Cyclic CMP ELISA Kit

Item No. 502480

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage Temperature
401107	Cyclic CMP-AChE Tracer	1 vial/100 dtn	-20°C
401106	Cyclic CMP ELISA Monoclonal Antibody	1 vial/100 dtn	-20°C
401108	Cyclic CMP ELISA Standard	1 ea	-20°C
400008/400009	Anti-Mouse IgG-Coated 96-Well Solid/Strip Plate	1 plate	4°C
400060	ELISA Buffer Concentrate (10X)	1 vial/10 ml	RT
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	RT
400050	Ellman's Reagent	3 vials/100 dtn	-20°C
400035	Polysorbate 20	1 vial/3 ml	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 Cyclic CMP 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 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 between 405-420 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 11)

INTRODUCTION

Background

Cyclic cytidine 3'5'-monophosphate (cCMP) is a non-canonical cyclic pyrimidine nucleotide and second messenger.[1,2] It is found in many organisms, including bacteria, plants, and mammals.[1,3] In bacteria, cCMP is produced from CTP by clade E pyrimidine cytidylate cyclase (PycC) in response to phage infection as part of the pyrimidine cyclase system for antiphage resistance (Pycsar).[2] Once produced, it interacts with effectors, such as the transmembrane receptor PycTM, to induce abortive infection *via* growth arrest. In mammalian systems, cCMP is produced from CTP by soluble guanylate or adenylate cyclases in response to bacterial toxins, including *B. anthracis* edema factor and *B. pertussis* adenylate cyclase (CyaA), and interacts with the effectors PKA, PKG, and hyperpolarization-activated cyclic nucleotide-gated channels (HCN).[1,4,5]

About This Assay

Cayman's Cyclic CMP ELISA Kit is a competitive assay that can be used for the quantification of cCMP in bacterial lysates. The assay has a range of 0.156-20 nM, an average sensitivity (80% B/B_0) of 0.41 nM, and a lower limit of detection (LLOD) of 0.1 nM.

Principle of This Assay

This assay is based on the competition between free cCMP and a cCMP-acetylcholinesterase conjugate (cCMP-AChE Tracer) for a limited number of cCMP monoclonal antibody binding sites. Because the concentration of the cCMP-AChE Tracer is held constant while the concentration of free cCMP varies, the amount of cCMP-AChE Tracer that is able to bind to the cCMP Monoclonal Antibody will be inversely proportional to the concentration of free cCMP in the well. This antibody-cCMP complex binds to anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and Ellman's Reagent (which contains the substrate for 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 cCMP-AChE Tracer bound to the well, which is inversely proportional to the amount of free cCMP present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{bound cCMP-AChE tracer}] \propto 1/[\text{cCMP}]$$

A schematic of this process is shown in Figure 1 below.

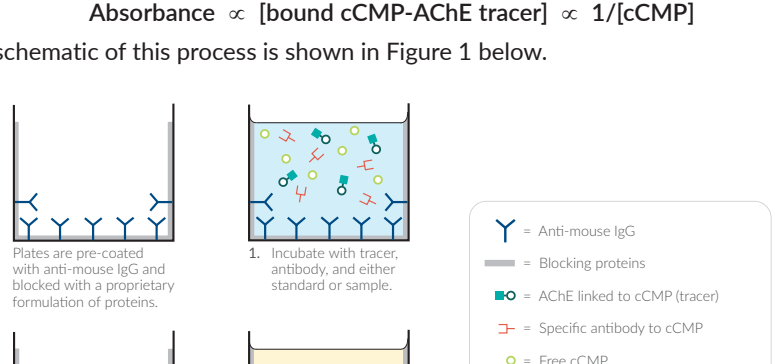


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by Ellman's Reagent. 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 AChE-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:

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

LLOD (Lower Limit of Detection): 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 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. **Assay 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.

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

   Dilute the contents of the 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 ultrapure water or B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific). 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, test one or two samples at a range of dilutions, then select 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 cCMP 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.

Preparation of Bacterial Lysates in Ultrapure Water

Weigh and resuspend pelleted bacterial cells in 4 ml of ultrapure water per gram of wet mass. Sonicate to disrupt the pellet for a homogenous mixture. Flash-freeze, then sonicate in a room temperature water bath until thawed. Repeat the freeze/thaw cycle four more times. Centrifuge the lysed sample at 21,000 x g for five minutes at 4°C and transfer the supernatant to a clean tube. Lysates should be stored at -20°C.

Preparation of Bacterial Cell Lysates in B-PER™

Weigh and resuspend pelleted bacterial cells in B-PER™ following the manufacturer's protocol. Lysates should be stored at -20°C. Please note that B-PER™ interferes with this assay at concentrations of ≥5% and above, which results in higher measured concentrations in samples. Therefore, at least a 1:20 dilution with Assay Buffer is recommended for lysates prepared in B-PER™. Alternatively, samples may be assayed at any dilution, provided the final B-PER™ concentration remains constant across all samples and standards.

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 -20°C.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Cyclic CMP ELISA Standard

Reconstitute the lyophilized Cyclic CMP ELISA Standard (Item No. 401108) with 1 ml of Assay Buffer and mix gently. The concentration of this solution (the bulk standard) will be 200 nM. The reconstituted standard will be stable for three weeks when stored at 4°C.

NOTE: If assaying samples in >5% B-PER™, prepare the standard curve in Assay Buffer containing the same percentage of B-PER™ as the samples.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl of Assay Buffer to tube #1 and 500 µl of Assay Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (200 nM) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing it 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 be used within four hours.

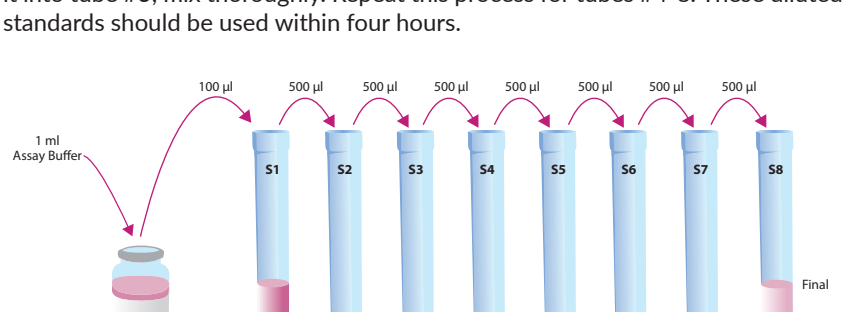


Figure 2. Preparation of the cCMP standards

Cyclic CMP-AChE Tracer

Reconstitute the Cyclic CMP-AChE Tracer (Item No. 401107) with 6 ml of Assay Buffer. Store the reconstituted Cyclic CMP-AChE Tracer at 4°C (*do not freeze!*). It will be stable for three weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Cyclic CMP ELISA Monoclonal Antibody

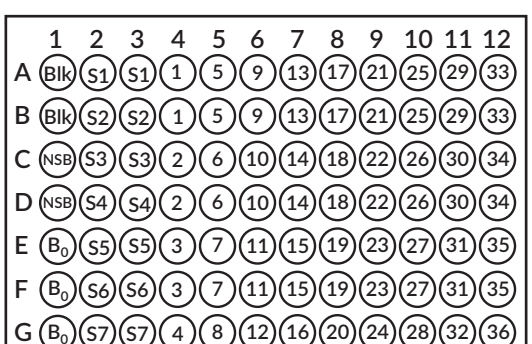
Reconstitute the Cyclic CMP ELISA Monoclonal Antibody (Item No. 401106) with 6 ml of Assay Buffer. Store the reconstituted Cyclic CMP ELISA Monoclonal Antibody at 4°C (*do not freeze!*). It will be stable for at least three weeks. A 20% surplus of antibody has been included to account for any incidental losses.

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 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 at least in duplicate.

A suggested plate format is shown in Figure 3, on page 15. The user may vary the location of wells as necessary for each particular experiment. The plate format provided has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 18 for more details). It is suggested that the contents of each well be recorded on the template sheet provided (see page 26).



Blk = Blank Wells
TA = Total Activity Wells
NSB = Non-Specific Binding Wells
B_0 = Maximum Binding Wells
S1-S8 = Standard Wells
1-36 = Sample Wells

Figure 3. 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. **Assay Buffer**

   Add 100 µl of Assay Buffer to NSB wells. Add 50 µl of Assay Buffer to B_0 wells. If a solution of B-PER™ was used to prepare the standard curve, substitute 50 µl of that solution for Assay Buffer in the NSB and B_0 wells.

2. **Cyclic CMP 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.

4. **Cyclic CMP-AChE Tracer**

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

5. **Cyclic CMP 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 for two hours at room temperature on an orbital shaker.

Development of the Plate

1. Reconstitute 100 dtn vial of Ellman's Reagent (Item No. 400050) with 20 ml of ultrapure water immediately before use.

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 on the same day or multiple assays be run on different days.

2. Empty the wells and rinse five times with ~300 µl per well of Wash Buffer (1X).
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of the reconstituted tracer to the TA wells.
5. Cover the plate with the 96-Well Cover Sheet. 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. (blk subtracted)) in 60-90 minutes at room temperature.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, *etc.*
2. Remove the cover sheet being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings.*
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. (Blk subtracted). The plate should be read when the absorbance of the B_0 wells is in the range of 0.3-2.0 A.U. (Blk subtracted). If the absorbance of the wells exceeds 2.0 A.U., wash the plate, add fresh Ellman's Reagent, and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plots 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/analysisTools/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. 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* cCMP 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 by identifying the %B/B_0 on the standard curve and reading the corresponding values on the x-axis. *NOTE: Remember to account for any dilution of the original sample concentration prior to its addition to the well.* Samples with %B/B_0 values outside of the linear portion of the standard curve should be re-assayed. 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

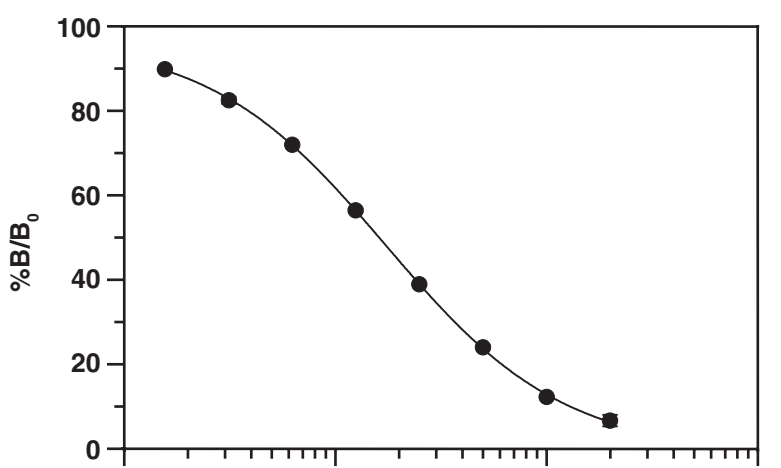
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

cCMP Standards (nM)	Blk-Substracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
NSB	0.008	--	--	--	--
B_0	1.166	1.158	--	--	--
TA	1.435	1.427	--	--	--
20.0	0.087	0.079	6.8	10.2	5.4
10.0	0.152	0.144	12.4	6.6	3.9
5.0	0.287	0.279	24.1	6.4	4.5
2.5	0.460	0.452	39.0	4.4	2.5
1.25	0.663	0.655	56.5	7.1	4.0
0.625	0.842	0.834	72.0	7.7	4.1
0.313	0.964	0.956	82.6	19.4	7.2
0.156	1.049	1.041	89.9	22.5**	9.4

Table 1. Typical results

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

**Evaluate data in this range with caution



Assay Range = 0.156-20 nM
Sensitivity (defined as 80% B/B_0) = 0.39 nM
Mid-point (defined as 50% B/B_0) = 1.62 nM
Lower Limit of Detection (LLOD) = 0.1 nM

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

Figure 4. Typical standard curve

Compound	Cross Reactivity
3'5-cCMP	100%
Cytidine 5' monophosphate	0.02%
Cytidine 3' monophosphate	0.01%
Cytidine	<0.01%
Cytosine	<0.01%
CTP	<0.01%
3'5'-cGMP	<0.01%
3'5'-cAMP	<0.01%
3'5'-cUMP	<0.01%
2'3'-cCMP	<0.01%

Table 2. Cross reactivity of the cCMP ELISA

RESOURCES

Troubleshooting

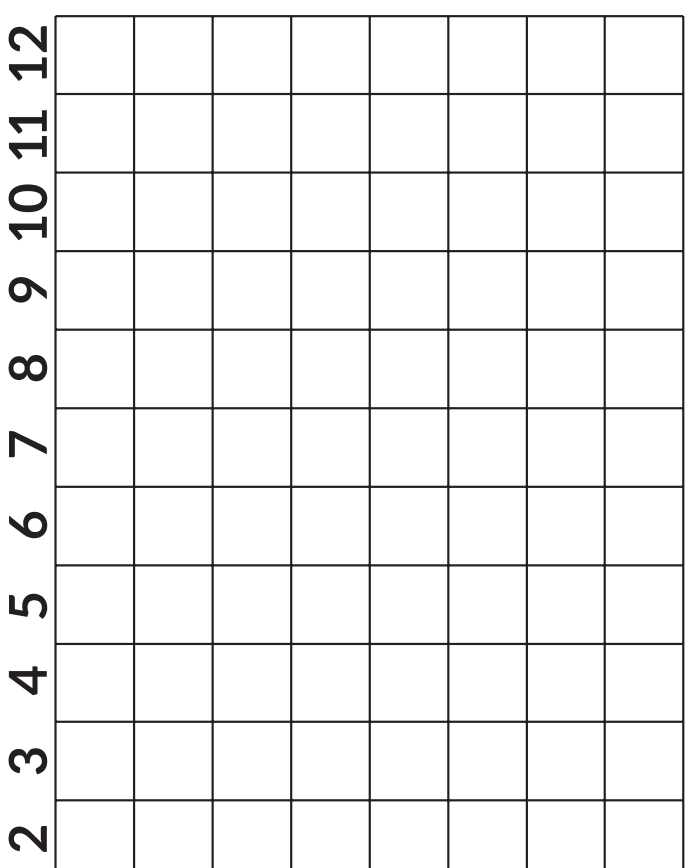
Problem	Possible Causes
Erratic values; dispersion of replicates	A. Trace organic contaminants in the water B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water B. Plate requires additional development time C. Dilution error in preparing reagents
50% B/B_0 is significantly outside of the expected range	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 the sample wells (below the range of the standard curve)	A. AChE inhibitors are present; ensure that the samples and buffers are free of AChE inhibitors B. Sample requires further dilution
Only TA wells develop	A. Trace organic contaminants in the water B. The tracer or antibody was not added to the wells

References

1. Seifert, R. cCMP and cUMP across the tree of life: From cCMP and cUMP generators to cCMP- and cUMP-regulated cell functions. *Handb. Exp. Pharmacol.* **238**, 3-23 (2017).
2. Tal, N., Morehouse, B.R., Millman, A., *et al.* Cyclic CMP and cyclic UMP mediate bacterial immunity against phages. *Cell* **184**, 5728-5739 (2021).
3. Marondedze, C., Wong, A., Thomas, L., *et al.* Cyclic nucleotide monophosphates in plants and plant signaling. *Handb. Exp. Pharmacol.* **238**, 87-103 (2017).
4. Göttle, M., Dove, S., Kees, F., *et al.* Cytidylyl and uridylyl cyclase activity of *Bacillus anthracis* edema factor and *Bordetella pertussis* CyaA. *Biochemistry* **49**, 5494-5503 (2010).
5. Zong, X., Krause, S., Chen, C.-C., *et al.* Regulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channel activity by cCMP. *J. Biol. Chem.* **287(32)**, 26506-26512 (2012).

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Reconstitute and mix	Mix all reagents gently				
Assay Buffer	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--		50 µl
Tracer	--	--	50 µl	50 µl	50 µl
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 Ellman's Reagent	200 µl				
TA- Apply Tracer	--	5 µl	--	--	--
Develop	Seal the plate and incubate for 60-90 minutes at room temperature on an orbital shaker protected from light				
Read	Read absorbance at 405-420 nm				

Table 3. Assay summary



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.