2’3’-cGMP ELISA Kit

Item No. 502510

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	Storage Temperature
400704	2'3'-cGMP-HRP Tracer	1 vial/100 dtn	4°C
400706	2'3'-cGMP ELISA Monoclonal Antibody	1 vial/100 dtn	4°C
400705	2'3'-cGMP ELISA Standard	1 vial	4°C
400009/ 400008	Goat Anti-Mouse IgG Coated Strip/Solid Plate	1 plate	4°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400035	Polysorbate 20	1 vial/3 ml	RT
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	RT
400040	ELISA Tracer Dye	1 ea	RT
400042	ELISA Antiserum Dye	1 ea	RT
400012	96-Well Cover Sheet	1 ea	RT

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 2'3'-cGMP 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.

The stop solution provided with this kit is an acid solution. Please wear appropriate personal protective equipment (*e.g.*, safety glasses, gloves, and lab coat) when using this material.

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 in the **Materials Supplied** section (see page 3) 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 at 450 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. 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).*
5. Materials used for **Sample Preparation** (see page 13)

INTRODUCTION

Background

3'5'-cGMP is a cyclic nucleotide monophosphate (cNMP) and key intracellular second messenger involved in the transduction of cellular signaling events in response to a variety of hormones and small molecules.[1] 2'3'-cGMP is an non-canonical cNMP with the phosphate attached at the 2' and 3' positions of the ribose sugar moiety.[2] It is found in bacteria, various mammalian cells and tissues, plants, and algae. 2'3'-cGMP is formed in *E. coli via* RNase I-mediated degradation of cytoplasmic RNA and accumulates in the cytoplasm and periplasm.[3] It binds to purified *E. coli* 70S ribosomes and inhibits reporter activity in a coupled transcription/translation reporter assay.[4] An overall reduction of 2'3'-cNMPs in bacteria is associated with an increase in biofilm formation, while in plants 2'3'-cGMP and 2'3'-cAMP levels increase rapidly after wounding stress.[1,3]

About This Assay

Cayman's 2'3'-cGMP ELISA Kit is a competitive assay that can be used for the quantification of 2'3'-cGMP in cell lysates, plasma, serum, and tissue samples. The assay has a range of 0.7-200 nM, an average sensitivity (80% B/B_0) of 2.2 nM, and a lower limit of detection (LLOD) of 0.6 nM.

Principle of This Assay

This assay is based on the competition between free 2'3'-cGMP and a 2'3'-cGMP-horseradish peroxidase conjugate (2'3'-cGMP-HRP Tracer) for a limited number of 2'3'-cGMP monoclonal antibody binding sites. Because the concentration of the 2'3'-cGMP-HRP Tracer is held constant while the concentration of free 2'3'-cGMP varies, the amount of 2'3'-cGMP-HRP Tracer that is able to bind to the 2'3'-cGMP Monoclonal Antibody will be inversely proportional to the concentration of free 2'3'-cGMP in the well. This antibody-2'3'-cGMP complex binds to goat anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and TMB Substrate Solution (which contains the substrate to HRP) is added to the well. After a sufficient period of time, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 2'3'-cGMP-HRP Tracer bound to the well, which is inversely proportional to the amount of free 2'3'-cGMP present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto \text{[bound 2'3'-cGMP-HRP tracer]} \propto 1/\text{[2'3'-cGMP]}$$

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

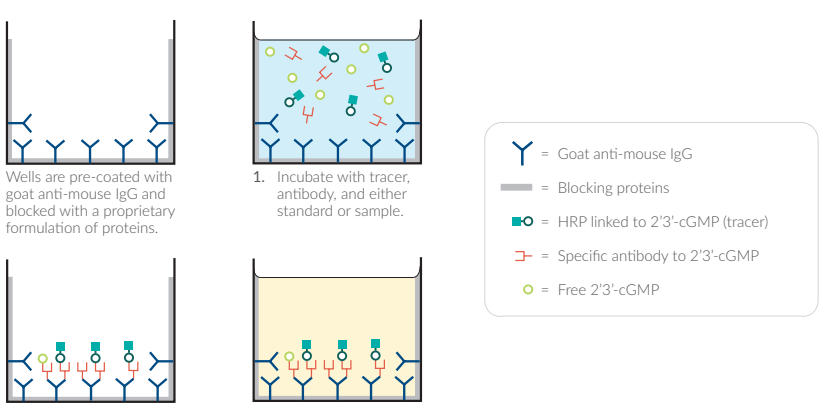


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and the HRP Stop Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells, including the non-specific binding (NSB) wells.

TA (Total Activity): total enzymatic activity of the 2'3'-cGMP HRP-linked 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.

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 sample or standard well to the average absorbance of the maximum binding (B_0) wells.

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): 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 midpoint (50% B/B_0) value of the tested molecule to the midpoint (50% B/B_0) value of the primary analyte when each is measured in assay buffer using the following formula:

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

Lower Limit of Detection (LLOD): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they will be stable for approximately two months. NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with ultrapure water.

1. **Immunoassay Buffer C (1X) Preparation**

   Dilute the contents of one vial of Immunoassay Buffer C (10X) (Item No. 401703) with 90 ml of ultrapure water. Be certain to rinse the vial to remove any salts that may have precipitated.

2. **Wash Buffer (1X) Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with ultrapure water to a total volume of 2 L and add 1 ml of Polysorbate 20 (Item No. 400035). Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding 0.5 ml of Polysorbate 20 per 1 L of Wash Buffer (1X). *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

Testing for Interference

This assay has been validated in bacterial cell lysates prepared in B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific), plasma, serum, and tissue homogenates. Other sample types should be tested for interference to evaluate the need for sample purification before embarking on a large number of sample measurements. To test for interference, dilute one or two samples to obtain at least two different dilutions of each sample within the linear portion of the standard curve. If two different dilutions of the same sample show good correlation (differ by 20% or less) in the final calculated 2'3'-cGMP concentration, sample purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the user and tested for compatibility in the assay.

Bacterial Cell Lysates

Bacterial cells can be lysed in B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific) following the manufacturer's protocol prior to use in the assay.

Plasma and Serum

Collect blood in vacutainers containing heparin, EDTA, or sodium citrate for plasma or without an anticoagulant for serum. Centrifuge the whole blood at 1,500 x g at 4°C for 20 minutes and collect the clear upper layer. The sample can be tested immediately or stored at -80°C.

Plasma/Serum Purification

Plasma and serum samples can be extracted using the following protocol. Alternative protocols may be used based on the experimental requirements, sample type, and the user's expertise.

1. Prepare an extraction solution by mixing acetonitrile:methanol:ultrapure water at a ratio of 2:2:1 and keep it on ice.
2. Aliquot a known amount of sample into a clean test tube and add cold extraction solution (approximately four times the amount of the sample volume). Vortex to mix thoroughly.
3. Incubate for 10 minutes on ice. Centrifuge at 5,000 x g for 5 minutes at 4°C. Transfer the supernatant to a clean tube. Repeat this extraction procedure two more times and combine the supernatants.
4. Evaporate the supernatant under a gentle stream of nitrogen. Samples can be heated to 37°C to promote evaporation.
5. Resuspend the extract in Immunoassay Buffer C (1X) to its original volume and use for ELISA analysis. Samples can be concentrated in this step by using smaller volumes of buffer compared to the original sample volumes.

Tissue Samples

Tissue samples can be purified using the following protocol. Alternative protocols may be used based on the experimental requirements, sample type, and the user's expertise.

1. Prepare an extraction solution by mixing acetonitrile:methanol:ultrapure water at a ratio of 2:2:1 and keep it on ice.
2. Weigh 100 mg of frozen tissue and add 2 ml of the ice-cold extraction solution. Homogenize the sample.
3. Immediately aliquot a known amount of sample into a clean test tube and add the cold extraction solution (approximately four times the amount of sample volume). Vortex to mix thoroughly.
4. Incubate for 10 minutes on ice. Centrifuge at 5,000 x g for 5 minutes at 4°C. Transfer the supernatant to a clean tube. Repeat this extraction procedure two more times and combine the supernatants.
5. Evaporate the supernatant under a gentle stream of nitrogen. Samples can be heated to 37°C to promote evaporation.
6. Resuspend the extract in Immunoassay Buffer C (1X) to its original volume and use this for ELISA analysis. It is recommended to dilute the tissue sample at least two-fold to avoid interferences.

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 mouse origin may contain antibodies that interfere with the assay by binding to the goat anti-mouse IgG plate. We recommend that all mouse samples be purified prior to use in the assay.

Sample Matrix Properties

Parallelism

To assess parallelism, bacterial lysates, mouse liver, mouse kidney, and rat brain extracts were processed as described in the **Sample Preparation** section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated using the 2'3'-cGMP ELISA Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

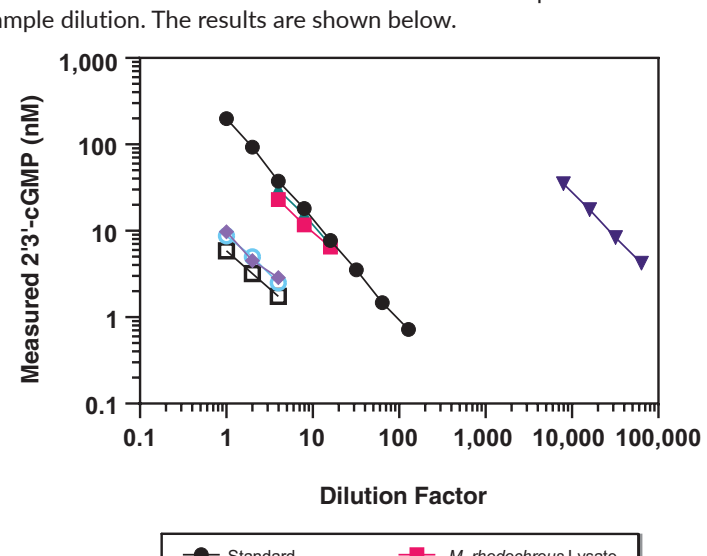


Figure 2. Parallelism of bacterial lysates and tissue extracts in the 2'3'-cGMP ELISA

Spike and Recovery

Pooled human plasma and mouse kidney extract were spiked with 2'3'-cGMP, processed as described in the **Sample Preparation** section, serially diluted with Immunoassay Buffer C (1X), and evaluated using the 2'3'-cGMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

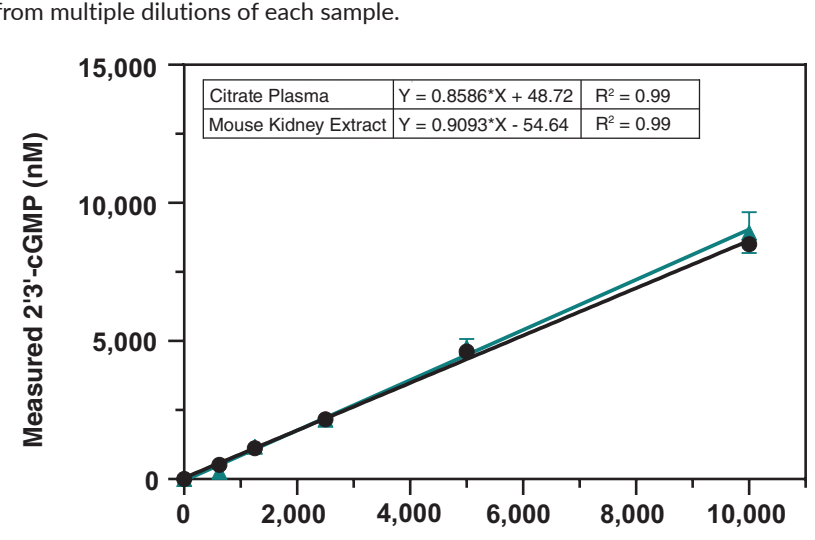


Figure 3. Spike and recovery of 2'3'-cGMP in various matrices

P. aeruginosa lysate was spiked with 2'3'-cGMP, serially diluted with Immunoassay Buffer C (1X), and evaluated using the 2'3'-cGMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

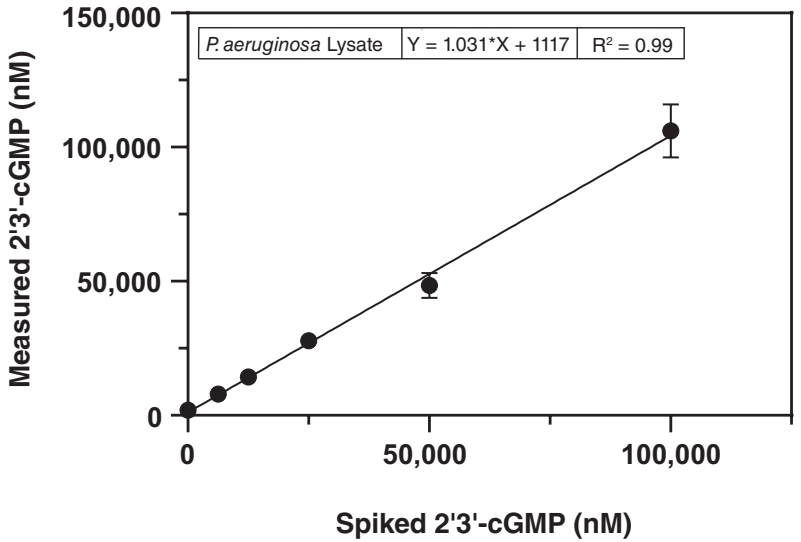


Figure 4. Spike and recovery of 2'3'-cGMP in bacterial lysates

Linearity

P. aeruginosa lysate, pooled human plasma and serum, and mouse liver extract were spiked with 2'3'-cGMP, processed as described in the Sample Preparation section, serially diluted with Immunoassay Buffer C (1X), and evaluated for linearity using the 2'3'-cGMP ELISA Kit.

Dilution Factor	Measured Concentration (nM)	Linearity (%)
P. aeruginosa lysate, spiked with 10 µM 2'3'-cGMP		
500	9,767	100
1,000	9,840	101
2,000	9,638	99
Human citrate plasma, spiked with 10 µM of 2'3'-cGMP		
400	8,267	100
800	8,469	102
1,600	8,783	106
Human serum, spiked with 300 nM of 2'3'-cGMP		
12	247	100
24	236	96
48	254	103
Mouse kidney tissue homogenate, spiked with 10 µM of 2'3'-cGMP		
400	8,292	100
800	8,577	103
1,600	8,841	107

Table 1. Linearity in various matrices

NOTE: Linearity has been calculated using the following formula:
*%Linearity = (Observed concentration value, dilution adjusted / First observed concentration value in the dilution series, dilution adjusted)*100*

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

2’3’-cGMP ELISA Standard

Reconstitute the lyophilized 2’3’-cGMP ELISA Standard (Item No. 400705) with 0.5 ml of Immunoassay Buffer C (1X) and mix gently. The concentration of this solution (the bulk standard) will be 500 nM. The reconstituted standard will be stable for four weeks when stored at 4°C.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 240 µl of Immunoassay Buffer C (1X) to tube #1 and 200 µl of Immunoassay Buffer C (1X) to tubes #2-8. Transfer 160 µl of the bulk standard (500 nM) to tube #1 and mix thoroughly. Serially dilute the standard by removing 160 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 160 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should be used within three hours.

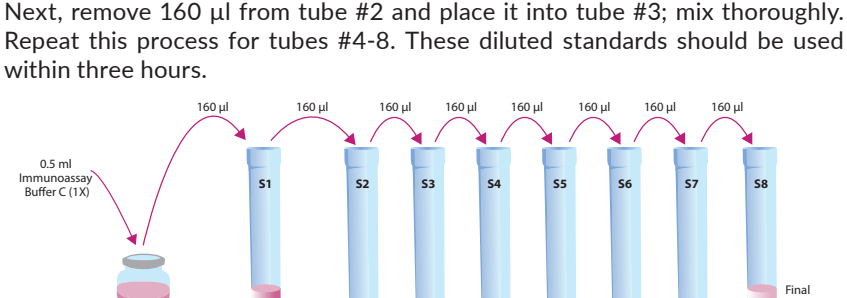


Figure 5. Preparation of the 2’3’-cGMP Standards

2’3’-cGMP-HRP Tracer

Dilute the 2’3’-cGMP-HRP Tracer (Item No. 400704) with 5 ml of Immunoassay Buffer C (1X). Store the diluted 2’3’-cGMP-HRP Tracer at 4°C (*do not freeze!*). It will be stable for at least four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the diluted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer). *NOTE: Do not store tracer with dye for more than three weeks at 4°C.*

2’3’-cGMP ELISA Monoclonal Antibody

Dilute the 2’3’-cGMP ELISA Monoclonal Antibody (Item No. 400706) with 5 ml of Immunoassay Buffer C (1X). Store the diluted 2’3’-cGMP-Monoclonal Antibody at 4°C (*do not freeze!*). It will be stable for at least four weeks. A 20% surplus of antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the diluted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). *NOTE: Do not store antibody with dye for more than three weeks at 4°C.*

Plate Set Up

The 96-well plate(s) included with this kit must be pre-washed five times with Wash Buffer (1X) (~300 µl/well) prior to use in the ELISA. *NOTE: If you do not need to use all the strips at once, place the unwashed 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 Blk, two NSB, and three B_0 wells, 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 a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, assaying the samples in triplicate is recommended.

A suggested plate format is shown in Figure 6, 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 25 for more details). We suggest you record the contents of each well on the template sheet provided (see page 33).

	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 Wells
TA = Total Activity Wells
NSB = Non-Specific Binding Wells
B_0 = Maximum Binding Wells
S1-S8 = Standard Wells
1-24 = Sample Wells

Figure 6. Sample plate format

Performing the Assay

Equilibrate all reagents at room temperature prior to addition to the plate.

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.

Pre-Wash the Plate

Rinse the plate (or strips to be used) five times with ~300 µl Wash Buffer (1X).

Addition of the Reagents

1. **Immunoassay Buffer C (1X)**

   Add 100 µl of Immunoassay Buffer C (1X) to NSB wells. Add 50 µl of Immunoassay Buffer C (1X) to B_0 wells.

2. **2'3'-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 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. **2'3'-cGMP-HRP Tracer**

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

5. **2'3'-cGMP ELISA Monoclonal Antibody**

   Add 50 µl to each well, *except* the TA, NSB, and Blk wells, within 15 minutes of addition of the tracer.

Incubation of the Plate

Cover each plate with a 96-Well Cover Sheet (Item No. 400012) and incubate two hours at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl of Wash Buffer (1X).
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Dilute 10 µl of the previously diluted tracer with 40 µl of Immunoassay Buffer C (1X). Add 5 µl of this solution to the TA wells.
4. Cover the plate with the 96-Well Cover Sheet. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes protected from light.
5. Remove the cover sheet being careful to keep TMB Substrate Solution from splashing on the cover. *NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.*
6. **DO NOT WASH THE PLATE.** Add 75 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. *NOTE: The stop solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.*

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Read the plate at a wavelength of 450 nm.

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 Blk 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. Low or no absorbance from a TA well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B_0 for standards S1-S8 *versus* 2'3'-cGMP concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized 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 dilution of the sample prior to its 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.

NOTE: If there is an error in the B_0 wells, plot the absorbance values instead of %B/B_0 to calculate sample concentrations.

Performance Characteristics

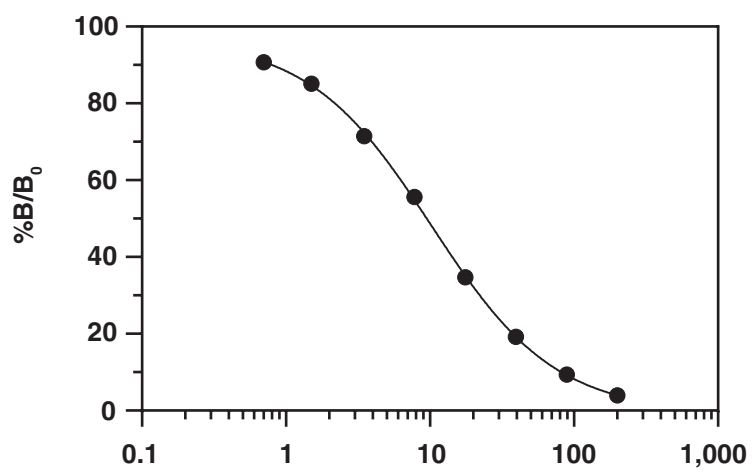
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Absorbance at 450 nm

2'3'-cGMP Standards (nM) and Controls	Blk-Subtracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
NSB	0.03	--	--	--	--
B_0	1.555	1.525	--	--	--
TA	0.579	--	--	--	--
200	0.065	0.035	2.3	11.7	2.4
88.9	0.148	0.118	7.7	5.2	2.5
39.5	0.301	0.271	17.8	7.8	1.8
17.6	0.542	0.512	33.6	8.7	1.3
7.8	0.867	0.837	54.9	7.9	2.9
3.5	1.113	1.083	71.0	9.7	4.2
1.5	1.324	1.294	84.9	12.4	5.0
0.7	1.411	1.381	90.6	15.2	4.7

Table 2. Typical results

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.



Assay Range = 0.7-200 nM
Sensitivity (defined as 80% B/B_0) = 2.2 nM
Mid-point (defined as 50% B/B_0) = 9.5 nM
Lower Limit of Detection (LLOD) = 0.6 nM

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted in Immunoassay Buffer C (1X).

Figure 7. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (bacterial lysates) in a single assay.

Matrix Control	Measured 2'3'-cGMP (nM)	%CV
High Control	$1.8x10^5$	7.9
Medium Control	1,039	9.4
Low Control	84.4	6.2

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing lysates of three matrix controls (bacterial lysates) in eight separate assays on different days.

Matrix Control	Measured 2'3'-cGMP (nM)	%CV
High Control	$1.6x10^5$	5.2
Medium Control	863	8.7
Low Control	117	14.1

Table 4. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity
2'3'-cGMP	100%
2'3'-cIMP*	34%
cAAG	1.070%
3'2'-cGAMP	0.350%
Cyclic di-GMP	0.210%
2'3'-cGAMP	0.140%
2'2'-cGAMP	0.110%
3'-GMP	0.100%
3'5'-cGMP	0.065%
Guanosine	0.040%
2'-GMP	0.030%
pGpG	0.019%
Guaninine	0.004%
GMP	0.002%
GDP	0.001%
GTP	0.001%
2'3'-cAMP	<0.001%

Table 5. Cross reactivity of the 2'3'-cGMP ELISA
**The biological relevance is unknown*

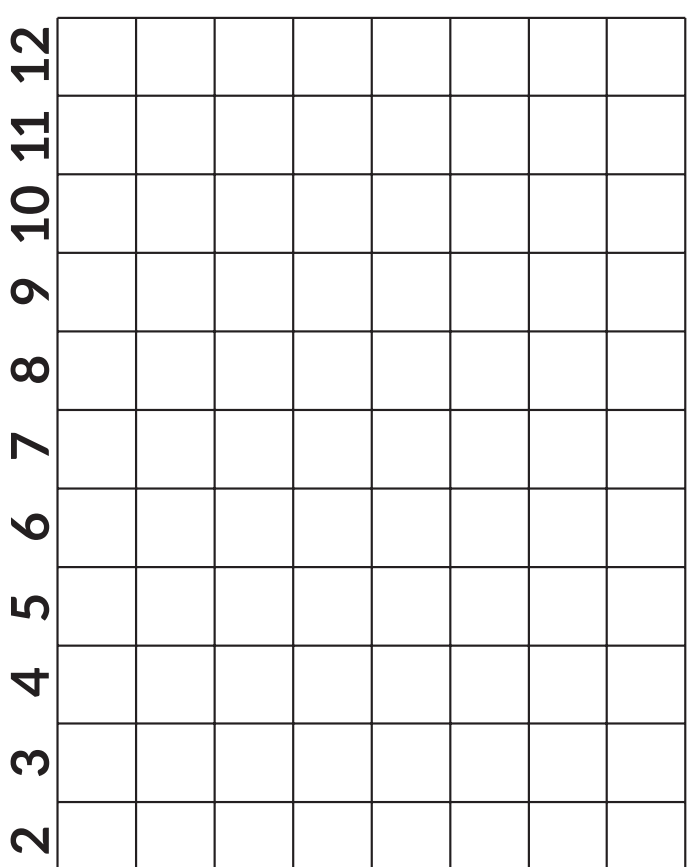
RESOURCES

References

1. Van Damme, T., Zhang, Y., Lynen, F., *et al.* Determination of cyclic guansosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry. *J. Chromatogr. B* **909**, 14-21 (2012).
2. Jackson, E.K., Mi, Z., Janesko-Feldman, K., *et al.* 2',3'-cGMP exists in vivo and comprises a 2',3'-cGMP-guanosine pathway. *Am. J. Phys. Regul. Integr. Comp, Physiol.* **316(6)**, R783-R790 (2019).
3. Fontaine, B.M., Martin, K.S., Garcia-Rodrguez, J.M., *et al.* RNase I regulates *Escherichia coli* 2',3'-cyclic nucleotide monophosphate levels and biofilm formation. *Biochem. J.* **475(8)**, 1491-1506 (2019).
4. Chauhan, S.S., Marotta, N.J., Karls, A.C., *et al.* Binding of 2',3'-cyclic nucleotide monophosphates to bacterial ribosomes inhibits translation. *ACS Cent. Sci.* **8(11)**, 1518-1526 (2022).

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Plate Preparation	Rinse the plate (or strips to be used) five times with ~300 µl Wash Buffer (1X)				
Dilute and Mix	Mix all reagents gently				
Immunoassay Buffer C (1X)	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--	--	50 µl
2'3'-cGMP-HRP Tracer	--	--	50 µl	50 µl	50 µl
2'3'-cGMP ELISA Monoclonal Antibody	--	--	--	50 µl	50 µl
Incubate	Seal the plate and incubate for 2 hours at room temperature on an orbital shaker				
Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Apply TMB Substrate	175 µl				
TA - Apply Tracer (dilute the pre-diluted tracer 1:5)	--	5 µl	--	--	--
Develop	Seal the plate and incubate for 30 minutes at room temperature on an orbital shaker protected from light				
Apply HRP Stop Solution	75 µl				
Read	Read absorbance at 450 nm				

Table 6. Assay summary



Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	A. Standard is degraded or contaminated B. Dilution error in preparing standards
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present
Low signal in sample wells (below the range of the standard curve)	A. HRP inhibitors present: ensure that the samples and buffers are free of HRP inhibitors, such as azide B. Sample requires further dilution
Only TA wells develop	A. Trace organic contaminants in the water source B. The tracer or the antibody were not added to the wells

NOTES

Warranty and Limitation of Remedy

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