Cyclic GMP ELISA Kit

Item No. 581021

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
481022	Cyclic GMP ELISA Antiserum	1 vial/100 dtn	1 vial/500 dtn
481020	Cyclic GMP AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
481024	Cyclic GMP ELISA Standard	1 vial	1 vial
400060	ELISA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
400035	Polysorbate 20	1 vial/3 ml	1 vial/3 ml
400004/400006	Mouse Anti-Rabbit IgG Coated Plate	1 plate	5 plates
400012	96-Well Cover Sheet	1 cover	5 covers
400050	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
400031	Acetic Anhydride	1 vial/2.5 ml	1 vial/12.5 ml
400029	Potassium Hydroxide	1 vial	1 vial

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Cyclic GMP ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Materials used for **Sample Preparation** (see page 14).

INTRODUCTION

Background

Guanosine 3',5'cyclic monophosphate (cGMP) is a key intracellular second messenger molecule which transduces cellular signaling events in response to a variety of hormones, autacoids and drugs. cGMP is synthesized from GTP by both membrane-bound and soluble guanlyate cyclase enzymes.[1,2] Characterization of the particulate guanylate cyclase indicates that the protein contains both the cGMP catalytic domain and a cell surface receptor function. In mammals, six membrane forms (GC-A-F) have been identified, of which only three have ligands that are known. Soluble guanylate cyclase is a heterodimer consisting of α (α_1 or α_2) and β (β_1 or β_2) subunits. The well-characterized α_1/β_1 heterodimer is activated several hundred fold by nM concentrations of endogenous nitric oxide, resulting in accumulation of intracellular cGMP.[3,4] The downstream mediators of cGMP-controlled events include cGMP-gated channels, cGMP-dependent kinases and cGMP-regulated phosphodiesterases. The relative abundance of cGMP within a given cell can serve as a marker for activation by agonists acting through particulate guanylate cyclase at the cell surface, or intracellular activation of soluble guanylate cyclase by nitric oxide.

About This Assay

Cayman's cGMP Assay is a competitive ELISA that can be used for quantification of cGMP directly obtained from cell lysates, tissue homogenates, plasma, or urine. The assay has a range from 0.23-30 pmol/ml and a sensitivity (80% B/B_0) of approximately 1 pmol/ml. Since the antibody used in this assay was prepared against a cGMP-carrier protein conjugate, antibody binding is increased if an acetyl group is present on the 2' hydroxyl group of the cGMP. The optional acetylation procedure for both samples and standards increases the sensitivity of the assay approximately ten-fold. A protocol for acetylating both the standards and samples prior to performing the assay is provided (see protocol on page 21). Basal levels of cGMP in cell lysates can often be measured without acetylation, but results will depend on the type and number of cells being utilized. Platelets produce approximately 1.5-2.5 pmole cGMP/10^9 cells under basal conditions.[5,6,7] Cells such as NG108-15 cells and monocytes produce considerably more cGMP than platelets (approximately 0.1-1 pmol/10^6 cells).[8,9]

Description of AChE Competitive ELISAs[10]

This assay is based on the competition between free cGMP and a cGMP-acetylcholinesterase (AChE) conjugate (cGMP tracer) for a limited amount of cGMP-specific rabbit antibody binding sites. Because the concentration of the cGMP tracer is held constant while the concentration of cGMP varies, the amount of cGMP tracer that is able to bind to the rabbit antibody will be inversely proportional to the concentration of cGMP in the well. This rabbit antibody-cGMP complex binds to the mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of cGMP tracer bound to the well, which is inversely proportional to the amount of free cGMP present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound cGMP Tracer}] \propto 1/[\text{cGMP}]$$

A schematic of this process is shown in Figure 1, on page 9.

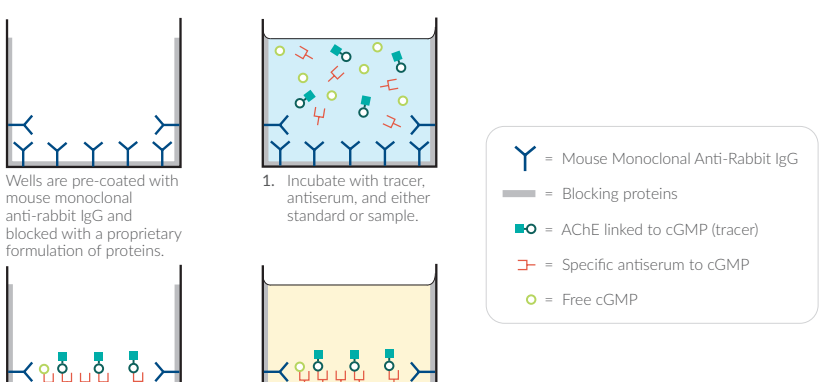


Figure 1. Schematic of the AChE ELISA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *E. electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in AChE enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 2, on page 11). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

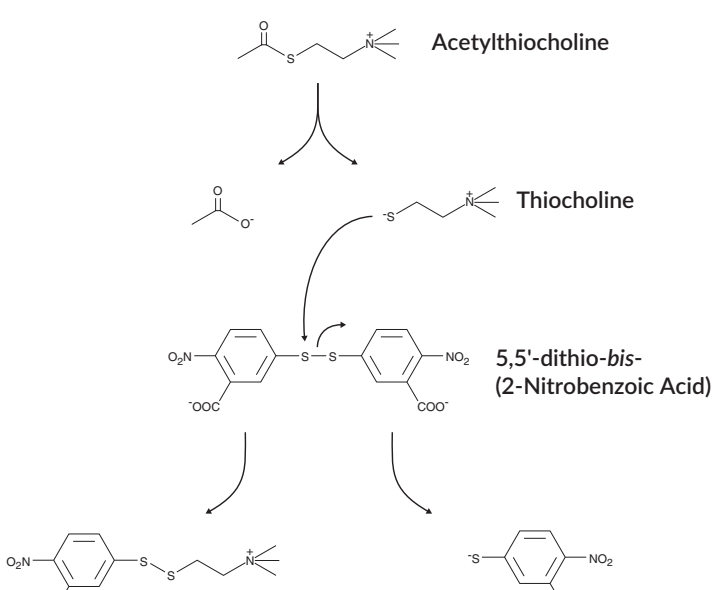


Figure 2. Reaction catalyzed by acetylcholinesterase

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including NSB wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding. Do not forget to subtract the Blank absorbance values.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Cross Reactivity: numerical representation of the relative reactivity of this assay towards structurally related molecules as compared to the primary analyte of interest. Biomolecules that possess similar epitopes to the analyte can compete with the assay tracer for binding to the primary antibody. Substances that are superior to the analyte in displacing the tracer result in a cross reactivity that is greater than 100%. Substances that are inferior to the primary analyte in displacing the tracer result in a cross reactivity that is less than 100%. Cross reactivity is calculated by comparing the mid-point (50% B/B_0) value of the tested molecule to the mid-point (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

$$\%\ \text{Cross Reactivity} = \left[\frac{50\%\ B/B_0 \text{ value for the primary analyte}}{50\%\ B/B_0 \text{ value for the potential cross reactant}}\right] \times 100\%$$

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA. UltraPure water may be purchased from Cayman (Item No. 400000).

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) (Item No. 400060) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X) (96-well kit; Item No. 400062): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20 (Item No. 400035).

   OR

   12.5 ml vial Wash Buffer Concentrate (400X) (480-well kit; Item No. 400062): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Polysorbate 20 (Item No. 400035).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

In general, urine and tissue culture supernatant samples may be diluted with ELISA Buffer and added directly to the assay well. Plasma, serum, whole blood, and tissue homogenates, as well as other heterogeneous mixtures such as lavage fluids and aspirates often contain contaminants which can interfere in the assay. The presence of rabbit IgG in the sample may cause interference in the assay. It is best to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between ~20-80% B/B_0 on the standard curve. If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated cGMP concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Due to the presence of phosphodiesterases in many samples (*i.e.*, tissues and cell lysates), sample purification is mandatory to prevent enzymatic hydrolysis of cGMP. Protocols for sample preparation are provided below.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.
- Samples of rabbit origin may contain antibodies which interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.

Urine

Urine samples may be diluted in ELISA Buffer and assayed directly. Dilutions of between approximately 1:200 and 1:500 may be required for measurement of cGMP in urine.

Plasma

To 500 µl plasma add 2 ml ice cold ethanol and vortex. Leave the sample at room temperature for five minutes. Remove the precipitate by centrifugation at 1,500 x g for 10 minutes. Transfer the supernatant to a clean 10 ml test tube. Dry the supernatant by vacuum centrifugation or under a stream of nitrogen and then resuspend in 500 µl of ELISA Buffer (vacuum centrifugation can be used to remove the final aqueous portion of the extract). Ensure that all of the ethanol is removed as trace amounts can affect the performance of the assay.

Culture Medium Samples

Tissue and cell culture supernatants may be assayed directly without purification. If the cGMP concentration in the medium is high enough to dilute the sample ten-fold with ELISA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted with ELISA Buffer), dilute the standard curve in the same culture medium as that used in the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in a particular medium.

Cell Culture Extraction for cGMP Assay

a. Aspirate medium from plate/flask.

b. Add 1 ml of 0.1 M HCl for every 35 cm^2 of surface area (*e.g.*, for a 150 mm plate, add 5 ml).

c. Incubate at room temperature for 20 minutes.

d. Scrape cells off the surface with a cell scraper or rubber policeman.

e. Dissociate the mixture by pipetting up and down until the suspension is homogeneous and transfer to appropriately sized centrifuge tube.

f. Centrifuge at 1,000 x g for 10 minutes.

g. Decant the supernatant into a clean test tube.

Dilute the supernatants at least 1:2 in ELISA buffer to neutralize the acid prior to performing the assay. A supernatant protein concentration of at least 1 mg/ml is recommended for reproducible results.

If acetylation is required, follow the procedure for sample acetylation on page 21.

Tissue Samples

a. Cyclic nucleotides may be metabolized quickly in tissue, so it is important to rapidly freeze (*i.e.*, using liquid nitrogen) the sample immediately after collection.

b. Weigh the frozen tissue and drop into 5-10 volumes (ml of solution/gram of tissue) of 5% trichloroacetic acid (TCA) in water. Homogenize the sample on ice (0-4°C) using a Polytron-type homogenizer. *NOTE: Alternatively the frozen sample can be pulverized prior to addition of TCA.*

c. Remove the precipitate by centrifugation at 1,500 x g for 10 minutes. Carefully transfer the supernatant to a clean test tube.

d. Extract the TCA from the sample using water-saturated ether. *NOTE: To make water-saturated ether, add water to ether until layers form; mix and use the top (ether) layer.* Add five volumes of ether to one volume of supernatant, mix for 10 seconds, and then allow the organic and aqueous phases to separate. Carefully remove the top (ether) layer and discard. Repeat the extraction two more times.

e. Remove the residual ether from the aqueous layer by heating the sample to 70°C for five minutes. It is imperative that all the ether be removed as even trace amounts can interfere with the assay.

The supernatants from the tissue extraction can be assayed directly without dilution provided the standard curve is prepared in the same matrix as the samples. To prepare the standard curve matrix solution, extract about 20 ml of the 5% TCA preparation with ether in the same manner as used for sample extraction. Remove the residual ether by heating and use the remaining solution to prepare the standard curve.

Preparation of Assay-Specific Reagents

cGMP AChE Tracer

Reconstitute the cGMP AChE Tracer as follows:

100 dtn cGMP AChE Tracer (96-well kit; Item No. 481020): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn cGMP AChE Tracer (480-well kit; Item No. 481020): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted cGMP AChE Tracer at 4°C *(do not freeze!)* and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

cGMP ELISA Antiserum

Reconstitute the cGMP ELISA Antiserum as follows:

100 dtn cGMP ELISA Antiserum (96-well kit; Item No. 481022): Reconstitute with 6 ml ELISA Buffer.

OR

500 dtn cGMP ELISA Antiserum (480-well kit; Item No. 481022): Reconstitute with 30 ml ELISA Buffer.

Store the reconstituted cGMP ELISA Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

cGMP Standards and Samples

Do you need to acetylate?

The ELISA is able to detect lower concentrations of cGMP if the samples are first acetylated. If the expected concentration is less than ~1 pmol/ml, the acetylation procedure should be performed. If the expected concentration is greater than ~1 pmol/ml no acetylation is necessary. *NOTE: The acetylation procedure may be affected by proteins or sugars in the sample matrix. In these cases, the sample should be purified before acetylation.*

1. **Preparation of Standards and Samples - No Acetylation**

   Standard Curve Preparation

   Reconstitute the cGMP ELISA Standard (Item No. 481024) with 1 ml of ELISA Buffer. The concentration of this solution will be 300 pmol/ml. Store this solution at 4°C; it will be stable for approximately six weeks. We have included enough cGMP to run ten standard curves. This surplus should accomodate any experimental design.

   NOTE: If the samples are prepared from TCA-extracted tissue and cannot be diluted at least 1:5 in ELISA Buffer for analysis, use ether-extracted 5% TCA for preparation of the standard curve. Any dilution of samples should then be performed in this solution.

   To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 500 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (300 pmol/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

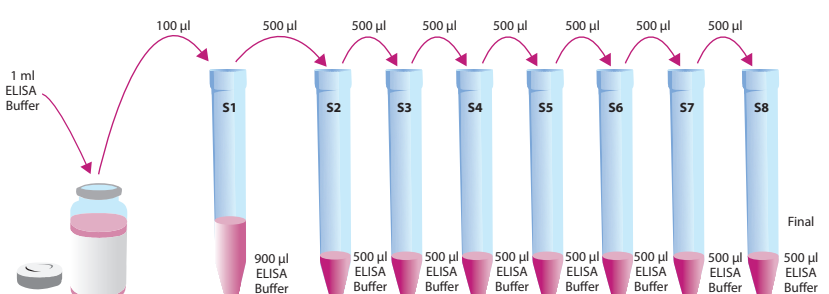


Figure 3. Preparation of non-acetylated cGMP Standards

Sample preparation

If samples require purification, please refer to the protocols on pages 14-17. Upon purification, no further sample preparation is necessary; however, the samples may require dilution to ensure that they will fall on the linear portion of the standard curve (20-80% B/B_0).

Proceed to Performing the Assay

2. Preparation of Standards and Samples - Acetylation

Standard Curve Preparation

Reconstitute the cGMP ELISA Standard (Item No. 481024) with 1 ml of ELISA Buffer (label this Standard A). Aliquot 100 µl of Standard A (300 pmol/ml) into 9.9 ml of ELISA Buffer (label this Standard B). The concentration of this standard is 3 pmol/ml.

NOTE: If the samples are prepared from TCA-extracted tissue and cannot be diluted at least 1:5 in ELISA Buffer for analysis, use ether-extracted 5% TCA for preparation of the standard curve. Any dilution of samples should then be performed in this solution.

To prepare the standard for use in ELISA: Obtain nine clean test tubes and number them #0 through #8. Aliquot 500 µl ELISA Buffer to tube #0 (this tube will contain only buffer) and 500 µl ELISA Buffer to tubes #2-8. Transfer 1 ml of Standard B (3 pmol/ml) to tube #1. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. Discard 500 µl of the solution in tube #8 so each tube contains 500 µl. These diluted standards should not be stored for more than 24 hours.

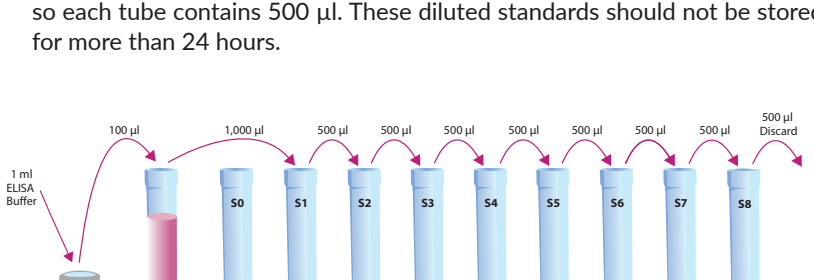


Figure 4. Preparation of acetylated cGMP Standards

Sample preparation

If samples need to be purified, do so before proceeding with the acetylation procedure (see page 14-17 for Purification Protocol). Although purification may not be necessary, we recommend that samples be purified to ensure assay integrity. If you are acetylating less than 500 µl of sample, you must adjust the amounts of Potassium Hydroxide (KOH) and acetic anhydride proportionally.

Preparation of KOH

Prepare a 4 M solution of KOH:

100 dtn Potassium Hydroxide (96-well kit; Item No. 400029): Dissolve with 10 ml UltraPure water.

OR

500 dtn Potassium Hydroxide (480-well kit; Item No. 400029): Dissolve with 50 ml UltraPure water.

Acetylation procedure *(based on 500 µl sample size)*

All samples, as well as standard tubes #0-8, must be acetylated. Each sample/standard should be acetylated individually. It is important to be consistent in the acetylation technique as differences in vortex time and/or delayed addition of KOH may result in irreproducible results.

To 500 µl of sample, add 100 µl of 4 M KOH and 25 µl Acetic Anhydride in quick succesion. Vortex for 15 seconds. Add 25 µl of 4 M KOH and vortex. Repeat for all samples and standard tubes.

NOTE: If the samples contain sugars at concentration >250 mM, it may be necessary to proportionately increase the amount of KOH and acetic anhydride added to ensure complete acetylation of cGMP.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 28, for more details). We suggest you record the contents of each well on the template sheet provided (see page 38).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **ELISA Buffer**

   Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B_0 wells. If culture medium or ether-saturated 5% TCA was used to dilute the standard curve, substitute 50 µl of that matrix for ELISA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl ELISA Buffer to NSB wells). If standards and samples were acetylated, substitute 50 µl of acetylated sample matrix (tube #0) for ELISA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl of tube #0 to NSB and B_0 wells and 50 µl of ELISA Buffer to NSB wells).

2. **cGMP ELISA Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **cGMP AChE Tracer**

   Add 50 µl to each well *except* the TA and the Blk wells.

5. **cGMP ELISA Antiserum**

   Add 50 µl to each well *except* the TA, the NSB, and the Blk wells.

Well	ELISA Buffer	Standard/Sample	Tracer	Antiserum
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate 18 hours at room temperature (optional: incubation for 18 hours at 4°C will increase the sensitivity of the assay slightly).

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): Reconstitute with 50 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays be run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes. If incubation was done at 4°C, development will take approximately 90-120 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either %B/B_0 *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/elisa) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B_0 for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the %Bound. This value should approximate the %Bound that can be calculated from the ***Sample Data*** *(see pages 30 and 33). Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 36 for* ***Troubleshooting****).*

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* cGMP concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. *NOTE: Do not use %B/B_0 in this calculation.*

$$\text{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with %B/B_0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

Performance Characteristics

The standard curves presented here are examples of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially.

Sample Data (Non-Acetylated)

	Raw Data		Average	Corrected
Total Activity	3.908	3.908	3.908	
NSB	0	0	0	
B_0	0.520	0.511		
	0.507		0.513	0.513

Dose (pmol/ml)	Raw Data		Corrected		%B/B_0	
30	0.086	0.085	0.086	0.085	16.8	16.6
15	0.132	0.137	0.132	0.137	25.8	26.7
7.5	0.207	0.203	0.207	0.203	40.4	39.6
3.75	0.277	0.274	0.277	0.274	54.0	53.5
1.87	0.353	0.358	0.353	0.358	68.9	69.8
0.93	0.407	0.419	0.407	0.419	79.4	81.7
0.46	0.455	0.454	0.455	0.454	88.8	88.6
0.23	0.465	0.474	0.465	0.474	90.7	92.5

Table 2. Typical results for non-acetylated cGMP

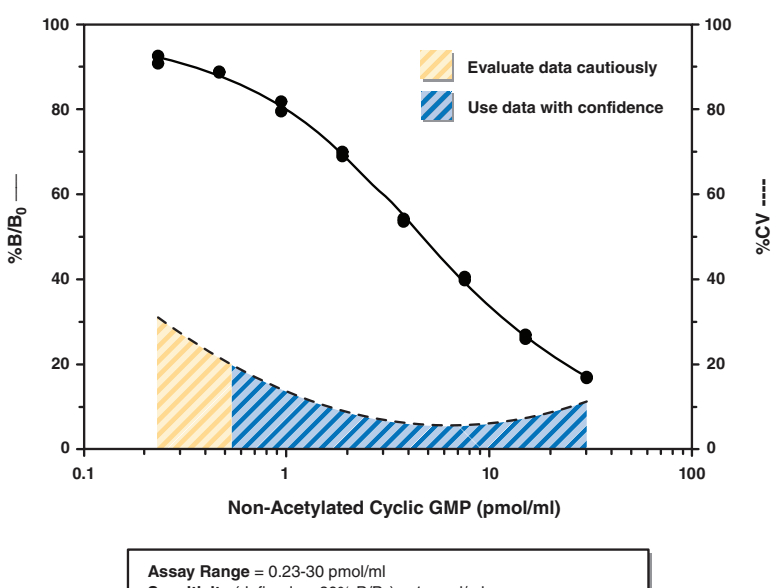


Figure 6. Typical standard curve for non-acetylated cGMP

Precision:

The intra- and inter-assay CVs have been determined at multiple points on the standard curve. These data are summarized in the graph on page 31 and in the table below.

Dose (pmol/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
30	5.7	11.3
15	3.8	6.9
7.5	4.7	7.3
3.75	1.8	4.7
1.87	2.5	5.3
0.93	7.3	11.9
0.46	16.3	31.4
0.23	24.0	25.6

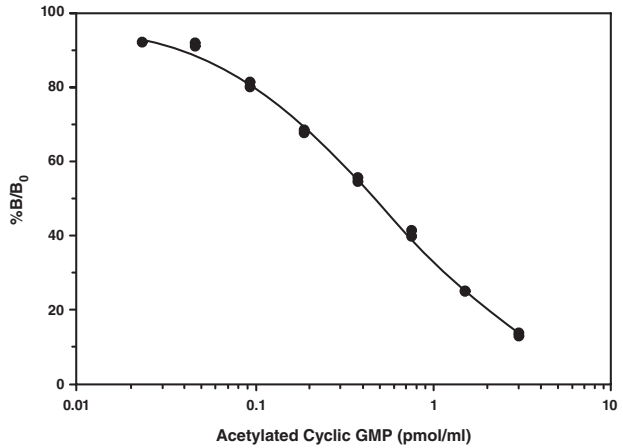
Table 3. Intra- and inter-assay variation of the non-acetylated cGMP assay
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

Sample Data (Acetylated)

	Raw Data		Average	Corrected
Total Activity	2.806	2.796	2.801	
NSB	0.032	0.032	0.032	
B_0	0.405	0.416		
	0.406	0.385	0.403	0.371

Dose (pmol/ml)	Raw Data		Corrected		%B/B_0	
3	0.063	0.063	0.031	0.031	8.36	8.36
1.5	0.125	0.126	0.093	0.094	25.07	25.34
0.75	0.180	0.186	0.148	0.154	39.89	41.51
0.375	0.239	0.235	0.207	0.203	55.80	54.72
0.187	0.284	0.287	0.252	0.255	67.92	68.73
0.093	0.335	0.330	0.303	0.298	81.67	80.32
0.046	0.373	0.376	0.341	0.344	91.91	92.72
0.023	0.375	0.363	0.343	0.331	92.45	89.22

Table 4. Typical results for acetylated cGMP



Assay Range = 0.023-3 pmol/ml
Sensitivity (defined as 80% B/B_0) = 0.1 pmol/ml
Mid-point (defined as 50% B/B_0) = 0.46 pmol/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Figure 7. Typical standard curve for acetylated cGMP

Cross Reactivity:

Compound	Cross Reactivity
cGMP	100%
Dibutyryl cGMP	0.8%
AMP	<0.01%
ATP	<0.01%
cAMP	<0.01%
8-bromo-cGMP	<0.01%
2'3'-cGMP	<0.01%

Table 5. Cross Reactivity of the non-acetylated cGMP ELISA

Compound	Cross Reactivity
Acetylated cGMP	100%
Acetylated cAMP	0.05%
Acetylated Adenosine	<0.01%
Acetylated AMP	<0.01%
Acetylated Cytidine	<0.01%
Acetylated Guanosine	<0.01%
Acetylated Uridine	<0.01%

Table 6. Cross Reactivity of the acetylated cGMP ELISA

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[11]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

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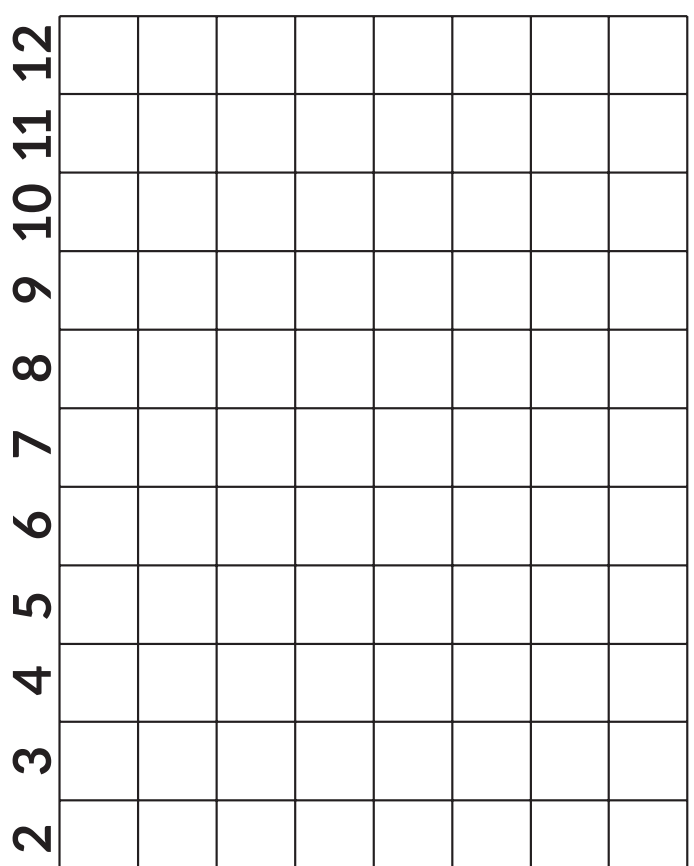

NOTES

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