GE Healthcare

Amersham cAMP Fluorescence Polarization (FP) Biotrak Immunoassay System

Product booklet

Code: RPN3596

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1. Legal

http://www.gehealthcare.com/lifesciences

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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.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product and components are 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

Assay buffer concentrate:
On dilution this bottle contains 0.05 M Sodium Acetate buffer, pH 5.8, containing 0.02% Bovine Serum Albumin and 0.01% preservative, 2 vials.

cAMP standard:
2560 pmol, for assays in the range

0.2–51.2 pmol/well, lyophilized. On reconstitution the bottle contains 2560 pmol/ml.

Antiserum:
Rabbit anti-succinyl cAMP serum, lyophilized.

Cy3B conjugate:
cAMP-Cy™3B conjugate, lyophilized.

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

Lysis reagent 2:
Solid, 5 g.

4. Other materials required

- Fluorimeter capable of reading fluorescence polarization fitted with suitable polarization filters. For this assay, fluorescence is activated at 535 nm and emission is detected at 590 nm. This assay was developed on the FARCyte™ fluorimeter that is available from GE Healthcare. This instrument is optimal for detection of fluorescence from cyanine dyes.
- Disposable polypropylene test tubes for the preparation of the working standards.
- Test tube rack to hold tubes.
- Pipettes or pipetting equipment with disposable tips.
- Vortex mixer.
- Glass measuring cylinders.
- Distilled or deionized water.
- Magnetic stirrer and stirrer bars.
- Centrifuge and microplate holders for centrifuge (if using suspension cells).
- Use the recommended plates only. For protocols 1 & 2, use Corning™ Costar™ NBS plates, catalogue code 3654; for protocols 3 & 4 use solid, black 384 tissue culture (TC) plates, catalogue code 3709, also available from Corning Inc.

5. Description

The cAMP Fluorescence Polarization (FP) Biotrak™ Immunoassay System from GE Healthcare is specifically designed for research purposes. FP technology obviates the need to separate antibody bound from free analyte, and thus unlike conventional immunoassays, is homogeneous. The cAMP FP Immunoassay System requires only the simple mixing of a sample with the immunochemical components supplied with the kit, followed by detection. This greatly improves precision and enables complete automation of the assay. The assay is based on competition between unlabeled cAMP and a fixed quantity of CyDye™-labeled cAMP, for a limited number of binding sites on a cAMP specific antibody (Figure 1).

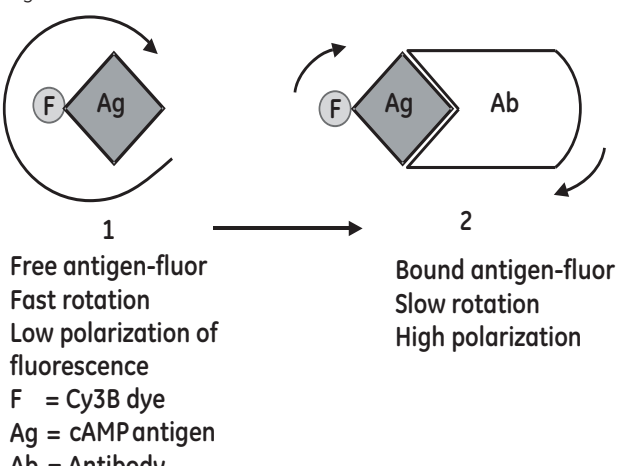


Figure 1. Principle of the assay

The present assay uses Cy3B†. This molecule is a bright version of the standard Cy3 dye, ideally suited to FP applications due to an increased lifetime when compared with conventional fluors (1).
The kit also includes 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, and ensure cAMP is directly available for analysis. Indeed, the method allows the extraction and measurement of cAMP to be carried out directly in the microplate used to culture the cells. Lysis reagent 1 hydrolyzes cell membranes to release intracellular cAMP. Lysis reagent 2 sequesters the key component in lysis reagent 1, and ensures cAMP is free for subsequent measurement. 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.
The procedure may be carried out in one of four ways (see Figure 2, page 12). Firstly, a traditional cAMP assay method is described, where the kit components are prepared in the absence of the cell lysis reagents. Secondly, 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. Thirdly, the one-stage method for intracellular cAMP measurement allows extraction and measurement to be carried out in the microtiter plate used for culturing cells. Finally, the fourth method is a one-stage technique, which measures the combined amount of intracellular and cell supernatant cAMP. This fraction is referred to as 'total' cellular cAMP.
cAMP may be measured in the range 0.2–51.2 pmol/well (3.29–842.75 ng/ml).
The sensitivity of the assay system is 0.1 pmol/well (1.65 ng/ml).

† and †† see legal section on page 3.

Each pack contains sufficient material for 384 wells. This allows the construction of one standard curve and the measurement of 180 unknowns in duplicate.

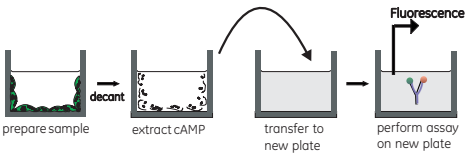
- Specific for cAMP
- Non-radioactive
- Homogeneous method
- Bright Cy3B labeled probe
- Few pipetting steps
- Automatable
- Elimination of inconvenient, time-consuming extraction procedures
- Direct measurement of intracellular levels of cAMP
- Flexible method - choice of assay protocols

6. Critical parameters

The following points are critical:

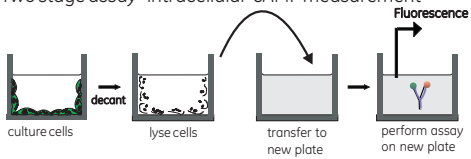
- It is essential to read the instructions before starting work.
- Do not agitate the plates during the course of the assay incubation period.
- A separate standard curve must be run on each plate.
- Allow all reagents to reach room temperature prior to performing the assay.
- Mix reagents thoroughly before use.
- Avoid excessive foaming of reagents.
- Avoid handling the tops of the wells before and after filling.
- Keep the wells covered with lids except when adding reagents and reading.
- Standards should be assayed in duplicate.
- Incubate the plates in the dark. Tin foil can be used for this.

Protocol 1.
Measurement of cAMP with traditional extraction methods



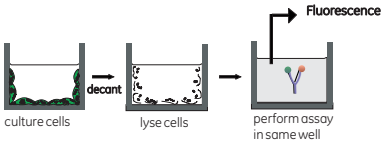
Benefits
- Traditional extraction
- Uses standard microtitre plates
- Uses a wide range of biological samples

Protocol 2.
Two stage assay -intracellular cAMP measurement



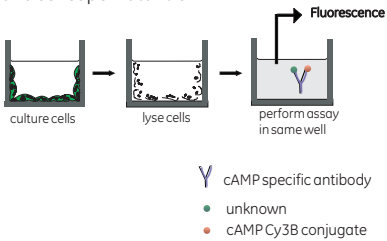
Benefits
- Traditional extraction
- Uses standard microtitre plates
- If unsure of cAMP concentration - can reassay

Protocol 3.
One stage intracellular cAMP measurement



Benefits
- High throughput
- Quicker
- Fewer manipulations

Protocol 4.
One stage total cAMP measurement- intracellular and cell supernatant cAMP



Benefits
- High throughput
- No decantation
- Rapid screen

Figure 2. cAMP FP Direct protocols

7. Selection of the appropriate protocol

Protocol 1. The normal assay used for the measurement of cAMP in urine, tissue extracts, from membrane preparations and from cell cultures prepared with traditional sample extraction methods such as acid, solvent and solid-phase methods (see page 14).

Protocol 2. This method describes an 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 (see page 23).

Protocol 3. This one-stage method for intracellular cAMP measurement allows extraction and direct measurement to be carried in the microplate used for culturing cells. Cells are simply lysed before the addition of antisera and conjugate prepared in Lysis reagent 2 (see page 30).

Protocol 4. This method is a one-stage technique, which measures the combined amount of intracellular and cell supernatant cAMP. This fraction is referred to as 'total' cellular cAMP and has the additional benefit of no decantation. As with protocol 3 measurement of cAMP is carried out in the microplate used for culturing cells (see page 38).

8. Protocol 1. Measurement of cAMP in samples using traditional extraction procedures

8.1. 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 (2–5).

Some investigators also recommend the use of ion exchange chromatography (6) following one of these extraction techniques. However, it remains the responsibility of the investigator to validate the chosen extraction procedure.

Representative procedures are described on page 15 for the extraction of cAMP from tissues and cell cultures. This information is provided for guidance only.

Urine

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

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

It is not necessary to extract or deproteinize urine before analysis. Urine should be diluted with assay buffer before measurement.

Tissue

Tissue sections must be rapidly frozen immediately after collection so as to prevent alterations to cAMP levels and its 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* (6).

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°C to give a 10% (w/v) homogenate.

2. Centrifuge at 2000 × g for 15 minutes at 4°C.

3. Recover the supernatant and discard the pellet.

4. Wash the supernatant four times with five 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 method 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.

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

Amprep extraction of cyclic AMP

Column conditioning

1. Rinse an Amprep SAX 500 mg minicolumn (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

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 × g at 4°C.

3. Dilute the 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 × g at 4°C. Apply 1 ml of supernatant to the column.

Interference removal

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.

Cell suspension

Methods of freezing biological samples for cyclic nucleotide assays are reviewed by Mayer *et al* (6).

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

Liquid phase extraction method

1. Add ice-cold Ethanol to cell suspension to give a final suspension volume of 65% (v/v) Ethanol. Allow to settle.

2. Draw of the supernatant into a test tube.

3. Wash the precipitate remaining with ice cold 65% (v/v) Ethanol and add the washings to the appropriate tube.

4. Centrifuge the extracts at 2000 × 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.

Prof. B.L. Brown, University of Sheffield kindly provided the above protocol.

Solid phase extraction method
Amprep extraction of cyclic AMP
Column conditioning

1. Rinse an Amprep SAX 500 mg minicolumn (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

1. Apply directly to the column.

Interference removal

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 can be used to elute the analyte.

8.2. Reagent preparation

Stability before reconstitution

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

Stability after reagent reconstitution

All kit reagents are stable for 14 days when reconstituted and stored at 2–8°C.

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 pH 5.8 containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Standard

1. Carefully add 1 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 2560 pmol/ml in 0.05 M acetate buffer, pH 5.8, containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Cy3B-cAMP conjugate

1. Add 5 ml of diluted assay buffer and replace the stopper. Mix until the contents are completely dissolved.

2. The final solution will contain the Cy3B-cAMP conjugate in 0.05 M acetate buffer, pH 5.8, containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

Antiserum

1. Add 5 ml of diluted assay buffer and replace the stopper. Mix until the contents are completely dissolved.

2. The final solution will contain the anti-cAMP serum in 0.05 M acetate buffer, pH 5.8, containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative.

8.3. Preparation of working standards

For the measurement of cAMP in the range 0.2–51.2 pmol/well.

1. Label 8 polypropylene or polystyrene tubes (12 × 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol, 12.8 pmol, and 25.6 pmol.

2. Pipette 0.5 ml of diluted assay buffer into all the tubes.

3. Into the 25.6 pmol tube, pipette 0.5 ml of stock standard (2560 pmol/ml) and mix thoroughly.

4. Transfer 0.5 ml from the 25.6 pmol tube to the 12.8 pmol tube and mix thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 20 µl aliquots from each serial dilution will give rise to 8 standard levels of cAMP ranging from 0.2–25.6 pmol/well.

7. 20 µl from the stock standard vial will give rise to the 51.2 pmol standard.

8. All working standards will contain different standard levels of cAMP dissolved in assay buffer.

8.4. Assay procedure

1. Prepare assay components and standards ranging from 0.2–51.2 pmol/well as described in the previous section.

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

3. Set up the microplate with sufficient wells for assaying of all blanks, conjugate only wells, zero standard, and standard wells. Recommended positioning of buffer blank (Bu), conjugate only (C), zero standard (0) and standard (0.2–51.2 pmol) wells are shown in Figure 3.

4. Pipette 40 µl of diluted assay buffer into the buffer only wells.
5. Pipette 30 µl of diluted assay buffer into the conjugate only wells.
6. Pipette 20 µl of diluted assay buffer into the zero standard wells.
7. Pipette 20 µl of each standard (0.2–51.2 pmol/well) into the appropriate wells, using a clean pipette tip for each standard.
8. Pipette 20 µl of unknown sample into the appropriate wells (see previous section on preparation of samples).
9. Pipette 10 µl of antiserum into all standard, zero standard and sample wells. Do not add antiserum to the conjugate only and buffer blank wells.
10. Carefully pipette 10 µl of the diluted Cy3B-cAMP conjugate into all wells (including the conjugate only), except for the buffer blank.
11. Cover the plate, gently mix, cover in tin foil and incubate for at least 4 hours at room temperature. Improved results are obtained with an overnight incubation step.
12. Measure the fluorescence polarization on a fluorimeter suitable for use with 384 microplates, using the appropriate polarization programme, with activation at 535 nm and emission at 590 nm.

This assay kit has been developed on the FARCyte instrument (GE Healthcare) which is optimal for use with the CyDye fluors. Instrument settings for this machine are as follows:

1. Select polarization.
2. Select the plate from the appropriate plate definition file.
3. Set up the instrument with optimal gain and 0 ms lag time. Set the integration time for 60 µs with the number of flashes set at 30. Time between move and flash -0 ms.

4. Determine the 'Z' value for the instrument using wells A1 and B1

5. Calibrate G-factor. Reference is calculated from wells A11 to wells A11 (conjugate only well), with a polarization value of **50**. Check buffer from well A12 to well A12 (buffer only wells). Check blank reduction and check same as buffer.

6. Start calibration measurement (results should be approximately 1).

7. Start polarization measurement.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
B	51.2	25.5	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
D	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
E	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
F	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
H	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
J	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
L	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
O	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Figure 3. Recommended positioning of standard (0.2–51.2 pmol) wells.

0 = zero standard, C = conjugate only, Bu = buffer blank, S = sample wells.

9. Protocol 2. Two stage assay - Intracellular cAMP measurement using cell lysis reagents

9.1. Reagent preparation

Stability before reconstitution

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

Stability after reagent reconstitution

All kit reagents are stable for 14 days when reconstituted and stored at 2–8°C.

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 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. This is readily achieved using a beaker and magnetic stirrer.

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% (w/v) 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% (w/v) 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

1. Carefully add 1 ml of lysis reagent 1B (prepared as described above) and replace the stopper.

2. Mix the contents of the bottle until completely dissolved. The final solution should contain cAMP at a concentration of 2560 pmol/ml in lysis reagent 1B.

Cy3B-cAMP conjugate

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

2. The final solution will contain the Cy3B-cAMP conjugate in lysis reagent 2B.

Antiserum

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.
2. The final solution will contain the anti-cAMP serum in lysis reagent 2B.

9.2. Cell lysis methods intracellular cAMP measurement

Note: two methods are described for intracellular cAMP measurement (protocols 2 and 3). For 'total' cellular cAMP measurement, see protocol 4).

Adherent cells

1. Culture cells, using suitable volumes, on standard 96 or 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml.
2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note:** do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation. Aspirate or decant the cell culture media.
3. Add drug, agonist etc dissolved in a suitable volume of Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step.** Incubate for a suitable time period.
4. Decant or aspirate the excess buffer.
5. Add 30–50 µl/well of diluted lysis reagent 1B.
6. Agitate the 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. Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure').

A 20 µl aliquot of lysate should be transferred from the tissue culture plate to a second plate for assay.

Suspension cells

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

1. Culture cells, using suitable volumes, on standard 96 or 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml.

2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note:** do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation.

3. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well. **Note: the actual centrifugal speed is dependent on the cells under study and should be validated by the investigator.**

4. Aspirate or decant the cell culture media.

5. Add drug, agonist etc dissolved in a suitable volume of Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step.** Incubate for a suitable time period.

6. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well.

7. Gently decant or aspirate excess buffer and resuspend the pellet in 30–50 µl of lysis reagent 1B.

8. Agitate the 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.

Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure'). A 20 µl aliquot of lysate should be transferred from the tissue culture plate to a second plate for assay.

9.3. Preparation of working standards

For the measurement of cAMP in the range 0.2–51.2 pmol/well

1. Label 8 polypropylene or polystyrene tubes (12 × 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol, 12.8 pmol, and 25.6 pmol.

2. Pipette 0.5 ml of lysis reagent 1B into all the tubes.

3. Into the 25.6 pmol tube, pipette 0.5 ml of stock standard (2560 pmol/ml) and mix thoroughly.

4. Transfer 0.5 ml from the 25.6 pmol tube to the 12.8 pmol tube and mix thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 20 µl aliquots from each serial dilution will give rise to 8 standard levels of cAMP ranging from 0.2–25.6 pmol/well.

7. 20 µl from the stock standard vial will give rise to the 51.2 pmol standard.

8. All working standards will contain different standard levels of cAMP dissolved in lysis reagent 1B.

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

9.4. Assay procedure

1. Prepare assay components and standards ranging from 0.2–51.2 pmol/well as described in the previous section.

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

3. Set up the microplate with sufficient wells for assaying of all blanks, conjugate only wells, zero standard, and standard wells. Recommended positioning of buffer blank (Bu), conjugate only (C), zero standard (0) and standard (0.2–51.2 pmol) wells are shown in Figure 4.

4. Pipette 20 µl of lysis reagent 1B and 20 µl lysis reagent 2B into the buffer only wells.
5. Pipette 20 µl of lysis reagent1B and 10 µl of lysis reagent 2B into the conjugate only wells.
6. Pipette 20 µl of lysis reagent 1B into the zero standard wells.
7. Pipette 20 µl of each standard (0.2–51.2 pmol/well), prepared in lysis reagent 1B into the appropriate wells, using a clean pipette tip for each standard.
8. Pipette 20 µl of unknown sample into the appropriate wells (see previous section on preparation of cell samples).
9. Pipette 10 µl of antiserum prepared in lysis reagent 2B into all standard, zero standard and sample wells. Do not add antiserum to the conjugate only and buffer blank wells.
10. Carefully pipette 10 µl of the diluted Cy3B-cAMP conjugate prepared in lysis reagent 2B into all wells (including the conjugate only), except for the buffer blank.
11. Cover the plate, gently mix, cover in tin foil and incubate for at least 4 hours at room temperature. Improved results are obtained with an overnight incubation step.
12. Measure the fluorescence polarization on a fluorimeter suitable for use with 384 microplates, using the appropriate polarization programme, with activation at 535 nm and emission at 590 nm.

This assay kit has been developed on the FARCyte instrument (GE Healthcare) which is optimal for use with the CyDye fluors. Instrument settings for this machine are as follows:

1. Select polarization.
2. Select the plate from the appropriate plate definition file.

3. Set up the instrument with optimal gain and 0 ms lag time. Set the integration time for 60 µs with the number of flashes set at 30. Time between move and flash -0 ms.

4. Determine the 'Z' value for the instrument using wells A1 and B1

5. Calibrate G-factor. Reference is calculated from wells A11 to wells A11 (conjugate only well), with a polarization value of **50**. Check buffer from well A12 to well A12 (buffer only wells). Check blank reduction and check same as buffer.

6. Start calibration measurement (results should be approximately 1).

7. Start polarization measurement.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
B	51.2	25.5	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
D	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
E	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
F	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
H	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
J	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
L	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
O	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Figure 4. Recommended positioning of standard (0.2–51.2 pmol) wells.

0 = zero standard, C = conjugate only, Bu = buffer blank, S = sample wells.

10. Protocol 3. 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 will normally be at least four weeks from the date of despatch.

Stability after reagent reconstitution

All kit reagents are stable for 14 days when reconstituted and stored at 2–8°C.

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 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. This is readily achieved using a beaker and magnetic stirrer.

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% (w/v) 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% (w/v) 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 reagent 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

1. Carefully add 1 ml of lysis reagent 1B (prepared as described above) and replace the stopper.

2. Mix the contents of the bottle until completely dissolved. The final solution should contain cAMP at a concentration of 2560 pmol/ml in lysis reagent 1B.

Cy3B-cAMP conjugate

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided

2. The final solution will contain the Cy3B-cAMP conjugate in lysis reagent 2B.

Antiserum

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

2. The final solution will contain the anti-cAMP serum in lysis reagent 2B.

10.2. Cell lysis methods intracellular cAMP measurement

Note 1: Two methods are described for intracellular cAMP measurement (protocols 2 and 3). For 'total' cellular cAMP measurement, see protocol 4).

Note 2: Leave empty wells on the microplate for preparation of the standard curve. Recommended positioning of standard wells is shown in Figure 5.

Adherent cells

1. Culture cells (20 µl volumes) in solid, black 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml **(384-well assay plates from Corning code 3709 are recommended for this assay. Do not use clear or clear-bottomed 384 well plates).**

2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation**. Aspirate or decant the cell culture media.

3. Add 20 µl of drug, agonist etc dissolved in Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step.** Incubate for a suitable time period.

4. Decant or aspirate the excess buffer.

5. Add 20 µl/well of diluted lysis reagent 1B.

6. Agitate the 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. Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure').

Suspension cells

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

1. Culture cells (20 µl volumes) in solid, black 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml. **(384-well assay plates from Corning code 3709 are recommended for this assay. Do not use clear or clear-bottomed 384 well plates).**

2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation.**

3. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well. **Note: the actual centrifugal speed is dependent on the cells under study and should be validated by the investigator.**

4. Aspirate or decant the cell culture media.

5. Add 20 µl of drug, agonist etc dissolved in Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step.** Incubate for a suitable time period.

6. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well.
7. Gently decant or aspirate excess buffer and resuspend the pellet in 20 µl of lysis reagent 1B.
8. Agitate the 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.
9. Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure').

10.3. Preparation of working standards

For the measurement of cAMP in the range 0.2–51.2 pmol/well

1. Label 8 polypropylene or polystyrene tubes (12 × 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol, 12.8 pmol, and 25.6 pmol.
2. Pipette 0.5 ml of lysis reagent 1B into all the tubes.
3. Into the 25.6 pmol tube, pipette 0.5 ml of stock standard (2560 pmol/ml) and mix thoroughly.
4. Transfer 0.5 ml from the 25.6 pmol tube to the 12.8 pmol tube and mix thoroughly.
5. Repeat this doubling dilution successively with the remaining tubes.
6. 20 µl aliquots from each serial dilution will give rise to 8 standard levels of cAMP ranging from 0.2–25.6 pmol/well.
7. 20 µl from the stock standard vial will give rise to the 51.2 pmol standard.
8. All working standards will contain different standard levels of cAMP dissolved in lysis reagent 1B.

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

10.4. Assay procedure

1. Prepare assay components and standards ranging from 0.2–51.2 pmol/well as described in the previous section.
2. Equilibrate reagents to room temperature and mix before use.
3. Set up the microplate with sufficient wells for assaying of all blanks, conjugate only wells, zero standard, and standard wells. Recommended positioning of buffer blank (Bu), conjugate only (C), zero standard (0) and standard (0.2–51.2 pmol) wells are shown in Figure 5.
4. Pipette 20 µl of lysis reagent 1B and 20 µl lysis reagent 2B into the buffer only wells.
5. Pipette 20 µl of lysis reagent1B and 10 µl of lysis reagent 2B into the conjugate only wells.
6. Pipette 20 µl of lysis reagent 1B into the zero standard wells.
7. Pipette 20 µl of each standard (0.2–51.2 pmol/well), prepared in lysis reagent 1B into the appropriate wells, using a clean pipette tip for each standard.
8. Wells containing 20 µl of cell lysate should already be prepared at this stage (see previous section on preparation of cell samples).
9. Pipette 10 µl of antiserum, prepared in lysis reagent 2B, into all standard, zero standard and sample wells. Do not add antiserum to the conjugate only and buffer blank wells.
10. Carefully pipette 10 µl of the diluted Cy3B-cAMP conjugate prepared in lysis reagent 2B into all wells (including the conjugate only), except for the buffer blank.

11. Cover the plate, gently mix, cover in tin foil and incubate for at least 4 hours at room temperature. Improved results are obtained with an overnight incubation step.

12. Measure the fluorescence polarization on a fluorimeter suitable for use with 384 microplates, using the appropriate polarization programme, with activation at 535 nm and emission at 590 nm.

This assay kit has been developed on the FARCyte instrument (GE Healthcare) which is optimal for use with the cyanine dyes. Instrument settings for this machine are as follows:

1. Select polarization.

2. Select the plate from the appropriate plate definition file.

3. Set up the instrument with optimal gain and 0 ms lag time. Set the integration time for 60 µs with the number of flashes set at 30. Time between move and flash -0 ms.

4. Determine the 'Z' value for the instrument using wells A1 and B1

5. Calibrate G-factor. Reference is calculated from wells A11 to wells A11 (conjugate only well), with a polarization value of **50**. Check buffer from well A12 to well A12 (buffer only wells). Check blank reduction and check same as buffer.

6. Start calibration measurement (results should be approximately 1).

7. Start polarization measurement.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
B	51.2	25.5	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
D	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
E	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
F	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
H	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
J	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
L	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
O	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Figure 5. Recommended positioning of standard (0.2–51.2 pmol) wells. 0 = zero standard, C = conjugate only, Bu = buffer blank, S = sample wells.

11. Protocol 4. One stage assay - 'total' cellular cAMP measurement

11.1. Reagent preparation

Stability before reconstitution

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

Stability after reagent reconstitution

All kit reagents are stable for 14 days when reconstituted and stored at 2–8°C.

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 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. This is readily achieved using a beaker and magnetic stirrer.

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. Stir for 30 minutes after storage. Take 20 ml of lysis buffer 1A and make up to 100 ml with assay buffer to give a final 0.5% solution of Dodecyltrimethylammonium Bromide in assay buffer. **This is lysis buffer 1A1, which is used in the cell lysis method for the total cellular cAMP assay**. Stir continuously when used. Stir continuously 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 reagent 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

1. Carefully add 1 ml of lysis reagent 1B (prepared as described above) and replace the stopper.

2. Mix the contents of the bottle until completely dissolved. The final solution should contain cAMP at a concentration of 2560 pmol/ml in lysis reagent 1B.

Cy3B-cAMP conjugate

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

2. The final solution will contain the Cy3B-cAMP conjugate in lysis reagent 2B.

Antiserum

1. Add 5 ml of lysis reagent 2B and replace the stopper. Mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

2. The final solution will contain the anti-cAMP serum in lysis reagent 2B.

11.2. Cell lysis methods intracellular cAMP measurement

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

Note 1: Two methods are described for intracellular cAMP measurement (protocols 2 and 3). For 'total' cellular cAMP measurement, see protocol 4). It is the user's responsibility to validate the extraction method for cAMP. If autofluorescence or compound interference is a problem, please use Protocol 3 which includes an aspiration step.

Note 2: Leave empty wells on the microplate for preparation of the standard curve.

Adherent cells

1. Culture cells (20 µl volumes) in solid, black 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml **(384-well assay plates from Corning code 3709 are recommended for this assay. Do not use clear or clear-bottomed 384 well plates).**

2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation.** Aspirate or decant the cell culture media.

3. Add 10 µl of drug, agonist etc dissolved in Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step**. Incubate for a suitable time period.

4. Add 10 µl/well of **lysis reagent 1A1.**

5. Agitate the cells after lysis reagent 1A1 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. Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure').

Suspension cells

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

1. Culture cells (20 µl volumes) in solid, black 384-well microplates (tissue culture grade), with cell concentrations of between 10^4 and 10^6 cells/ml. **(384-well assay plates from Corning code 3709 are recommended for this assay. Do not use clear or clear-bottomed 384 well plates).**

2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over confluent (e.g. 10^7 cells/ml) as cells may be lost during decantation.**

3. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well. **Note: the actual centrifugal speed is dependent on the cells under study and should be validated by the investigator.**

4. Aspirate or decant the cell culture media.

5. Add 10 µl of drug, agonist etc dissolved in Krebs-Henseleit buffer or PBS. **Do not use cell culture media for this step.** Incubate for a suitable time period.

6. Add 10 µl of lysis reagent 1A1.

7. Agitate the cells after lysis reagent 1A1 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.

8. Lysed cells are now ready for use in the fluorescence polarization assay and should be processed immediately in the immunoassay (see 'assay procedure').

11.3. Preparation of working standards

For the measurement of cAMP in the range 0.2–51.2 pmol/well

1. Label 8 polypropylene or polystyrene tubes (12 × 75 mm), 0.2 pmol, 0.4 pmol, 0.8 pmol, 1.6 pmol, 3.2 pmol, 6.4 pmol, 12.8 pmol, and 25.6 pmol.

2. Pipette 0.5 ml of lysis reagent 1B into all the tubes.

3. Into the 25.6 pmol tube, pipette 0.5 ml of stock standard (2560 pmol/ml) and mix thoroughly.

4. Transfer 0.5 ml from the 25.6 pmol tube to the 12.8 pmol tube and mix thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 20 µl aliquots from each serial dilution will give rise to 8 standard levels of cAMP ranging from 0.2–25.6 pmol/well.

7. 20 µl from the stock standard vial will give rise to the 51.2 pmol standard.

8. All working standards will contain different standard levels of cAMP dissolved in lysis reagent 1B.

NOTE: Care should be taken when preparing working standards.

Dodecyltrimethylammonium Bromide may case frothing. Vigorous pipetting should be avoided.

11.4. Assay procedure

1. Prepare assay components and standards ranging from 0.2–51.2 pmol/well as described in the previous section.
2. Equilibrate reagents to room temperature and mix before use.
3. Set up the microplate with sufficient wells for assaying of all blanks, conjugate only wells, zero standard, and standard wells. Recommended positioning of buffer blank (Bu), conjugate only (C), zero standard (0) and standard (0.2–51.2 pmol) wells are shown in Figure 6.
4. Pipette 20 µl of lysis reagent 1B and 20 µl lysis reagent 2B into the buffer only wells.
5. Pipette 20 µl of lysis reagent 1B and 10 µl of lysis reagent 2B into the conjugate only wells.
6. Pipette 20 µl of lysis reagent 1B into the zero standard wells.
7. Pipette 20 µl of each standard (0.2–51.2 pmol/well), prepared in lysis reagent 1B into the appropriate wells, using a clean pipette tip for each standard.
8. Wells containing 20 µl of cell lysate should already be prepared at this stage (see previous section on preparation of cell samples).
9. Pipette 10 µl of antiserum prepared in lysis reagent 2B into all standard, zero standard and sample wells. Do not add antiserum to the conjugate only and buffer blank wells.
10. Carefully pipette 10 µl of the diluted Cy3B-cAMP conjugate prepared in lysis reagent 2B into all wells (including the conjugate only), except for the buffer blank.
11. Cover the plate, gently mix, cover in tin foil and incubate for at least 4 hours at room temperature. Improved results are obtained

with an overnight incubation step.

12. Measure the fluorescence polarization on a fluorimeter suitable for use with 384 microplates, using the appropriate polarization programme, with activation at 535 nm and emission at 590 nm.

This assay kit has been developed on the FARCyte instrument GE Healthcare) which is optimal for use with the cyanine dyes. Instrument settings for this machine are as follows:

1. Select polarization.

2. Select the plate from the appropriate plate definition file.

3. Set up the instrument with optimal gain and 0 µs lag time. Set the integration time for 60 µs with the number of flashes set at 30. Time between move and flash -0 ms.

4. Determine the 'Z' value for the instrument using wells A1 and B1

5. Calibrate G-factor. Reference is calculated from wells A11 to wells A11 (conjugate only well), with a polarization value of **50**. Check buffer from well A12 to well A12 (buffer only wells). Check blank reduction and check same as buffer.

6. Start calibration measurement (results should be approximately 1).

7. Start polarization measurement.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
B	51.2	25.5	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0	C	Bu	S	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
D	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
E	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
F	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
H	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
J	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
L	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
O	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Figure 6. Recommended positioning of standard (0.2–51.2 pmol) wells. 0 = zero standard, C = conjugate only, Bu = buffer blank, S = sample wells.

12. Data processing

12.1. Calculation of results

The calculation is illustrated using representative data.

The assay data collected should be similar to that shown in Table 1.

1. Calculate the average millipolarization values (mP) for each set of replicate wells.

2. A standard curve may be generated by plotting mPs as a function of the log cAMP concentration. Plot mPs (y axis) against pmol cAMP standard per well (x axis). The curve shape should be similar to Figure 7, if plotted on semi-log paper. The pmol/well value of samples can be read directly from the graph.

12.2. Typical assay data

Table 1. Typical assay data.

Standard (pmol/well)	Millipolarization (mP) units, activating at 535 nm, emission at 590 nm		Average mP units
0	328	329	329
0.2	290	292	291
0.4	259	262	261
0.8	235	236	236
1.6	195	196	196
3.2	156	157	157
6.4	121	122	122
12.8	99	100	100
25.6	78	82	80
51.2	70	74	72

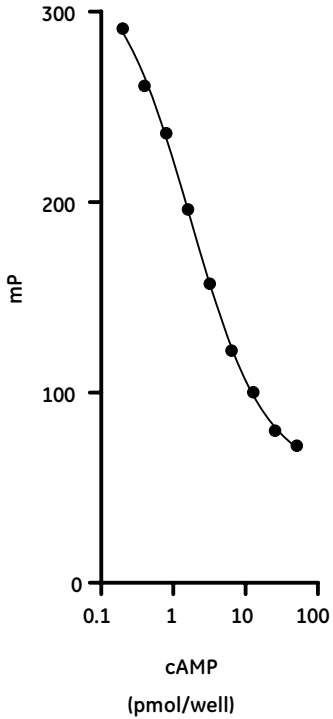


Figure 7. Typical standard curve

13. Additional information

13.1. Specificity

The cross-reactivity as determined with a number of related compounds is shown in Table 2.

Table 2.

Compound	% Cross-reactivity
cAMP	100
cIMP	<0.03
cGMP	<0.0018
cCMP	<0.001
cTMP	0.02
AMP	<0.05
ADP	0.002
ATP	<0.0002
EDTA	<0.0004
Theophylline	0.002
Iso-butyl-methyl-xanthine	<0.0007

13.2. 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 (1.65 ng/ml).

13.3. Precision

The within-assay precision for duplicate determinations was calculated by measuring crystalline controls in the assay. The results are shown in Table 3.

Table 3.

Sample	pmol cAMP/well	%CV	n
A	0.721±0.056	7.80	25
B	3.080±0.302	9.80	25
C	9.380±1.117	11.91	25

The between assay precision was assayed by repeated measurement of the same samples in successive assays. The results are shown Table 4.

Table 4

Sample	pmol cAMP/well	%CV	n
A	0.630±0.036	5.78	24
B	2.850±0.267	9.37	24
C	11.401±1.598	14.01	24

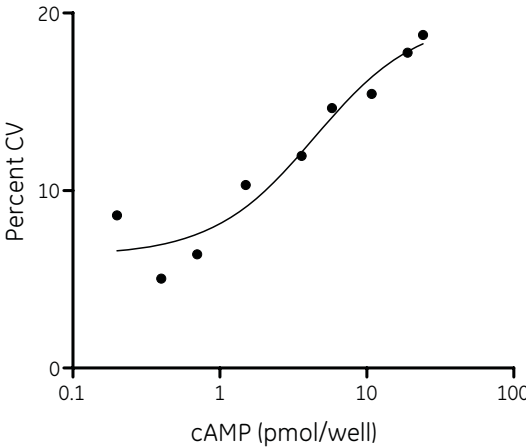
13.4. Precision profile

Preparing replicates of each of the standards and calculating the standard deviation and percent coefficient of variation at each concentration (Table 5) generated a precision profile.

Table 5.

Standard (pmol/well)	Standard deviation	%CV
51.2	4.54	18.76
25.6	3.82	17.76
12.8	1.66	15.44
6.4	1.18	14.63
3.2	0.61	11.94
1.6	0.15	10.30
0.8	0.05	6.40
0.4	0.03	5.04
0.2	0.02	8.60

Figure 8. Precision profile



13.5. Assay performance

Assay performance was monitored by the use of Z' as described by Zhang *et al* (7). Chinese hamster ovary cells stimulated with forskolin (n=90) gave a Z' value of 0.72.

13.6. Instrumentation

The cAMP FP Immunoassay System was developed for use with any microplate fluorescence reader, provided that the instrument can be used with 384-well plates, and is set up for fluorescence polarization. The recommended choice of filters is: excitation 525 nm, emission 580 nm. However, 535 nm/590 nm filter sets are equally suitable. Please consult with your instrument manufacturer for appropriate filters and specifications. However, it is the users' responsibility to validate the cAMP FP assay on their own instrumentation.

14. 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 (8).

cAMP was identified as playing a major role in the mode of action of adrenaline more than 40 years ago (9–12). 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 (ATCH), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), calcitonin, glucagon, vasopressin and parathyroid hormone (PTH).

cAMP has been shown to be involved in the cardiovascular (13) and nervous systems (14), in immune mechanisms (15), cell growth and differentiation (16), and general metabolism (17). 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 unlabeled ATP (18–21). The method allows high sensitivity without the interference from ATP to which other adenylate cyclase assays are prone (21).

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15. Related products

Amprep SAX Mini Column	RPN1918
FARCyte Fluorescence Plate Reader	VF129017
Cy3B Mono NHS Ester (1 mg)	PA63101
Cy3B Mono NHS Ester (5 mg)	PA63100
Cy3B Mono NHS Ester (25 mg)	PA63106
Cy3B Mono Maleimide (1 mg)	PA63131
Cy3B Mono Maleimide (5 mg)	PA63130
Cy3B Mono Maleimide (25 mg)	PA63136
Cy5 Mono NHS Ester (1 mg)	PA15101
Cy5 Mono NHS Ester (5 mg)	PA15100
Cy5 Mono NHS Ester (10 mg)	PA15104
Cy5 Mono NHS Ester (25 mg)	PA15106
Cy5 Mono Maleimide (1 mg)	PA15131
Cy5 Mono Maleimide (5 mg)	PA15130
Cy5 Mono Maleimide (25 mg)	PA15136
Cy5 Mono Hydrazide (1 mg)	PA15121
Cy5 Mono Hydrazide (5 mg)	PA15120
Cy5 Mono Hydrazide (25 mg)	PA15126
cAMP Fluorescence Polarization Immunoassay System (1 × 96 wells)	RPN3595

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