GE Healthcare

Amersham cAMP SPA Biotrak Direct Screening Assay System

5 x 96 wells

Product Booklet

Code: RPA559

Page finder

1. Legal

http//www.gehealthcare.com/lifesciences

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

2. Handling

2.1. Safety warnings and precautions

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

Caution: Radioactive material

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

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

vials containing 'hard' beta emitters such as phosphorus-32 or gamma emitting labelled compounds. Ampoules likely to contain volatile radioactive compounds should be opened only in a well ventilated fume cabinet.

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

should check local regulations.

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

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).

2.2. Storage

Store at 2–8°C.

2.3. Expiry

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

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 10 ml. On dilution the contents of this bottle will produce a 0.05 M acetate buffer containing 0.01% sodium azide. See safety data sheet supplied.

Standard, 2 vials
cAMP standard 512 pmol, for assays in the range 0.2–25.6 pmol/well, lyophilized. On reconstitution each bottle contains 512 pmol/ml.

Tracer, 2 vials
Adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I]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.

Lysis reagent 1
Dodecyltrimethylammonium bromide, 2 g, solid. See safety data sheet supplied.

Lysis reagent 2
Solid, 5 g. The lysis reagent 2 vial contains no chemicals classified as hazardous.

4. Description

cAMP SPA Biotrak™ Direct Screening Assay System from GE Healthcare utilizes novel lysis reagents in order to facilitate simple and rapid extraction of intracellular cAMP. These components avoid the requirement for removal of extraction reagents prior to measurement, by ensuring cAMP is available for subsequent analysis. Indeed, the method allows the extraction and measurement of cAMP to be carried out directly in the microplate used to culture the cells.

The procedure may be carried out in one of three ways. A two-stage method for intracellular cAMP measurement is described where cells are cultured in a separate vessel from the microplate used for assay. Here the cells are lysed and an aliquot of lysate is transferred to a second plate for assay. The one-stage method for intracellular cAMP measurement allows extraction and measurement to be carried in the microplate used for culturing cells. The third method is a one-stage technique which measures the combined amount of intracellular and cell super-natant cAMP. This fraction is referred to as 'total' cellular cAMP.

The kit makes use of scintillation proximity assay technology (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 enables complete automation of the assay.

cAMP may be routinely measured in the range
0.2–25.6 pmol/well (1.32–168.56 ng/ml).
The sensitivity of the assay system is 0.1 pmol/well (0.65 ng/ml).

Each pack contains sufficient material for 5 x 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.

- Elimination of inconvenient, time consuming extraction procedures
- No separation step
- Few pipetting steps
- Automatable
- Direct measurement of intracellular levels of cAMP
- Extracellular (cell culture media only) levels of cAMP may be measured with the cAMP SPA screening assay (RPA556)

This assay system has been specifically designed for research purposes.

5. Critical parameters

The following points are critical.

- It is essential to read the complete instruction booklet before starting work.
- Do not agitate the plates during the course of the assay incubation period.
- A separate standard curve must be run on each plate.
- Carry out a microscopic evaluation before and after lysing cells.

6. Additional equipment and reagents required

- Microplate liquid scintillation counter
- 96 well microplates compatible with the scintillation counter

  One-stage and total cAMP assays:- Viewplates™ are available from Packard Instrument Company, 800 Research Parkway, Meriden, CT 06450, USA

  Two-stage assay:- Disposable tissue-culture plates are supplied by a wide range of companies, eg. Costar, One Alewife Center, Cambridge, MA 02140, USA.

  96-well Microlite™ plates (for scintillation counting) are available from Dynatech Inc. Chantilly, Virginia 22021, USA
- Plate sealers
- Disposable polypropylene or polystyrene tubes (12x75 mm) for preparation of working standards, drugs, agonists and cell viability reagents.
- Pipettes or pipetting equipment (50 µl, 1 ml, 2 ml, 5 ml, 20 µl, 200 µl)
- Glass measuring cylinder (2000 ml)
- Distilled or deionized water
- Test tube rack to hold tubes
- Vortex mixer
- Magnetic stirrer and stirrer bars
- 0.4% Trypan blue solution
- Centrifuge and microplate holders for centrifuge (if using suspension cells)

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.

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.

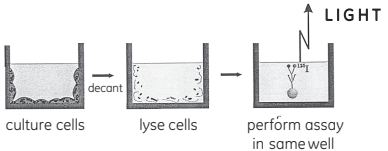
7. Selection of appropriate protocol

This kit offers a simple extraction and measurement method for intracellular levels of cAMP. The protocol may be carried out in one of 3 ways.

- A two-stage method is described where cells are cultured in a separate vessel from the microplate used for assay. Here the cells are lysed and an aliquot of lysate is transferred to a second plate for assay.
- In addition, a one-stage method is described whereby extraction and measurement is carried out in the same microplate that is used for culturing cells.
- Furthermore, for the one-stage method an alternative procedure for the measurement of both intracellular and extracellular (cAMP present in cell culture media) cAMP is described. This is referred to as 'total' cellular cAMP (see assay principle, figure 1).

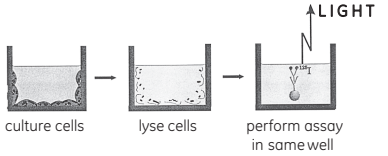
cAMP DIRECT SCREENING PROTOCOLS

One stage intracellular cAMP measurement (See pages 25-33)



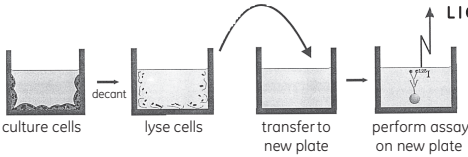
Benefits
- High throughput
- Quicker
- Fewer manipulations

One stage total cAMP measurement-intracellularand cell supernatant cAMP (See pages 34-43)



Benefits
- High throughput
- No decantation

Two stage assay-intracellularcAMP measurement (See pages 16-24)



Benefits
- High throughput
- Uses standard microplates
- If unsure of cAMP concentration – can reassay

SPA reagent
cAMP specific antibody
unknown
cAMP tracer

Figure 1. Assay principle

8. Assay procedure: Two stage assay - intracellular cAMP measurement

8.1. Reagent preparation

Stability before reconstitution

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.

Stability after reagent reconstitution

SPA beads: 4 weeks when stored at 2–8°C.

All others (excluding immunoreagent): 14 days when stored at 2–8°C.

The immunoreagent solution should be freshly prepared before each assay and not re-used.

After storage, thoroughly mix buffer A for 20 minutes before use.

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 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 pH5.8 containing 0.01%(w/v) sodium azide.

Lysis reagent 1

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.

1. Transfer the contents of the bottle (lysis reagent 1, solid) to a 50 ml graduated cylinder by repeated washing with assay buffer.

Dissolve in 15 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 20 ml with assay buffer and mix thoroughly. The final solution contains a 10% solution of dodecyltrimethylammonium bromide in assay buffer. This is buffer A.
3. Take 10 ml of buffer A and make up to 100 ml with assay buffer to give a final 1% solution of dodecyltrimethyl-ammonium bromide in assay buffer. This is lysis reagent 1 (working solution).

Buffer A is used in the 'total' cellular cAMP assay. Stir continuously when used. Lysis reagent 1 is used in the intracellular protocols (one and two stage).

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) to a 250 ml graduated cylinder by repeat washings with assay buffer.
2. Adjust the final volume to 200 ml with assay buffer and mix thoroughly. This is lysis reagent 2 (working solution).

Standard

1. Add 1 ml diluted lysis reagent 1 (working solution) and replace the stopper.
2. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 512 pmol/ml.

Tracer

1. Add 14 ml lysis reagent 2 (working solution) 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'-0-succinyl-3-[^{125}I]iodotyrosine methyl ester.

Antiserum

1. Add 15 ml diluted lysis reagent 2 (working solution) 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 lysis reagent 2 (working solution) to the bottle and replace the stopper.
2. Gently shake the bottle for 5 minutes.

Preparation of immunoreagent

This step involves adding tracer, antiserum and SPA anti-rabbit reagent together before use in the assay.

1. Add equal volumes of tracer, antiserum and SPA anti-rabbit reagent to a beaker, ensuring that a sufficient volume of this mixture is prepared for the desired number of wells (eg 1 ml tracer, 1 ml antiserum and 1 ml SPA anti-rabbit reagent will provide sufficient mixture for 20 wells).
2. Mix thoroughly.
3. **This immunoreagent solution should be freshly prepared before each assay and not re-used.**

NOTE: The SPA fluomicrospheres are insoluble. The beaker 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.

8.2. Preparation of working standards

For the measurement of cAMP in the range 0.2–25.6 pmol/well.

1. Label 7 polypropylene or polystyrene tubes (12x75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol and 12.8 pmol.
2. Pipette 500 µl of lysis reagent 1 into all the tubes.

3. Into the 12.8 pmol tube pipette 500 µl of stock standard (512 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 12.8 pmol tube to the 6.4 pmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 50 µl aliquots from each serial dilution will give rise to 7 standard levels of cAMP ranging from 0.2–12.8 pmol/well.
7. 50 µl from the stock standard vial will give rise to the 25.6 pmol standard.
8. All working standards will contain different standard levels of cAMP dissolved in assay buffer containing 1%(w/v) lysis reagent 1.

NOTE: Care should be taken when preparing working standards. Dodecyltrimethylammonium bromide may cause frothing. Vigorous pipetting should be avoided.

8.3. Cell lysis methods

Adherent cells

1. Culture cells (100 µl volumes) in flat-bottomed 96-well microplates (tissue culture grade), with cell concentrations between 10^4 and 10^6 cells/well.
2. Incubate plate overnight at 37°C, (5% CO_2 and 95% humidity).
3. Decant or aspirate excess culture media and add 100 µl of diluted lysis reagent 1 (working solution).

NOTE: Do not use cell cultures that are over-confluent (eg at 10^7 cells/ml) as cells may be lost during the decantation step.

4. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be readily achieved by vigorous, successive pipetting. Incubate cells for 5 minutes after adding the lysis reagent.

5. Carry out a microscopic evaluation to check cells have lysed. **Cell membranes may still be visible after cell lysis.**

   When cells are lysed, immediately process the extracted cAMP for measurement with the SPA radioimmunoassay (see 'assay protocol').

Suspension cells

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

1. Culture cells (100 µl volumes) in flat-bottomed 96-well microplates (tissue culture grade), with cell concentrations between 10^4 and 10^6 cells/well.
2. Incubate plate overnight at 37°C, (5% CO_2 and 95% humidity).
3. Centrifuge the microplate using a centrifugal microplate adapter (1500–2000 x g) for 3 minutes to form a pellet in each well.
4. Gently decant or aspirate excess media and resuspend pellet in 100 µl of lysis reagent 1 (working solution).
5. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be readily achieved by vigorous, successive pipetting. Incubate cells for 5 minutes after adding the lysis reagent.
6. Carry out a microscopic evaluation to check cells have lysed. Lysed cells are now ready for use with the SPA radioimmunoassay protocol. Immediately process the extracted cAMP for measurement with the SPA radioimmunoassay (see 'assay protocol').

8.4. Assay protocol - two stage method for the measurement of cAMP in the range 0.2–25.6 pmol/well

Equilibrate all reagents to room temperature and mix before use. Set up the plate to enable the running in duplicate of all zero standard

(B_0), standards and samples.

This method has fewer pipetting steps compared with standard assays. It involves adding tracer, antiserum and SPA anti-rabbit reagent together before use.

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 µl of lysis reagent 1 into the zero standard wells (B_0)
2. Starting with the most dilute, pipette 50 µl of each standard containing lysis reagent 1 into the appropriate wells.
3. Pipette 50 µl of each unknown sample into the appropriate wells. (See section on cell lysis methods).
4. Place the immunoreagent solution (containing equal volumes of tracer, antiserum and SPA anti-rabbit reagent, in lysis reagent 2) on to a magnetic stirrer and adjust the stirring speed to ensure a homogeneous suspension. Add 150 µl to all wells.

   All wells should contain a total volume of 200 µl.
5. Seal the plates and incubate at room temperature (15–30°C) for 15–20 hours.
6. Determine the amount of [^{125}I]cAMP bound to the fluomicrospheres by counting in a microplate β scintillation counter for 2 minutes.

See page 24 for typical data.

Table 1. Assay summary

	Zero standard (B_0)	Standards	Samples
Lysis reagent 1	50	–	–
Standard	–	50	–
Samples	–	–	50
Immunoreagent	150	150	150
Seal plate, incubate overnight and count			

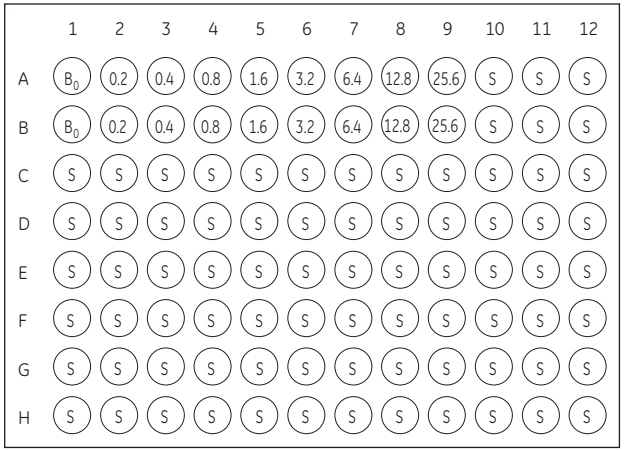


Figure 2. Plate map for the two stage assay

8.5. Counting

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

Window settings for Microbeta: 5–530

Table 2. 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

9. Data processing: Two stage assay - intracellular cAMP measurement

9.1. Calculation of results

The assay data collected should be similar to the data shown in table 3.

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:

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

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.

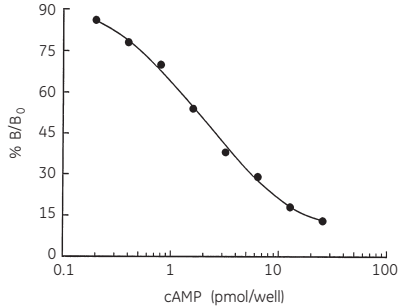


Figure 3. Typical standard curve (for two-stage protocol)

9.2. Typical assay data

Table 3. Typical assay data for two-stage protocol

Well	CPM*	Average CPM	% B/B_0
Zero standard (B_0)	5158 5202	5180	
0.2 pmol standard	4419 4510	4465	86
0.4 pmol standard	3926 4115	4021	78
0.8 pmol standard	3665 3769	3717	72
1.6 pmol standard	2810 2781	2796	54
3.2 pmol standard	1917 1950	1934	37
6.4 pmol standard	1470 1473	1472	29
12.8 pmol standard	890 957	924	18
25.6 pmol standard	647 672	660	13

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

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

10. Assay procedure: One stage assay - intracellular cAMP measurement

10.1. Reagent preparation

Stability before reconstitution

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.

Stability after reagent reconstitution

SPA beads: 4 weeks when stored at 2–8°C.

All others (excluding immunoreagent): 14 days when stored at 2–8°C.

The immunoreagent solution should be freshly prepared before each assay and not re-used.

After storage, thoroughly mix buffer A for 20 minutes before use.

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 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 pH5.8 containing 0.01%(w/v) sodium azide.

Lysis reagent 1

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.

1. Transfer the contents of the bottle (lysis reagent 1, solid) to a 50 ml graduated cylinder by repeated washing with assay buffer.

Dissolve in 15 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 20 ml with assay buffer and mix thoroughly. The final solution contains a 10% solution of dodecyltrimethylammonium bromide in assay buffer. This is buffer A.
3. Take 10 ml of buffer A and make up to 100 ml with assay buffer to give a final 1% solution of dodecyltrimethyl-ammonium bromide in assay buffer. This is lysis reagent 1 (working solution).

   Buffer A is used in the 'total' cellular cAMP assay. Stir continuously when used. Lysis reagent 1 is used in the intracellular protocols (one and two stage).

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) to a 250 ml graduated cylinder by repeat washings with assay buffer.
2. Adjust the final volume to 200 ml with assay buffer and mix thoroughly. This is lysis reagent 2 (working solution).

Standard

1. Add 1 ml diluted lysis reagent 1 (working solution) and replace the stopper.
2. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 512 pmol/ml.

Tracer

1. Add 14 ml lysis reagent 2 (working solution) 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'-0-succinyl-3-[^{125}I]iodotyrosine methyl ester.

Antiserum

1. Add 15 ml diluted lysis reagent 2 (working solution) 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 lysis reagent 2 (working solution) to the bottle and replace the stopper.
2. Gently shake the bottle for 5 minutes.

Preparation of immunoreagent

This step involves adding tracer, antiserum and SPA anti-rabbit reagent together before use in the assay.

1. Add equal volumes of tracer, antiserum and SPA anti-rabbit reagent to a beaker, ensuring that a sufficient volume of this mixture is prepared for the desired number of wells (eg 1 ml tracer, 1 ml antiserum and 1 ml SPA anti-rabbit reagent will provide sufficient mixture for 20 wells).
2. Mix thoroughly.
3. **This immunoreagent solution should be freshly prepared before each assay and not re-used.**

NOTE: The SPA fluomicrospheres are insoluble. The beaker 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.

10.2. Preparation of working standards

For the measurement of cAMP in the range 0.2–25.6 pmol/well.

1. Label 7 polypropylene or polystyrene tubes (12 x 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol and 12.8 pmol.
2. Pipette 500 µl of lysis reagent 1 into all the tubes.
3. Into the 12.8 pmol tube pipette 500 µl of stock standard (512 pmol/ml) and mix thoroughly.

4. Transfer 500 µl from the 12.8 pmol tube to the 6.4 pmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 50 µl aliquots from each serial dilution will give rise to 7 standard levels of cAMP ranging from 0.2–12.8 pmol/well.
7. 50 µl from the stock standard vial will give rise to the 25.6 pmol standard.
8. All working standards will contain different standard levels of cAMP dissolved in assay buffer containing 1%(w/v) lysis reagent 1.

NOTE: Care should be taken when preparing working standards. Dodecyltrimethylammonium bromide may cause frothing. Vigorous pipetting should be avoided.

10.3. Cell lysis methods

NOTE: Leave empty wells on the microplate for preparation of the standard curve.

Adherent cells

1. Culture cells (50 µl volumes) in clear bottomed 96-well microplates with opaque walls eg. Viewplates, (tissue-culture treated) using cell concentrations between 10^4 and 10^6 cells/well. Incubate plate overnight at 37°C, (5% CO_2 and 95% humidity).
2. On day 2 decant or aspirate excess culture media and add 50 µl of reconstituted lysis reagent 1 (working solution).

NOTE: Do not use cell cultures that are over-confluent (eg at 10^7 cells/ml) as cells may be lost during the decantation step.

3. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be readily achieved by vigorous, successive pipetting. Incubate cells for 5 minutes after adding the lysis reagent.

4. Carry out a microscopic evaluation to check cells have lysed. Lysed cells are now ready for use with the SPA radioimmunoassay protocol. Immediately process the extracted cAMP for measurement with the SPA radioimmunoassay (see 'assay protocol').

Suspension cells

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

1. Culture cells (50 µl volumes) in flat-bottomed 96-well microplates with opaque wells (tissue-culture treated) using cell concentrations between 10^4 and 10^6 cells/well. Incubate plate overnight at 37°C, (5% CO_2 and 95% humidity).
2. On day 2 centrifuge the plate using a centrifugal microplate adapter (1500–2000 x g) for 3 minutes to form a pellet in each well.
3. Gently decant or aspirate excess media and resuspend each pellet in 50 µl of lysis reagent 1 (working solution).
4. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be readily achieved by vigorous, successive pipetting. Incubate cells for 5 minutes after adding the lysis reagent.
5. Carry out a microscopic evaluation to check cells have lysed. Lysed cells are now ready for use with the SPA radioimmunoassay protocol. Immediately process the extracted cAMP measurement with the SPA radioimmunoassay (see 'assay protocol').

10.4. Assay protocol - one stage method for the measurement of cAMP in the range 0.2–25.6 pmol/well

Equilibrate all reagents to room temperature and mix before use. Use the microplate containing cultured cells (see day 1 of cell lysis methods) to enable the running in duplicate of all zero binding (B_0), standards and sample wells.

This method has fewer pipetting steps compared with standard assays. It involves adding tracer, antiserum and SPA anti-rabbit reagent buffer together before use.

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 µl of lysis reagent 1 into the zero standard wells (B_0).
2. Starting with the most dilute, pipette 50 µl of each standard containing lysis reagent 1 into the appropriate wells.
3. Place the immunoreagent solution (containing equal volumes of tracer, antiserum and SPA anti-rabbit reagent, in lysis reagent 2) on to a magnetic stirrer and adjust the stirring speed to ensure a homogeneous suspension. Add 150 µl to all wells.

   All wells should contain a total volume of 200 µl.
4. Seal the plates and incubate at room temperature (15–30°C) for 15–20 hours.
5. Determine the amount of [^{125}I]cAMP bound to the fluomicrospheres by counting in a microplate β scintillation counter for 2 minutes.

NOTE: Place a white card under the plate before counting. This is in order to enhance the number of counts detected.

See page 33 for typical data.

Table 4. Assay summary

	Zero standard (B_0)	Standards	Samples
Lysis reagent 1	50	–	–
Standard	–	50	–
Samples	–	–	50
Immunoreagent	150	150	150
Seal plate, incubate overnight and count			

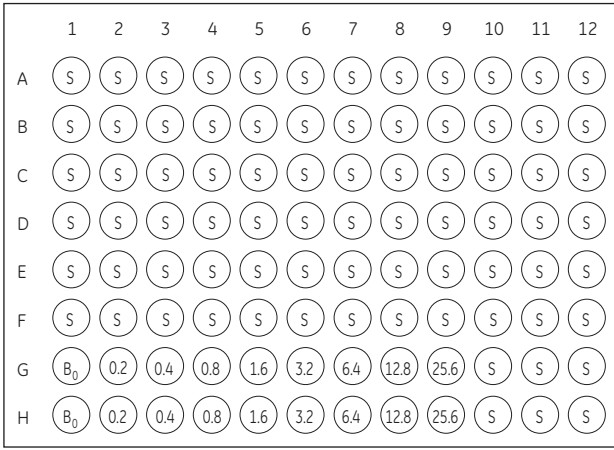


Figure 4. Plate map for one-stage and total cAMP assays

10.5. Counting

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

Window settings for Microbeta: 5–530

Table 5. 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

11. Data processing: One stage assay - intracellular cAMP measurement

11.1. Calculation of results

The assay data collected should be similar to the data shown in table 6.

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:

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

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.

11.2. Typical assay data

Table 6. Typical assay data for one-stage and total cAMP protocols

Well	CPM*	Average CPM	% B/B_0
Zero standard (B_0)	2329 2169	2249	
0.2 pmol standard	2184 2135	2160	96
0.4 pmol standard	1933 2000	1967	88
0.8 pmol standard	1806 1826	1816	81
1.6 pmol standard	1552 1568	1560	69
3.2 pmol standard	1177 1124	1151	51
6.4 pmol standard	730 769	750	33
12.8 pmol standard	627 554	591	26
25.6 pmol standard	426 367	397	18

These data were calculated using Viewplates counted on a TopCount microplate scintillation counter.

*Corrected for instrument blank

A white card was placed below the Viewplate when counting in order to enhance the counts.

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

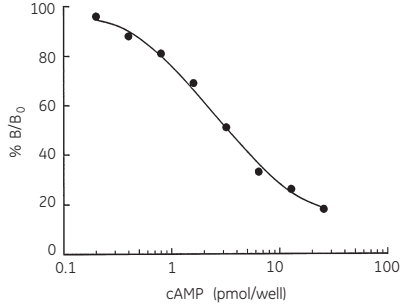


Figure 5. Typical standard curve (for one-stage protocol)

12. Assay procedure: -'Total' cellular cAMP measurement

NOTE: Do not use cell culture media containing phenol red dye. Leave empty wells on the microplate for preparation of the standard curve.

12.1. Reagent preparation

Stability before reconstitution

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.

Stability after reagent reconstitution

SPA beads: 4 weeks when stored at 2–8°C.

All others (excluding immunoreagent): 14 days when stored at 2–8°C.

The immunoreagent solution should be freshly prepared before each assay and not re-used.

After storage, thoroughly mix buffer A for 20 minutes before use.

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 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 pH5.8 containing 0.01%(w/v) sodium azide.

Lysis reagent 1

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.

1. Transfer the contents of the bottle (lysis reagent 1, solid) to a 50 ml graduated cylinder by repeated washing with assay buffer. Dissolve in 15 ml of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.
2. Adjust the final volume to 20 ml with assay buffer and mix thoroughly. The final solution contains a 10% solution of dodecyltrimethylammonium bromide in assay buffer. This is buffer A, which is used in the 'total' cellular cAMP assay in the cell lysis method. Stir continuously when used.
3. Take 10 ml of buffer A and make up to 100 ml with assay buffer to give a final 1% solution of dodecyltrimethyl-ammonium bromide in assay buffer. This is lysis reagent 1 (working solution), which is used for the preparation of standards.

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) to a 250 ml graduated cylinder by repeat washings with assay buffer.
2. Adjust the final volume to 200 ml with assay buffer and mix thoroughly. This is lysis reagent 2 (working solution).

Standard

1. Add 1 ml diluted lysis reagent 1 (working solution) and replace the stopper.
2. Gently mix until the contents are completely dissolved. The final solution contains cAMP at a concentration of 512 pmol/ml.

Tracer

1. Add 14 ml lysis reagent 2 (working solution) 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'-0-succinyl-3-[^{125}I]iodotyrosine methyl ester.

Antiserum

1. Add 15 ml diluted lysis reagent 2 (working solution) 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 lysis reagent 2 (working solution) to the bottle and replace the stopper.
2. Gently shake the bottle for 5 minutes.

Preparation of immunoreagent

This step involves adding tracer, antiserum and SPA anti-rabbit reagent together before use in the assay.

1. Add equal volumes of tracer, antiserum and SPA anti-rabbit reagent to a beaker, ensuring that a sufficient volume of this mixture is prepared for the desired number of wells (eg 1 ml tracer, 1 ml antiserum and 1 ml SPA anti-rabbit reagent will provide sufficient mixture for 20 wells).
2. Mix thoroughly.
3. **This immunoreagent solution should be freshly prepared before each assay and not re-used.**

NOTE: The SPA fluomicrospheres are insoluble. The beaker 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.

12.2. Preparation of working standards

For the measurement of cAMP in the range 0.2–25.6 pmol/well.

1. Label 7 polypropylene or polystyrene tubes (12 x 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol and 12.8 pmol.
2. Pipette 500 µl of **lysis reagent** 1 into all the tubes.
3. Into the 12.8 pmol tube pipette 500 µl of stock standard (512 pmol/ml) and mix thoroughly.
4. Transfer 500 µl from the 12.8 pmol tube to the 6.4 pmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 50 µl aliquots from each serial dilution will give rise to 7 standard levels of cAMP ranging from 0.2–12.8 pmol/well.
7. 50 µl from the stock standard vial will give rise to the 25.6 pmol standard.
8. All working standards will contain different standard levels of cAMP dissolved in assay buffer containing 1%(w/v) lysis reagent 1.

NOTE: Care should be taken when preparing working standards. Dodecyltrimethylammonium bromide may cause frothing. Vigorous pipetting should be avoided.

12.3. Cell lysis method - adherent and suspension cells

NOTE: Leave empty wells on the microplate for the preparation of the standard curve.

1. Culture adherent or suspension cells (40 µl/well) in clear bottomed 96-well microplates with opaque wells, eg. Viewplates (tissue culture grade), with cell concentrations between 10^4 and 10^6

cells/well. Incubate the plate overnight at 37°C, (5% CO_2 and 95% humidity).

2. Add 5 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.
3. Add 5 µl of **buffer A**, (10% dodecyltrimethylammonium bromide in assay buffer). The final volume in the wells should be 50 µl, each containing 1% buffer A (final concentration) which is equivalent to the lysis reagent 1 working solution.
4. Following the addition of buffer A, agitate cells to facilitate cell lysis. This can be readily achieved by vigorous, successive pipetting. Incubate the plate for 5 minutes at room temperature (in order to achieve cell lysis).
5. Carry out a microscopic evaluation. Lysed cells are now ready for use with the SPA radioimmunoassay protocol. Immediately process the extracted cAMP for measurement with the SPA radioimmunoassay (see 'assay protocol'). Do not transfer aliquots to a second plate for assay.

12.4. Assay protocol - total cAMP, for the measurement of cAMP in the range 0.2–25.6 pmol/well

Equilibrate all reagents to room temperature and mix before use. Use the microplate containing cultured cells (see day 1 of cell lysis methods) to enable the running in duplicate of all zero binding (B_0), standards and sample wells.

This method has fewer pipetting steps compared with standard assays. It involves adding tracer, antiserum and SPA anti-rabbit reagent buffer together before use.

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 µl of lysis reagent 1 (working solution) into the zero standard wells (B_0).
2. Starting with the most dilute, pipette 50 µl of each standard containing lysis reagent 1 into the appropriate wells.
3. Place the immunoreagent solution (containing equal volumes of tracer, antiserum and SPA anti-rabbit reagent, in lysis reagent 2) on to a magnetic stirrer and adjust the stirring speed to ensure a homogeneous suspension. Add 150 µl to all wells.

   All wells should contain a total volume of 200 µl.
4. Seal the plates and incubate at room temperature (15–30°C) for 15–20 hours.
5. Determine the amount of [^{125}I]cAMP bound to the fluomicrospheres by counting in a microplate β scintillation counter for 2 minutes.

NOTE: Place a white card under the plate before counting. This is in order to enhance the number of counts detected.

See page 42 for typical data

Table 7. Assay summary

	Zero standard (B_0)	Standards	Samples
Lysis reagent 1	50	–	–
Standard	–	50	–
Samples	–	–	50
Immunoreagent	150	150	150
Seal plate, incubate overnight and count			

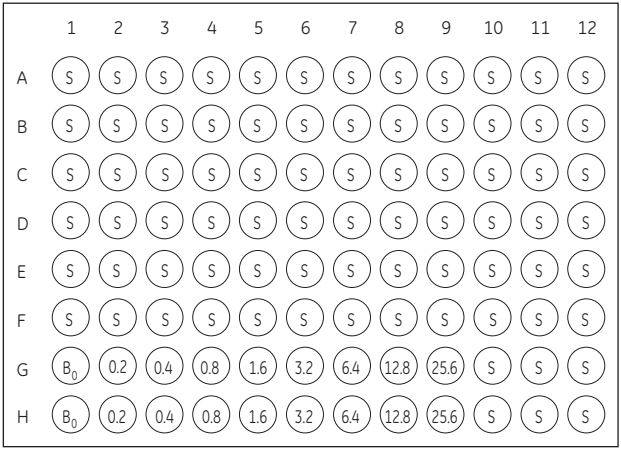


Figure 6. Plate map for one-stage and total cAMP assays

12.5. Counting

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

Window settings for Microbeta: 5–530

Table 8. 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

13. Data processing: 'Total' cellular cAMP measurement

13.1. Calculation of results

The assay data collected should be similar to the data shown in table 9.

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:

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

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.

13.2. Typical assay data

Table 9. Typical assay data for one-stage and total cAMP protocols

Well	CPM*	Average CPM	% B/B_0
Zero standard (B_0)	2329 2169	2249	
0.2 pmol standard	2184 2135	2160	96
0.4 pmol standard	1933 2000	1967	88
0.8 pmol standard	1806 1826	1816	81
1.6 pmol standard	1552 1568	1560	69
3.2 pmol standard	1177 1124	1151	51
6.4 pmol standard	730 769	750	33
12.8 pmol standard	627 554	591	26
25.6 pmol standard	426 367	397	18

These data were calculated using Viewplates counted on a TopCount microplate scintillation counter.

*Corrected for instrument blank

A white card was placed below the Viewplate when counting in order to enhance the counts.

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

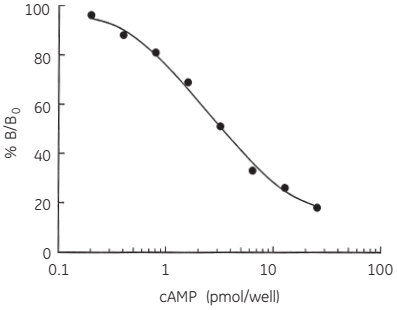


Figure 7. Typical standard curve (for 'total' protocol)

14. Additional information

14.1.Specificity

The cross-reactivity, as determined by the concentration giving 50% B/B_0 with a number of related compounds is shown in table 10.

Table 10.

Compound	Cross-reactivity %
cAMP	100
cIMP	0.006
cGMP	0.0015
cCMP	0.028
cTMP	0.0058
AMP	0.00008
ADP	0.0001
ATP	<0.00025
EDTA	<0.00025
Theophylline	0.0001
Iso-butyl-methyl-xanthine	0.0004

14.2. 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).

14.3. Precision

The within-assay precision for duplicate determinations was calculated by measuring crystalline controls in the assay. The results are shown in table 11.

Table 11.

Sample	pmol cAMP/well	% 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 12.

Sample	pmol cAMP/well	% 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

14.4. 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 13.

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

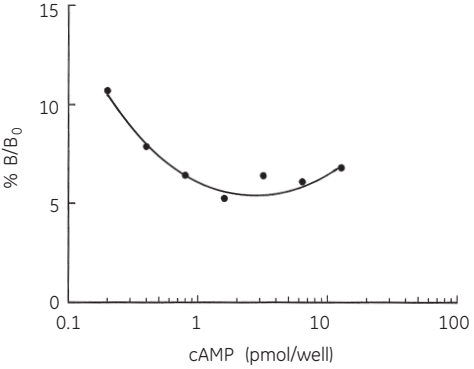


Figure 8. Precision profile

15. Troubleshooting guide

Problem	Check
1. Poor replication	**1.** Reduce the pipetting speed. **2.** Use a pre-wet cycle. This improves replicates when using small volumes (eg 50 µl). **3.** Ensure that a homogeneous suspension of SPA beads is maintained. **4.** If replication is still poor, calibrate tools. If no improvement is gained, contact supplier of equipment.
2. Cell lysis	**1.** Cell membranes may still be visible microscopically after cell lysis.

16. Background

The physiological responses to many biologically important compounds are mediated through 'second messengers'. This is a term described by Sutherland for molecules which are able to transmit intracellularly, the biological effects of compounds not able to enter the target cells themselves (1).

cAMP was identified as playing a major role in the mode of action of adrenaline 30 years ago (2–5). In response to receptor binding, the enzyme adenylate cyclase converts ATP to cAMP, which exerts its effect by activating a protein kinase capable of phosphorylating specific substrates. Numerous hormones are known to act through this mechanism including corticotrophin (ACTH), Luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), calcitonin, glucagon, vasopressin and parathyroid hormone (PTH).

cAMP has now been shown to be involved in the cardiovascular (6) and nervous systems (7), in immune mechanisms (8), cell growth and differentiation (9), and general metabolism (10). There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of the physiology and pathology of many disease states.

The assay system may be used in adenylate cyclase assays which determine cAMP formation from unlabelled ATP (11–14). The method allows high sensitivity without the interference from ATP to which other adenylate cyclase assays are prone (14).

17. References

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18. Related products

cAMP enzyme immunoassay (EIA) system (dual range)	RPN 225
cAMP [^{3}H]assay system	TRK 432
cAMP [^{125}I] assay system (dual range)	RPA 509
cAMP [^{125}I]scintillation proximity assay (SPA) system (dual range) 100 tubes	RPA 538
cAMP [^{125}I]scintillation proximity assay (SPA) system (dual range) 500 tubes	RPA 542
cGMP enzyme immunoassay (EIA) system (dual range)	RPN 226
cGMP [^{3}H] 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
sn-1,2-Diacylglycerol (DAG) assay system	RPN 200
D-*myo*-Inositol 1,4,5-trisphosphate (IP_3) [^{3}H] assay system	TRK 1000
MAP kinase enzyme assay	RPN 84
Protein kinase C enzyme assay	RPN 77
Amprep™ X 500mg (pack of 50)	RPN 1918
Amprep SAX 100mg (pack of 100)	RPN 1908

SPA reagents

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

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