centrifuge the extracts at 2000 x g for 15 minutes at 4°C and transfer the supernatant to a fresh tube.
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.

Solid phase extraction method

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.

Sample treatment - cell culture

1. Apply directly to the column

Removal of interference

1. Wash the column with 3 ml methanol

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 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 Protocol

8.1. Reagent preparation

Storage

Before reconstitution store the reagents at 2–8°C. The expiry date is stated on the package and will normally be at least four weeks from the date of despatch.

After reconstitution the scintillation proximity assay reagent will be stable for up to 4 weeks stored at 2–8°C and protected from light. All other reconstituted components should be stored at 2–8°C and may be re-used within 14 days of reconstitution.

Note: All reagents should be allowed to equilibrate to room temperature. Either distilled or deionized water may be used for reagent preparation.

Assay buffer

1. Transfer the contents of the bottle to a 2000 ml graduated cylinder by repeated washing with distilled water.
2. Adjust the final volume to 1250 ml with distilled water and mix thoroughly. The diluted buffer contains 0.05 M acetate buffer pH 5.8 containing 0.01% (w/v) sodium azide.

Standard

1. Add 2 ml diluted assay to the buffer and replace the stopper.
2. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 256 pmol/ml.

Tracer

1. Add 14 ml diluted assay buffer to each vial and replace the stoppers.
2. Gently mix until the contents are completely dissolved. The final solution contains adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[¹²⁵I]iodotyrosine methyl ester.

Antiserum

1. Add 15 ml diluted assay buffer to each vial and replace the stoppers.
2. Gently mix until the contents are completely dissolved. The final solution contains anti-cAMP serum.

SPA anti-rabbit reagent

1. Add 30 ml of diluted buffer to the bottle and replace the stopper.
2. Gently shake the bottle for 5 minutes.

8.2. Preparation of working standards

For the measurement of cAMP in the range 0.2–12.8 pmol/well

1. Label 6 polypropylene or polystyrene tubes (12 x 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol and 6.4 pmol.
2. Pipette 1 ml of assay buffer into all the tubes.
3. Into the 6.4 pmol tube pipette 1 ml of stock standard (256 pmol/ml) and mix thoroughly.
4. Transfer 1 ml from the 6.4 pmol tube to the 3.2 pmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 50 μ l aliquots from each serial dilution will give rise to 6 standard levels of cAMP ranging from 0.2–6.4 pmol/well.
7. 50 μ l from the stock standard vial will give rise to the 12.8 pmol standard.

8.3. Assay procedure

Note: The procedure can be modified to increase sensitivity and improve assay automation. These alternative protocols are described on pages 29–30 of the pack leaflet.

Measurement of cAMP in the range 0.2–12.8 pmol/well

1. Prepare assay buffer and standards ranging from 0.2–12.8 pmol/50 μ l as described in the previous section.
2. Equilibrate all reagents to room temperature and mix before use.
3. Set up the plate to enable the running in duplicate of all non-specific binding (NSB), zero standard (B_0), standards and samples.
4. Pipette 100 μ l of assay buffer into the non-specific binding wells (NSB).
5. Pipette 50 μ l of assay buffer into the zero standard wells (B_0).
6. Starting with the most dilute, pipette 50 μ l of each standard into the appropriate wells.
7. Pipette 50 μ l of each unknown sample into appropriate wells. See sample preparation section.
8. Pipette 50 μ l of [125 I]cAMP into all wells.
9. Pipette 50 μ l of antiserum into all wells except the non-specific binding (NSB).
10. Place the SPA anti-rabbit reagent on to a magnetic stirrer and adjust the stirring speed to ensure a homogeneous suspension. Then add 50 μ l to all wells.
11. All wells should now contain a total volume of 200 μ l.
12. Seal the plates and incubate at room temperature (15–30°C) for 15–20 hours.
13. Determine the amount of [125 I]cAMP bound to the fluomicrospheres by counting in the microplate β scintillation counter for 2 minutes.

Counting

- Scintillant should not be added to the wells.
- Instrument settings for microplate scintillation counters are described overleaf:

a. Window settings for Microbeta 5-530

b. Table 1 Instrument settings for TopCount

Scintillator	Energy range	Efficiency mode	Isotope	Window settings	
				Region A	Region B
Liquid	Low	High	Iodine-125	0-100	0-256

- Where assays are carried out in vials, and counted on a rack beta counter, for those fitted with spectrum analysis packages the suitable window opening may be determined and set accordingly. Windows for other counters should be set wide open. For example:-
- a. Beckman LS7800 where H# is set up at 0 and the window open from 10-999.
 - b. Packard TriCarb 460 with the window open from 0 to 999.
 - c. LKB 1209/1215 Rack Beta with the window open from 5 to 999. SQP(I) and SQP(E) are not used.

9. Data processing

9.1. Calculation of standard curve data

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

1. Calculate the average counts per minute (cpm) for each set of replicate wells.
2. Calculate the percent bound for each standard and sample using the following relationship:-

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

A standard curve may be generated by plotting the percent B/B_0 as a function of the log cAMP concentration.

3. Plot $\% B/B_0$ (y-axis) against the amount of standard per well (x-axis). The amount of cAMP per well of the unknown samples can be read directly from the graph.

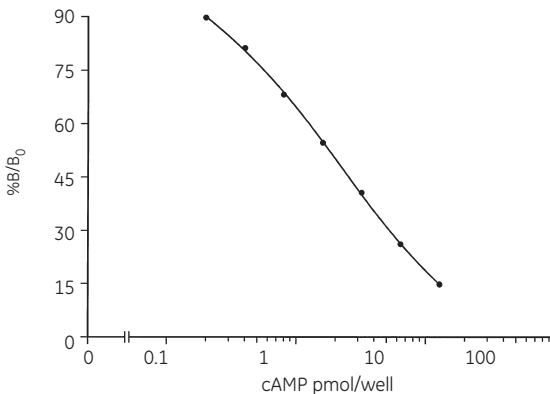


Figure 2. Typical standard curve

Table 2. Typical assay data

Well	CPM*	Average CPM	%B/B ₀ **
Non-specific binding (NSB)	259 237	248	
Zero standard (B ₀)	4916 4772	4844	
0.2 pmol standard	4490 4249	4370	89.7
0.4 pmol standard	4067 3885	3976	81.2
0.8 pmol standard	3388 3358	3373	68.1
1.6 pmol standard	2738 2768	2753	54.6
3.2 pmol standard	2143 2067	2105	40.6
6.4 pmol standard	1440 1395	1440	26.1
12.8 pmol standard	897 938	918	14.8

These data were calculated using Dynatech Microlite-1 plates counted on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this, and may give different results.

* Corrected for instrument blank

** For the calculation of % B/B₀ values, non-specific binding has been subtracted from the counts.

In order to express results from unknown samples in terms of pmol/ml, multiply obtained values from the standard curve by 20.

10. Additional information

10.1. Specificity

The cross-reactivity, as determined by the concentration giving 50% B/B₀ with a number of related compounds is shown in table 3.

Table 3

Compound	Cross-reactivity %
cAMP	100
cIMP	0.01
cGMP	0.0006
cCMP	0.04
cTMP	0.002
AMP	0.0001
ADP	0.0002
ATP	0.00002
EDTA	0.0000001
Theophylline	0.000002
Iso-butyl-methyl-xanthine	0.000008

10.2. Sensitivity

The sensitivity, defined as the amount of cAMP needed to reduce the zero dose binding by two standard deviations, was 0.1 pmol/well (0.65 ng/ml).

10.3. Precision

The within-assay precision for duplicate determinations was calculated by measuring samples in the assay. The results are shown overleaf.

Table 4

Sample	pmol cAMP/well ± SD	% CV	n
A	0.352 ± 0.040	11.3	20
B	0.999 ± 0.056	5.7	20
C	4.189 ± 0.123	2.9	20

The between assay precision was assayed by repeated measurement of the same samples in successive assays. The results are shown below.

Table 5

Sample	pmol cAMP/well ± SD	% CV	n
A	0.331 ± 0.036	10.8	20
B	0.884 ± 0.099	11.2	20
C	3.946 ± 0.355	9.0	20

10.4. Precision profile

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

Table 6

Standard (pmol/well)	Standard deviation	% CV
0.2	0.021	10.70
0.4	0.032	7.87
0.8	0.071	6.42
1.6	0.078	5.25
3.2	0.20	6.40
6.4	0.39	6.09
12.8	0.80	6.80

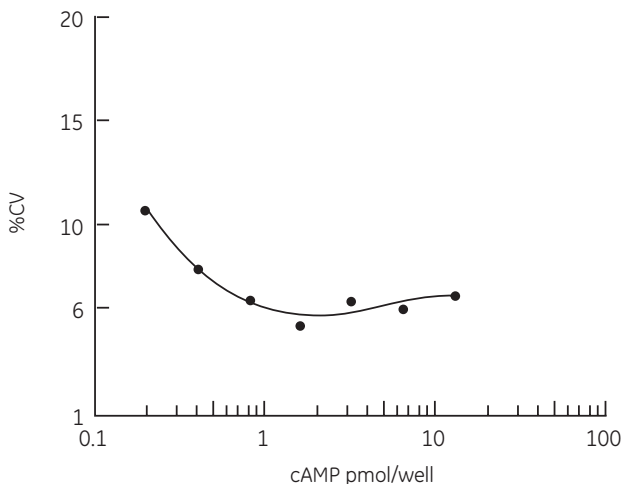


Figure 3. Precision profile

10.5. Troubleshooting guide

Table 7

Problem	Check
Poor replication	<ul style="list-style-type: none"> • Reduce the pipetting speed. Use ‘contain’ pipetting with tip touch at source and destination • Use a pre-wet cycle. This improves replicates when using small volumes (eg. 50 μl) • Ensure that a homogeneous suspension of fluomicrosphere is maintained • If replication is still poor, calibrate tools. If no improvement is gained, contact supplier of equipment

10.6. 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 (9).

cAMP was identified as playing a major role in the mode of action of adrenaline 30 years ago (10–13). 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 (14) and nervous systems (15), in immune mechanisms (16), cell growth and differentiation (17), and general metabolism (18). 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 (19–22). The method allows high sensitivity without the interference from ATP to which other adenylate cyclase assays are prone (22).

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10.7. Related products

*cAMP direct [^{125}I] SPA assay	RPA 559
*cAMP direct enzymeimmunoassay (EIA) system (dual range)	RPN 225
cAMP [^3H] assay system	TRK 432
cAMP [^{125}I] assay system (dual range)	RPA 509
*cAMP direct [^{125}I] scintillation proximity assay (SPA) system (dual range) 100 tubes	RPA 538
*cAMP direct [^{125}I] scintillation proximity assay (SPA) system (dual range) 500 tubes	RPA 542
cGMP enzymeimmunoassay (EIA) system (dual range)	RPN 226
cGMP [^3H] assay system	TRK 500
cGMP [^{125}I] assay system (dual range)	RPA 525
cGMP [^{125}I] scintillation proximity assay (SPA) system (dual range) 100 tubes	RPA 540
cGMP [^{125}I] scintillation proximity assay (SPA) system (dual range) 500 tubes	RPA 541
<i>sn</i> -1,2-Diacylglycerol (DAG) assay system	RPN 200
D- <i>myo</i> -Inositol 1,4,5-trisphosphate (IP_3) [^3H] assay system	TRK 1000
MAP kinase enzyme assay	RPN 84
Protein kinase C enzyme assay	RPN 77
Amprep SAX 500 mg (pack of 50)	RPN 1918
Amprep SAX 100 mg (pack of 100)	RPN 1908
Amprep column adaptor (pack of 15)	RPN 1927
Amprep reservoir (pack of 100)	RPN 1929
Amprep manifold-10	RPN 1930
Amprep super-separator-24	RPN 1940

* Contains novel lysis reagents which eliminate traditional time consuming extraction procedures and allow direct cAMP measurement

Scintillation proximity assay reagents

Anti-rabbit	RPN 140
Anti-mouse	RPN 141
Anti-sheep	RPN 142
Protein A	RPN 143

10.8. Alternative protocol for increased sensitivity

For the measurement of cAMP in the range 0.025–3.2 pmol/well.

Preparation of reagents

Assay reagents should be prepared as described previously on page 17. Prepare working standards over the range 0.025–6.4 pmol as outlined on page 20. Discard the 6.4 pmol standard, giving 8 standards ranging from 0.025–3.2 pmol/well.

Assay procedure

Perform the assay as described in the procedure on page 20 of the pack leaflet. However the plates should be incubated at 2–8°C for 15–20 hours instead of room temperature.

Important

It is imperative that the microplates are counted immediately or within a maximum of 3 hours following completion of the incubation period. Otherwise the assay will re-equilibrate to ambient laboratory temperature resulting in a loss of sensitivity and possible assay drift.

Note: This protocol is not routinely quality controlled.

10.9. Alternative protocol for improved assay automation

Preparation of reagents

Prepare assay reagents and working standards in the range of 0.2–12.8 pmol/well as described on pages 17 and 19. Add equal volumes of tracer, antiserum and SPA anti-rabbit reagent to a beaker, ensuring that sufficient reagent is prepared to run the desired number of wells (e.g. 1 ml tracer, 1 ml antiserum and 1 ml SPA anti-rabbit reagent should provide sufficient mixture for 20 wells). This working solution should be freshly prepared before each assay and not be re-used.

Assay procedure

Note: It is recommended that non-specific binding wells (NSB) are excluded from this protocol because of the addition of antiserum to the working solution.

1. Pipette 50 μ l of assay buffer into the zero standard wells (B_0).
2. Starting with the most dilute, pipette 50 μ l of each standard into the appropriate wells
3. Pipette 50 μ l of each unknown sample into appropriate wells. See sample preparation section.
4. Place the working solution (containing equal volumes of tracer, antiserum and SPA anti-rabbit reagent) on to a magnetic stirrer and adjust the stirring speed to ensure a homogeneous suspension. Add 150 μ l to all wells, which should now contain a total volume of 200 μ l.
5. Seal, incubate and count the plates as described previously on page 20 of the pack leaflet.
6. Calculate results as described previously.

Note: This protocol is not routinely quality controlled.

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