GE Healthcare

Amersham cGMP Enzymeimmunoassay Biotrak (EIA) System

96 wells

Now includes: Novel lysis reagents and protocols for use with cell cultures, eliminating the requirement for time consuming extraction methods

Product Booklet

Code: RPN226

Page finder

1. Legal

Biotrak cGMP Assays

The use of this product in a binding assay is the subject of patent numbers US 6900019 and EP 0863402 and equivalent patents and patent applications in other countries in the name of GE Healthcare UK Limited.

Previously published 1999.

http://www.gelifesciences.com

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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 is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements for specific advice.

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

Warning: Sulphuric acid is corrosive.

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

2.2. Storage

Store at 2–8°C.

2.3. Expiry

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

3. Components of the assay system

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

Assay buffer
Bottle contains 10 ml sodium acetate buffer which on dilution gives a 0.05 M sodium acetate buffer pH 6.0 containing 0.002% (w/v) bovine serum albumin and 0.01% (w/v) preservative.

Standard (for non-acetylation assay)
Bottle contains cGMP at a concentration of 1.28 nmol, lyophilized. On reconstitution this bottle contains cGMP at 128 pmol/ml.

Standard (for acetylation assay)
Bottle contains cGMP at a concentration of 25.6 pmol, lyophilised. On reconstitution this bottle contains cGMP at 10.24 pmol/ml.

Antibody
Bottle contains lyophilised cGMP antibody.

cGMP conjugate
Bottle contains lyophilised cGMP conjugated to horseradish peroxidase.

Wash buffer
Bottle contains 12.5 ml of phosphate buffer concentrate which on dilution gives 0.01 M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween™ 20.

TMB substrate
Bottle contains 3,3',5'5'-tetramethylbenzidine (TMB)/ hydrogen peroxide. Ready for use.

Acetic anhydride
3 ml, ready for use.

Triethylamine
5 ml, ready for use.

Lysis reagent 1
Dodecyltrimethylammonium bromide, 2 g, solid.

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

4. Critical parameters

Important

The following points are critical:
When carrying out cGMP enzyme immunoassays, please take particular note of the following instructions regarding critical steps:

- It is essential to read the complete instruction booklet before starting work.
- This is a delayed addition assay. Do not empty and wash the plate before adding the peroxidase conjugate.
- **Thoroughly** wash the plate before adding the **substrate**. You can do this either manually or automatically provided that the following points are noted:

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

Automatic plate washing
- Ensure that all wells are filled and emptied **completely** with each cycle.
- Uneven washing will cause poor results. If you doubt the effectiveness of your instrument, wash manually as described above.
- There is no difference in results when using either automated or hand washing procedures, if the instrument is carefully maintained.

- To aid efficient washing in automated plate washers, the strips should be levelled with the edge of the microplate lid before each washing stage.
- Allow samples and all reagents to reach room temperature prior to performing the assay.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents.
- Avoid handling the tops of the wells both before and after filling.
- Keep the wells covered with lids except when adding reagents and reading.
- Standards and samples should be assayed in duplicate.
- Run a separate standard curve for each microplate.
- Carry out a microscope evaluation before and after lysing cells.

5. Additional equipment and reagents required

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

6. Description

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

Each pack contains sufficient material for 96 wells. This allows the construction of one standard curve and the measurement of 36 unknowns in duplicate.

- Elimination of inconvenient, time-consuming extraction procedures
- Flexible method/choice of assay protocols (see page 13)
- Rapid assay protocol
- Dual range 50–12 800 fmol/well (non-acetylation protocol)
- Dual range 2–512 fmol/well (acetylation protocol)
- Non-radioactive
- Specific for cGMP
- Precise and accurate measurement
- Ready to use substrate
- Colour coded reagents

Lysis reagent 1 hydrolyses cell membranes to release intracellular cGMP. Lysis reagent 2 sequesters the key component in lysis reagent 1 and ensures cGMP is free for subsequent analysis. The detergent/sequestrant complex does not interfere with antigen:antibody

binding. Lysis reagent 1 is simply added to cultured cells, followed by a 5 minute incubation before assay (fig 1). The antiserum is reconstituted with lysis reagent 2. The assay is based on competition between unlabelled cGMP and a fixed quantity of peroxidase-labelled cGMP, for a limited number of binding sites on a cGMP specific antibody (fig 2).

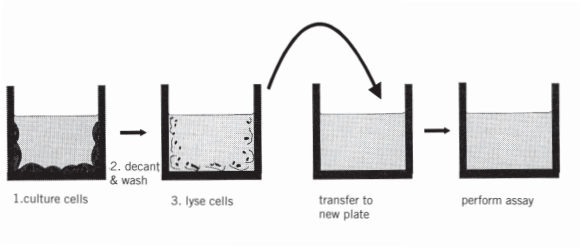


Fig 1. Cell lysis- protocol 3 intracellular method

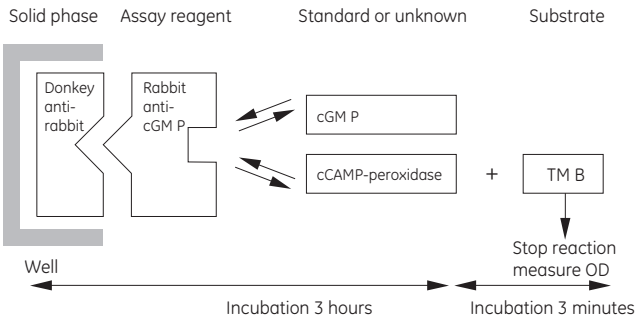


Fig 2. EIA principle

7. Protocol selection

The assay procedure can be carried out in one of four ways:

PROTOCOL 1. The normal non-acetylation assay (range 50–12 800 fmol/well) is used for the measurement of cGMP in urine and tissue extracts, prepared with traditional sample extraction methods such as acid, solvent and solid-phase methods.

PROTOCOL 2. The acetylation assay (range 2–512 fmol/well) is used for the measurement cGMP in plasma, cell culture or in tissue extracts where higher sensitivity is required. This protocol is the standard assay procedure.

PROTOCOL 3. This method describes a new direct, intracellular method for the measurement of cGMP in tissue culture lysates 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. cGMP is measured in the range 2–512 fmol/well.

PROTOCOL 4. This new method also uses the lysis reagents. Here the combined amount of intracellular and cell supernatant cGMP is measured. This fraction is referred to as 'total' cellular cGMP and has the additional benefit of not requiring decantation of the cell culture supernatant. cGMP is measured in the range 2–512 fmol/well.

Table 1. Summary of protocols

Curve range	Urine samples. No sample extraction needed	Tissue samples. Sample extraction required	Tissue samples where higher sensitivity is needed. Sample extraction required	Cell cultures. Lysis reagents and protocols provided	Plasma samples. No sample extraction required
50–12 800 fmol/well	Protocol 1 (see page 14)	Protocol 1 (see page 14)			
2–512 fmol/well			Protocol 2 (see page 23)	Protocol 3 Intracellular cGMP (see page 31) Protocol 4 'Total' cellular cGMP (see page 40)	Protocol 2 (see page 23)

8. Protocol 1. NON-ACETYLATION EIA PROCEDURE (for urine and tissue samples. Curve range 50–12 800 fmol/well)

8.1. Specimen collection and sample purification

Several methods are available for purifying cyclic nucleotides. These include both acidic extraction and extraction with aqueous ethanol (1–4).

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

Urine

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

Tissue sample collection

Tissue sections must be rapidly frozen immediately after collection so as to prevent alteration to the cGMP and associated enzymes

before analysis. This is usually achieved by immersion of the fresh tissue in liquid nitrogen at -196°C. Samples should be stored at -15°C to -30°C until the assay is carried out.

Tissue sample purification

1. Homogenise frozen tissue in cold 6% (w/v) trichloroacetic acid at 2–8°C to give a 10% (w/v) homogenate.
2. Centrifuge at 2000 ×g for 15 minutes at 4°C.
3. Recover the supernatent and discard the pellet.
4. Wash the supernatent 4 times with 5 volumes of water saturated diethyl ether. The upper ether layer should be discarded after each wash.
5. The aqueous extract remaining should be lyophilised or dried under a stream of nitrogen at 60°C.
6. Dissolve the dried extract in a suitable volume of assay buffer prior to analysis.

8.2. EIA procedure

8.2.1. Reagent preparation

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

Assay buffer

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

Standard

1. Carefully add 10 ml of diluted assay buffer to the non-acetylation standard bottle.
2. Gently mix until the contents are completely dissolved.

Antibody

1. Add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.
3. Take an aliquot of the diluted antibody and dilute with an equal volume of diluted assay buffer and gently mix.

cGMP conjugate

1. Add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should avoided.
3. Take an aliquot of the diluted conjugate and dilute with an equal volume of diluted assay buffer and gently mix.

Wash buffer

1. Transfer the contents of the bottle to a 500 ml cylinder by repeated washing with distilled water.
2. Adjust the final volume to 500 ml with distilled water and mix thoroughly. Reconstituted reagents should be stored at 2–8°C and re-used within 2 weeks. The reconstituted antiserum may precipitate on storage at 2–8°C. Equilibrate to room temperature and ensure that the precipitation is not allowed to settle when dispensing. This will not affect assay performance.

8.2.2. Preparation of working standards

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

1. Label 8 polypropylene tubes 50, 100, 200, 400, 800, 1600, 3200 and 6400 fmol.
2. Pipette 500 µl diluted assay buffer into these tubes.
3. Transfer 500 µl of the 128 pmol standard into the 6400 fmol tube and mix thoroughly.
4. Repeat the doubling dilution successively with the remaining tubes.
5. 100 µl aliquots from each of the serial dilutions will give rise to 8 standard levels ranging from 50 to 6400 fmol/well.

Note: 100 µl of the reconstituted stock standard provided, serves as the top standard (12 800 fmol/well).

8.2.3. Assay method

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

Day 1

1. Prepare the reagents and working standards as described in the 'reagent preparation' section.
2. Set up the microplate with sufficient wells for running of all blanks, standards and samples in duplicate (see fig 3).
3. Pipette 200 µl of diluted assay buffer into the non-specific binding (NSB) wells.
4. Pipette 100 µl of diluted assay buffer into the zero standard (0) wells.
5. Starting with the most dilute, pipette 100 µl of each standard into the appropriate wells.

6. Pipette 100 µl of each unknown sample into the appropriate wells.
7. Pipette 100 µl of diluted antibody into all wells except the blank (B) and NSB. **REMEMBER THE EXTRA DILUTION OF THIS COMPONENT** (see page 16).
8. Cover the plate with the lid provided and gently mix.
9. Incubate the plate at 3–5°C for between 15–18 hours.

Day 2

10. Prepare cGMP peroxidase conjugate as described in the previous section. **REMEMBER THE EXTRA DILUTION OF THIS COMPONENT** (see page 16).
11. Pipette 50 µl of diluted conjugate into all wells except the blank.
12. Cover the plate with the lid provided.
13. Incubate the plate at 3–5°C for 3 hours.
14. Aspirate and wash all wells 4 times with wash buffer ensuring that all wells are completely filled and emptied at each wash.
15. Blot the plate on tissue ensuring any residual buffer is removed. **Thorough washing is essential for good performance.**
16. Immediately pipette 200 µl of room temperature equilibrated enzyme substrate into all wells.
17. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 minutes at room temperature (15–30°C)
18. The blue colour that develops may be read at 630 nm. However it is recommended that the reaction is halted prior to end point determination by the addition of 100 µl of 1 M sulphuric acid to all wells. The optical density can be read at 450 nm within 30 minutes.

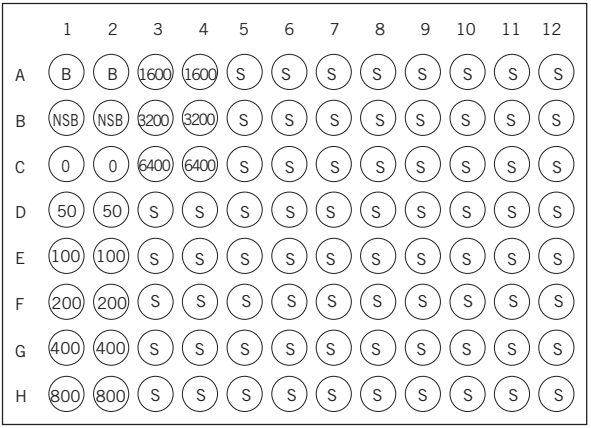


Fig 3. Recommended positioning of standard (50–12 800 fmol/well) and sample (S) well).

8.3. Data processing

8.3.1. Calculation of results

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

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

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

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

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

8.3.2. Typical assay data

Table 2. Typical assay data (protocol 1)

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD at 450 nm	Mean OD-NSB	%B/B$_0$
Blank	0.039	0.040		
	0.040			
NSB	0.066	0.065		
	0.063			
0	2.129	2.134	2.069	100
	2.139			
50	1.920	1.914	1.849	89
	1.907			
100	1.754	1.754	1.689	82
	1.753			
200	1.457	1.452	1.387	67
	1.447			
400	1.287	1.268	1.203	58
	1.249			
800	0.945	0.942	0.877	42
	0.939			
1600	0.787	0.755	0.690	33
	0.723			
3200	0.587	0.578	0.513	25
	0.568			
6400	0.446	0.435	0.370	18
	0.424			
12 800	0.375	0.365	0.300	14
	0.355			

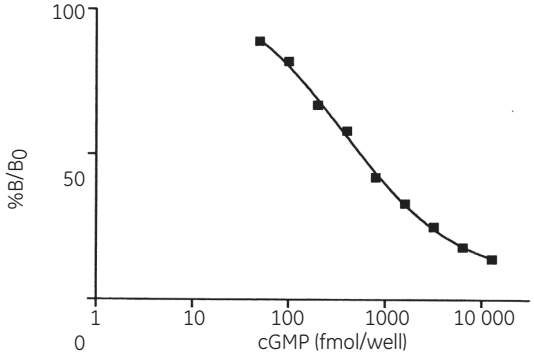


Fig 4. Typical standard curve for protocol 1 (non-acetylation procedure)

9. Protocol 2. ACETYLATION EIA PROCEDURE (for plasma, tissues and cell culture supernatants. Curve range 2–512 fmol/well)

9.1. Specimen collection, sample preparation and purification

- Representative procedures are described on pages 14–15 for the extraction of cGMP from tissues. Procedures for the extraction of cGMP from cell culture samples are described below. This information is provided for guidance only.

Plasma

Measurements should be made in plasma not serum. Blood should be collected into tubes containing 7.5 mM EDTA. Blood should be immediately centrifuged to remove cells and the plasma stored at -15°C to -30°C prior to analysis. If blood samples cannot be rapidly processed they should be stored in ice until it is possible to centrifuge.

It is not necessary to extract or deproteinise plasma samples before analysis. Plasma should be diluted 1:10 with assay buffer.

Cell culture

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

1. Add ice-cold ethanol to cell suspension to give a final suspension volume of 65% (v/v) ethanol. Allow to settle for 2–3 minutes and then pull off the supernatent into a test tube.

2. Wash the remaining precipitate with 2–3 mls of ice cold 65% (v/v) ethanol, allow to settle, and add the washings to the test tube in step 1.
3. Centrifuge the remining extracts at 2000 × g for 15 minutes at 4°C and transfer the remaining supernatant into the test tube from step 1.
4. Dry the combined extracts (washings and supernatants) under a stream of nitrogen at 60°C or in a vacuum oven.
5. Dissolve the dried extracts in a suitable volume of assay buffer prior to analysis.

9.2. EIA procedure

9.2.1. Reagent preparation

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

Assay buffer

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

Standard

1. Carefully add 2.5 ml of diluted assay buffer to the acetylation standard bottle.
2. Gently mix until the contents are completely dissolved.

Antibody

1. Add 11 ml diluted assay buffer and replace the stopper.

2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

cGMP conjugate

1. Add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should avoided.

Wash buffer

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

Reconstituted reagents should be stored at 2–8°C and re-used within 2 weeks. The reconstituted antiserum may precipitate on storage at 2–8°C. Equilibrate to room temperature and ensure that the precipitation is not allowed to settle when dispensing. This will not affect assay performance.

9.2.2. Preparation of working standards

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

1. Label 9 polypropylene tubes 2, 4, 8, 16, 32, 64, 128, 256 and 512 fmol.
2. Pipette 1 ml diluted assay buffer into all tubes except the 512 fmol.
3. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 512 fmol tube.
4. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 256 fmol tube and mix thoroughly.
5. Transfer 1 ml from the 256 fmol tube to the 128 fmol tube and mix thoroughly.

6. Repeat this doubling dilution successively with the remaining tubes.
7. 50 µl aliquots from each of the serial dilutions will give rise to 9 standard levels ranging from 2 to 512 fmol/well.

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

9.2.3. Assay method

Note: Steps 7–12 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–6. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5ºC during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare the assay buffer and working standards as described in the 'reagent preparation' section.
2. Label polypropylene or glass tubes (12 x 75 mm) for zero standard and unknowns. These will subsequently be known as acetylation tubes.
3. Set up the microplate with sufficient wells for running of all blanks, standards and samples in duplicate (see fig 5).
4. Prepare the acetylation reagent by adding 1 volume acetic anhydride to 2 volumes of triethylamine in a glass vessel and mixing well (sufficient reagent for 60 acetylations may be obtained by mixing 2 ml acetic anhydride with 4ml triethylamine).
5. Pipette 1 ml of diluted assay buffer into the zero standard acetylation tube.

6. Pipette 1 ml of each unknown (see sample preparation section) into the appropriately labelled acetylation tubes. Tubes containing 1 ml of each working standard should have already been prepared (see reagent preparation section).
7. Carefully add 100 µl of the acetylation reagent to all acetylation tubes containing standards and unknowns. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylating reagents.
8. Pipette 100 µl of antiserum into all wells except the blank and non-specific binding (NSB) wells.
9. Starting with the most dilute, pipette duplicate 50 µl aliquots from all acetylation tubes including the zero standard into the appropriate wells.
10. Pipette 150 µl of assay buffer into the non-specific binding wells.
11. Cover the plate with the lid provided and gently mix.
12. Incubate the plate at 3–5°C for exactly 2 hours.
13. Pipette 100 µl of diluted conjugate into all wells except the blank.
14. Cover the plate with the lid provided and gently mix.
15. Incubate the plate at 3–5°C for 60 minutes.
16. Aspirate and wash all wells 4 times with wash buffer ensuring that all wells are completely filled and emptied at each wash.
17. Blot the plate on tissue ensuring any residual buffer is removed. **Thorough washing is essential for good performance.**
18. Immediately pipette 200 µl of room temperature equilibrated enzyme substrate into all wells.

19. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 minutes at room temperature (15–30°C).

20. The blue colour that develops may be read at 630 nm. However it is recommended that the reaction is halted prior to end point determination by the addition of 100 µl of 1M sulphuric acid to all wells. The optical density can be read at 450 nm within 30 minutes.

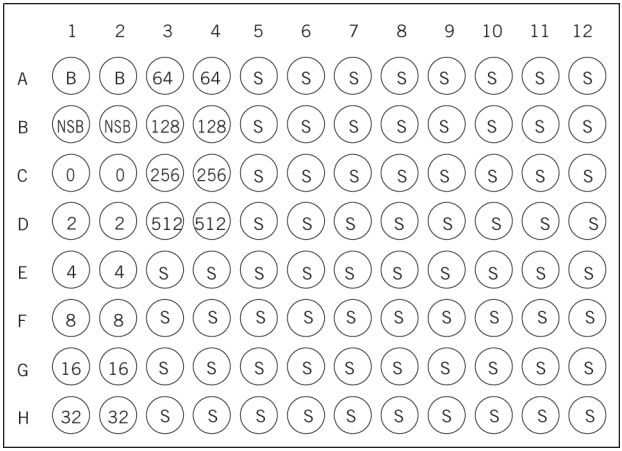


Fig 5. Recommended positioning of standard (2–512 fmol/well) and sample (S) wells

9.3. Data processing

9.3.1. Calculation of results

The method for calculating results is shown on page 20. The assay data should be similar to that shown in table 3. Fig 6 shows a standard curve generated from the data in table 3.

9.3.2. Typical assay data

Table 3. Typical assay data, protocol 2 (acetylation procedure)

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD at 450 nm	Mean OD-NSB	%B/B_0
Blank	0.040	0.041		
	0.041			
NSB	0.069	0.070		
	0.071			
0	1.382	1.369	1.299	100
	1.356			
2	1.319	1.325	1.255	97
	1.331			
4	1.251	1.270	1.200	92
	1.289			
8	1.146	1.156	1.089	84
	1.165			
16	0.976	0.971	0.901	69
	0.965			
32	0.746	0.745	0.675	52
	0.743			
64	0.578	0.582	0.512	39
	0.586			
128	0.429	0.423	0.353	27
	0.416			
256	0.302	0.305	0.235	18
	0.308			
512	0.228	0.218	0.148	11
	0.207			

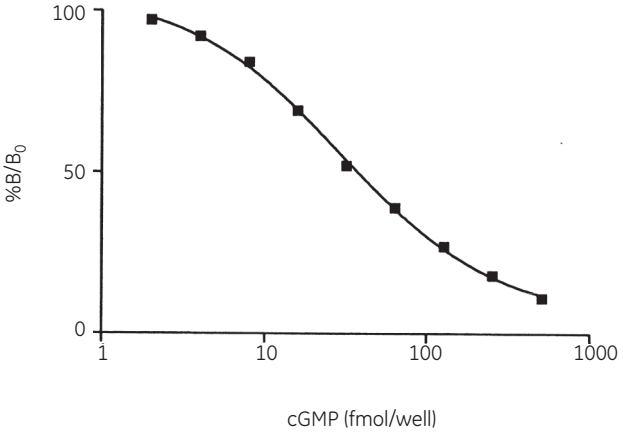


Fig 6. Typical standard curve for protocol 2 (acetylation procedure)

10. New protocol 3 INTRACELLULAR cGMP MEASUREMENT USING NOVEL LYSIS REAGENTS (for cell culture samples. Curve range 2–512 fmol/well)

10.1. EIA procedure

10.1.1. Reagent preparation

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

Assay buffer

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

NOTE: lysis reagents 1 and 2 (solids) require 30 minutes mixing at room temperature to dissolve in assay buffer. This is readily achieved using a beaker and magnetic stirrer. Lysis reagent buffer A and buffer B may appear slightly opaque after mixing. When lysis reagent 1(working solution) and lysis reagent 2 (working solution) are prepared these should be clear. This will not affect assay performance.

Lysis reagent 1

1. Transfer the contents of the bottle (lysis reagent 1, solid) into a beaker using repeated washing with 30 ml of diluted assay buffer. Use continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 40 ml with assay buffer and mix thoroughly. The final solution contains 5% dodecytrimethylammonium bromide. This is buffer A.
3. Take 10 ml of buffer A and make up to 100 ml with diluted assay buffer to give a final 0.5% solution of dodecyltrimethylammonium bromide. Mix thoroughly. This is lysis reagent 1 (working solution).

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) into a beaker using repeated washing with 80 ml of diluted assay buffer. Use 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. The final solution contains 5% lysis reagent 2. This is buffer B.
3. Take 40 ml of buffer B and carefully make up to 100 ml in a 100 ml measuring cylinder with diluted assay buffer to give a final 2.0% solution of lysis reagent 2. Mix thoroughly. This is lysis reagent 2 (working solution).

Standard

1. Carefully add 2.5 ml of diluted lysis reagent 1 (working solution) to the acetylation standard bottle.
2. Gently mix until the contents are completely dissolved.

Antibody

1. Add 11 ml diluted lysis reagent 2 (working solution) and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

cGMP conjugate

1. Add 11 ml diluted assay buffer and replace the stopper.

2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

Wash buffer

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

Reconstituted reagents should be stored at 2–8°C and re-used within two weeks. The reconstituted antiserum may precipitate on storage at 2–8°C. Equilibrate to room temperature and ensure that the precipitation is not allowed to settle when dispensing. This will not affect assay performance.

10.1.2. Preparation of working standards

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

1. Label 9 polypropylene tubes 2, 4, 8, 16, 32, 64, 128, 256 and 12 fmol.
2. Pipette 1ml diluted lysis reagent 1 into all tubes except the 512 fmol.
3. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 512 fmol tube.
4. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 256 fmol tube and mix thoroughly.
5. Transfer 1 ml from the 256 fmol tube to the 128 fmol tube and mix thoroughly.
6. Repeat this doubling dilution successively with the remaining tubes.
7. 50 µl aliquots from each of the serial dilutions will give rise to 9 standard levels ranging from 2 to 512 fmol/well.

Note: these tubes will be referred to as acetylation tubes containing working standards in the assay method section. Working standards should be freshly prepared before each assay and not re-used. Care should be taken when preparing working standards as dodecyltrimethylammonium bromide may cause frothing. Vigorous pipetting should be avoided.

10.1.3. Cell lysis methods

Adherent cells

1. Culture cells (100 µl) in standard 96-well microplates (tissue-culture grade) with cell concentrations of between 10^4-10^6 cells/ml.
2. Incubate plate overnight at 37°C (5% CO_2 and 95% humidity). **Note: do not use cell cultures that are over-confluent (eg at 10^7 cells/ml) as cells may be lost during decantation.**
3. Add 100 µl of drug, agonist etc. under study. Incubate for suitable time period.
4. Decant or aspirate excess culture media.
5. Add 200 µl/well of diluted lysis reagent 1 (working solution).
6. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be achieved by shaking the plate on a microplate shaker for 10 minutes after adding the lysis reagent.
7. Carry out a microscopic evaluation using Trypan blue to check cells have lysed. Cell membranes may still be visible after cell lysis. Lysed cells are now ready for use in the enzymeimmunoassay protocol and should be processed immediately in the immunoassay (see 'assay method').

Suspension cells

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

1. Culture cells (100 µl) in standard 96-well microplates (tissue-culture grade) with cell concentrations of between 10^4–10^6 cells/ml.

2. Incubate plate overnight at 37°C (5% CO_2 and 95% humidity).
3. Add 100 µl of drug, agonist etc. under study. Incubate for a suitable time period.
4. Using a centrifugal microplate adapter, centrifuge the microplate at 1000–1500 × g for 3 minutes to form a pellet in each well.

Note: the actual centrifugal speed required is dependent on the cells under study and should be validated by the investigator.

5. Gently decant or aspirate excess media and resuspend pellet in 200 µl of lysis reagent 1 (working solution).
6. Agitate cells after lysis reagent 1 is added. This is to facilitate cell lysis and can be achieved by shaking the plate on a microplate shaker for 10 minutes after adding the lysis reagent.
7. Carry out a microscopic evaluation using Trypan blue to check cells have lysed. Cell membranes may still be visible after cell lysis. Lysed cells are now ready for use in the enzymeimmunoassay protocol and should be processed immediately in the immunoassay (see 'assay method').

10.1.4. Assay method

Note: Steps 6–13 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–6. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare the reagents and working standards as described in the 'reagent preparation' section.
2. Label polypropylene or glass tubes (12 × 75 mm) for zero standard and unknowns. These will subsequently be known as acetylation tubes.

3. Set up the microplate with sufficient wells for running of all blanks, standards and samples in duplicate (see fig 5).
4. Prepare the acetylation reagent by adding 1 volume acetic anhydride to 2 volumes of triethylamine in a glass vessel and mixing well (sufficient reagent for 60 acetylations may be obtained by mixing 2 ml acetic anhydride with 4 ml triethylamine).
5. Pipette 1 ml of diluted lysis reagent 1 into the zero standard acetylation tube.
6. Carefully add 100 µl of the acetylation reagent to all acetylation tubes containing standards. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylation reagent.
7. Pipette 20 µl of the acetylation reagent into all the sample wells of the cell culture plate and mix on a microplate shaker for 5 minutes. If necessary, depending on the quality of the tissue culture plate used, transfer the 200 µl aliquot of cell culture lysate, prior to the addition of the acetylation reagent, to a polypropylene or glass tube. In this case add 20 µl of the acetylation reagent to the lysate in the polypropylene or glass tube and mix for 5 minutes. Transfer a 50 µl aliquot to the immunoassay microplate as described in step 11.
8. Pipette 100 µl of antiserum into all wells of the immunoassay microplate except the blank and non-specific binding (NSB) wells.
9. Pipette 100 µl of lysis reagent 2 and 50 µl of lysis reagent 1 (working solutions) into the NSB wells.
10. Starting with the most dilute, pipette 50 µl aliquots from all acetylation tubes including the zero standard into the appropriate wells.

11. Transfer 50 µl of each unknown acetylated sample from the cell culture plate into the appropriate wells of the immunoassay microplate.

12. Cover the plate with the lid provided and gently mix.

13. Incubate the plate at 3–5°C for exactly 2 hours.

14. Pipette 100 µl of diluted conjugate into all wells except the blank.

15. Cover the plate with the lid provided.

16. Incubate the plate at 3–5°C for 60 minutes.

17. Aspirate and wash all wells 4 times with wash buffer ensuring that all wells are completely filled and emptied at each wash.

18. Blot the plate on tissue ensuring any residual buffer is removed. **Thorough washing is essential for good performance.**

19. Immediately pipette 200 µl of room temperature equilibrated enzyme substrate into all wells.

20. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 minutes at room temperature (15–30°C).

21. The blue colour that develops may be read at 630 nm. However it is recommended that the reaction is halted prior to end point determination by the addition of 100 µl of 1 M sulphuric acid to all wells. The optical density can be read at 450 nm within 30 minutes.

10.2. Data processing

10.2.1. Calculation of results

The method for calculating results is shown on page 20. Typical results are shown in table 4. Fig 7 shows a standard curve generated from the data in table 4.

10.2.2. Typical assay data

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

Standard fmol/well	Optical density (OD) at 450 nm	Mean OD at 450 nm	Mean OD-NSB	%B/B_0
Blank	0.039	0.039		
	0.038			
NSB	0.067	0.073		
	0.079			
0	1.431	1.481	1.408	100
	1.531			
2	1.463	1.428	1.355	96
	1.392			
4	1.341	1.342	1.269	90
	1.343			
8	1.252	1.267	1.194	85
	1.282			
16	1.038	1.044	0.971	69
	1.050			
32	0.830	0.817	0.744	53
	0.804			
64	0.599	0.547	0.474	34
	0.535			
128	0.398	0.397	0.324	23
	0.395			
256	0.279	0.288	0.215	15
	0.297			
512	0.205	0.204	0.131	9
	0.203			

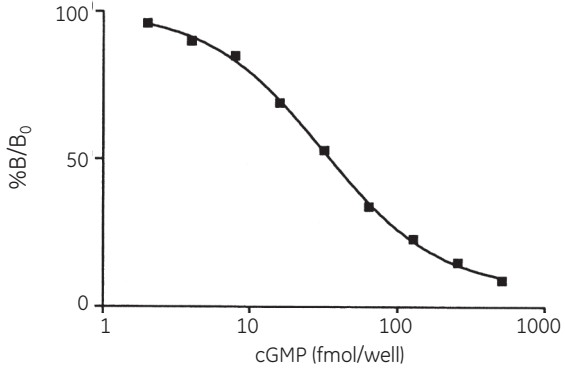


Fig 7. Typical standard curve for intra- and total cellular assays (protocols 3 and 4).

11. New protocol 4. TOTAL CELLULAR cGMP MEASUREMENT USING NOVEL LYSIS REAGENTS (for cell culture samples. Curve range 2–512 fmol/well)

11.1. EIA procedure

11.1.1. Reagent preparation

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

Assay buffer

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

NOTE: lysis reagents 1 and 2 (solids) require 30 minutes mixing at room temperature to dissolve in assay buffer. This is readily achieved using a beaker and magnetic stirrer. Lysis reagent buffer A and buffer B may appear slightly opaque after mixing. When lysis reagent 1 (working solution) and lysis reagent 2 (working solution) are prepared these should be clear. This will not affect assay performance.

Lysis reagent 1

1. Transfer the contents of the bottle (lysis reagent 1, solid) into a beaker using repeated washing with 30 ml of diluted assay buffer. Use continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.

2. Adjust the final volume to 40 ml with assay buffer and mix thoroughly. The final solution contains 5% dodecytrimethylammonium bromide. This is buffer A which is used in the cell lysis method for the 'total' cellular cGMP assay.
3. Take 10 ml of buffer A and make up to 100 ml with diluted assay buffer to give a final 0.5% solution of dodecyltrimethylammonium bromide. Mix thoroughly. This is lysis reagent 1 (working solution), and is used for the preparation of standards.

Lysis reagent 2

1. Transfer the contents of the bottle (lysis reagent 2, solid) into a beaker using repeated washing with 80 ml of diluted assay buffer. Use 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. The final solution contains 5% lysis reagent 2. This is buffer B.
3. Take 40 ml of buffer B and carefully make up to 100 ml in a 100 ml measuring cylinder with diluted assay buffer to give a final 2.0% solution of lysis reagent 2. Mix thoroughly. This is lysis reagent 2 (working solution).

Standard

1. Carefully add 2.5 ml of diluted lysis reagent 1 (working solution) to the acetylation standard bottle.
2. Gently mix until the contents are completely dissolved.

Antibody

1. Add 11 ml diluted lysis reagent 2 (working solution) and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

cGMP conjugate

1. Add 11 ml diluted assay buffer and replace the stopper.
2. Gently mix until the contents are completely dissolved. Vigorous agitation and foaming should be avoided.

Wash buffer

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

Reconstituted reagents should be stored at 2–8°C and re-used within two weeks. The reconstituted antiserum may precipitate on storage at 2–8°C. Equilibrate to room temperature and ensure that the precipitation is not allowed to settle when dispensing. This will not affect assay performance.

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

1. Label 9 polypropylene tubes 2, 4, 8, 16, 32, 64, 128, 256 and 512 fmol.
2. Pipette 1 ml diluted lysis reagent 1 into all tubes except the 512 fmol.
3. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 512 fmol tube.
4. Transfer 1 ml of the 10.24 pmol/ml stock standard into the 256 fmol tube and mix thoroughly.
5. Transfer 1 ml from the 256 fmol tube to the 128 fmol tube and mix thoroughly.
6. Repeat this doubling dilution successively with the remaining tubes.

7. 50 µl aliquots from each of the serial dilutions will give rise to 9 standard levels ranging from 2 to 512 fmol/well.

Note: These tubes will be referred to as acetylation tubes containing working standards in the assay method section. Working standards should be freshly prepared before each assay and not re-used. Care should be taken when preparing working standards as dodecyltrimethylammonium bromide may cause frothing. Vigorous pipetting should be avoided.

11.1.3. Cell lysis method

Adherent and suspension cells

1. Culture cells (100 µl) in standard 96-well microplates (tissue-culture grade) with cell concentrations of between 10^4-10^6 cells/ml.
2. Incubate the plate overnight at 37°C (5% CO_2 and 95% humidity).
3. Stimulate cells with (eg 80 µl) agonist, cell stimulant, inhibitor under study as required. Do not decant or aspirate the cell culture media. Incubate agonist/cell stimulant with cultures depending on the required experimental conditions.
4. Add 20 µl of buffer A (5% dodecyltrimethylammonium bromide in assay buffer, see page 40). The final volume in the wells should be 200 µl, each containing 0.5% buffer A (final concentration) and agonist/stimulant which is equivalent to the lysis reagent 1 working solution.
5. Following the addition of buffer A, agitate cells to facilitate cell lysis. This can be achieved by shaking the plate on a microplate shaker for 10 minutes after adding the lysis reagent.
6. Carry out a microscopic evaluation with Trypan blue to check the cells have lysed. Cell membranes may still be visible after cell lysis. Lysed cells are now ready for use in the enzymeimmunoassay protocol and should be processed immediately in the immunoassay (see 'assay method').

11.1.4. Assay method

Note: Steps 6–13 should be performed as quickly as possible. Only polypropylene or glass tubes should be used for steps 1–6. Equilibrate all reagents to room temperature and mix before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5°C during the course of the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the course of the assay.

1. Prepare the reagents and working standards as described in the 'reagent preparation' section.
2. Label polypropylene or glass tubes (12 × 75 mm) for zero standard and unknowns. These will subsequently be known as acetylation tubes.
3. Set up the microplate with sufficient wells for running of all blanks, standards and samples in duplicate (see fig 5).
4. Prepare the acetylation reagent by adding 1 volume acetic anhydride to 2 volumes of triethylamine in a glass vessel and mixing well (sufficient reagent for 60 acetylations may be obtained by mixing 2 ml acetic anhydride with 4 ml triethylamine).
5. Pipette 1 ml of diluted lysis reagent 1 into the zero standard acetylation tube.
6. Carefully add 100 µl of the acetylation reagent to all acetylation tubes containing standards. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylation reagent.

7. Pipette 20 µl of the acetylation reagent into all the sample wells of the cell culture plate and mix on a microplate shaker for 5 minutes. If necessary, depending on the quality of the tissue culture plate used, transfer the 200 µl aliquot of cell culture lysate, prior to the addition of the acetylation reagent, to a polypropylene or glass tube. In this case add 20 µl of the acetylation reagent to the lysate in the polypropylene or glass tube and mix for 5 minutes. Transfer a 50 µl aliquot to the immunoassay microplate as described in step 11.
8. Pipette 100 µl of antiserum into all wells of the immunoassay microplate except the blank and non-specific binding (NSB) wells.
9. Pipette 100 µl of lysis reagent 2 and 50 µl of lysis reagent 1 (working solutions) into the NSB wells.
10. Starting with the most dilute, pipette 50 µl aliquots from all acetylation tubes including the zero standard into the appropriate wells.
11. Transfer 50 µl of each unknown acetylated sample from the cell culture plate into the appropriate wells of the immunoassay microplate.
12. Cover the plate with the lid provided and gently mix.
13. Incubate the plate at 3–5°C for exactly 2 hours.
14. Pipette 100 µl of diluted conjugate into all wells except the blank.
15. Cover the plate with the lid provided and gently mix.
16. Incubate the plate at 3–5°C for 60 minutes.
17. Aspirate and wash all wells 4 times with wash buffer ensuring that all wells are completely filled and emptied at each wash.
18. Blot the plate on tissue ensuring any residual buffer is removed. **Thorough washing is essential for good performance.**

19. Immediately pipette 200 µl of room temperature equilibrated enzyme substrate into all wells.

20. Cover the plate with the lid provided and mix on a microplate shaker for exactly 30 minutes at room temperature (15–30°C).

21. The blue colour that develops may be read at 630 nm. However it is recommended that the reaction is halted prior to end point determination by the addition of 100 µl of 1M sulphuric acid to all wells. The optical density can be read at 450 nm within 30 minutes.

11.2. Data processing

For calculation of results and typical data see pages 20, 37 and 38.

12. Additional information

12.1. Specificity

The cross-reactivity, as determined by the concentration giving 50% B/B_0, is shown in the table below:

Table 5. Cross-reactivity

Compound	**% Cross-reactivity (50% B/B_0 displacement)**	
	Non-acetylation (protocol 1)	Acetylation (protocols 2–4)
cGMP	100	100
cAMP	<0.0005	<0.00008
AMP	<0.0000001	<0.000000133
ADP	<0.0000001	<0.000000133
ATP	<0.0000001	<0.000000133
GMP	<0.0005	<0.000004
GDP	<0.00025	<0.000002
GTP	<0.00025	<0.00000133

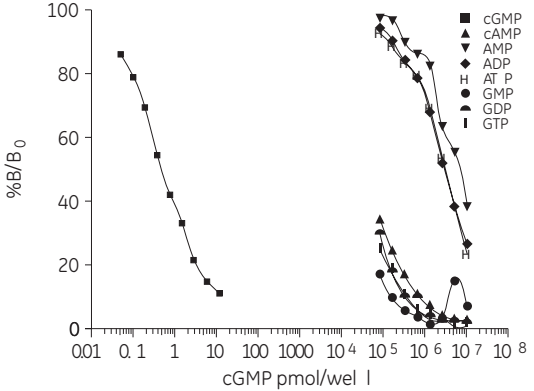
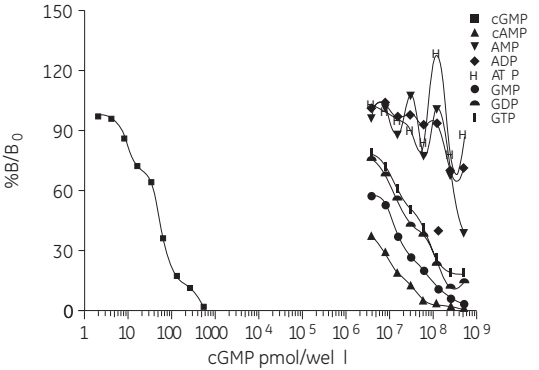


Fig 8. (a) Cross-reactivity profile for protocol 1 (non-acetylation assay)



(b) Cross-reactivity profile for protocols 2–4 (acetylation assay)

12.2. Reproducibility

Within-assay precision

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

	Protocol 1 non-acetylation assay (mean values as fmol/well)			Protocols 2–4 acetylation assay (mean values as fmol/well)		
Control	Mean ± SD	%CV	n	Mean ± SD	%CV	n
A	871.8 ± 65.1	7.5	15	9.9 ± 0.8	9.0	17
B	1712.0 ± 192.5	11.2	15	61.0 ± 6.9	11.4	17
C	10759.5 ± 497.2	4.6	15	156.3 ± 12.3	6.5	17

Between-assay precision

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

	Protocol 1 non-acetylation assay (mean values as fmol/well)			Protocols 2–4 acetylation assay (mean values as fmol/well)		
Control	Mean ± SD	%CV	n	Mean±SD	%CV	n
A	240.2 ± 22.9	9.5	10	9.0 ± 1.1	12.7	16
B	1003.0 ± 122.8	12.2	10	30.7 ± 3.0	9.8	16
C	5426.0 ± 614.7	11.3	19	109.7 ± 13.0	11.8	16

Precision profile

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

Protocol 1 non-acetylation assay			Protocols 2–4 acetylation assay		
Standard	Standard deviation	%CV	Standard	Standard deviation	%CV
50	9.1	17.4	2	0.2	11.0
100	15.9	17.6	4	0.4	10.4
200	32.7	11.2	8	0.5	7.4
400	24.1	6.9	16	0.9	5.5
800	96.4	10.0	32	1.8	5.3
1600	127.4	8.0	64	3.1	3.7
3200	276.5	8.3	128	7.1	7.0
6400	539.8	5.9	256	17.4	6.3
12800	986.6	7.7	512	43.5	8.5

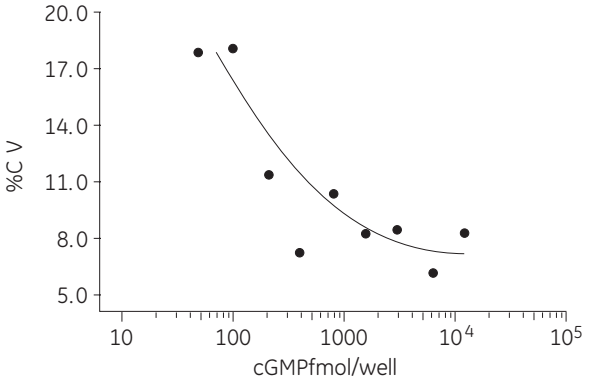
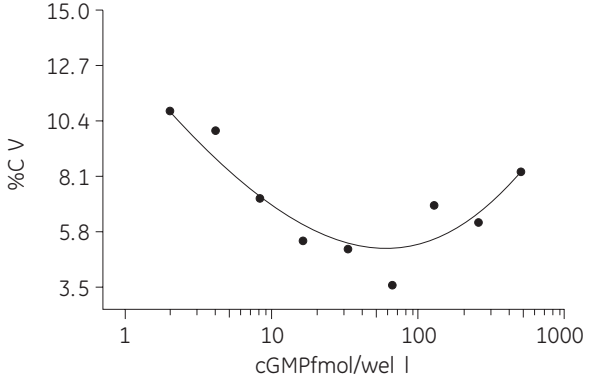


Fig 9. (a) Precision profile – protocol 1 (non-acetylation assay)



(b) Precision profile – protocols 2–4 (acetylation assay)

12.3. Sensitivity

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

Protocol 1 (non-acetylation assay)	46 fmol/well	161 pg/ml
Protocols 2–4 (acetylation assay)	2 fmol/well	14 pg/ml

13. Troubleshooting guide

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

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15. Related products

Biotrak signal transduction assay range

Cyclic AMP, direct enzyme immunoassay*	EIA	RPN 225
Cyclic AMP, [^{3}H]	RIA	TRK 432
D-myo-Inositol 1,4,5-trisphosphate (IP_3), [^{3}H]	RIA	TRK 1000
Amprep SAX 500 mg (pack of 50)		RPN 1918
Amprep SAX 100 mg (pack of 100)		RPN 1908

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