Cyclic di-AMP ELISA Kit

Item No. 501960

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	Storage Temperature
401960	Cyclic di-AMP-HRP Tracer	1 vial/100 dtn	4°C
401962	Cyclic di-AMP ELISA Monoclonal Antibody	1 vial/100 dtn	4°C
401964	Cyclic di-AMP ELISA Standard	1 vial	4°C
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml	4°C
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml	Room temperature
400035	Polysorbate 20	1 vial/3 ml	Room temperature
400008/400009	Goat Anti-Mouse IgG-Coated Plate	1 plate	4°C
400074	TMB Substrate Solution	2 vials/12 ml	4°C
10011355	HRP Stop Solution	1 vial/12 ml	Room temperature
400040	ELISA Tracer Dye	1 ea	Room temperature
400042	ELISA Antiserum Dye	1 ea	Room temperature
400012	96-Well Cover Sheet	1 ea	Room temperature

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.

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 at 450 nm
2. Adjustable pipettes and a repeating pipettor
3. An orbital microplate shaker
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 12)

INTRODUCTION

Background

Cyclic diadenosine monophosphate (cyclic di-AMP) is a second messenger in bacteria involved in a variety of bacterial cellular processes.[1,2] It is comprised of two AMP moieties linked by a 5'-3'-macrocyclic ring and is synthesized from two molecules of ATP by diadenylate cyclase.[3] Cyclic di-AMP is degraded to either the linear 5'-phosphoadenylyl-(3'-5')-adenosine (pApA) dinucleotide or two molecules of AMP by cyclic di-AMP-specific phosphodiesterases (PDEs). Cyclic di-AMP has several mechanisms by which it mediates its effects, including binding to transporters, riboswitches, transcription factors, and enzymes to regulate diverse processes, including sporulation, potassium homeostasis, cell wall homeostasis, biofilm formation, environmental stress management, and virulence.[3,4] For example, the *B. subtilis* DNA integrity scanning protein DisA synthesizes cyclic di-AMP *via* its diadenylate cyclase domain following DNA scanning to signal that the DNA is undamaged and sporulation can occur.[1] Low levels of cyclic di-AMP following DNA scanning inhibit sporulation. Cyclic di-AMP is essential for the viability of the bacteria that produce it but excess accumulation leads to bacterial cell death.[3] In addition to its role in prokaryotes, cyclic di-AMP is detected by the pattern recognition receptor DDX41 in eukaryotes to induce signaling by the transmembrane protein stimulator of interferon genes (STING) following infection, leading to activation of the innate immune system.[2,5,6]

About This Assay

Cayman's Cyclic di-AMP ELISA Kit is a competitive assay that can be used for quantification of cyclic di-AMP in bacterial cell lysates. The assay has a range of 15.6-2,000 pg/ml (23.7-3,038 pM) with an average sensitivity (80% B/B_0) of 52 pg/ml (79 pM) and a Lower Limit of Detection (LLOD) of 21 pg/ml (32 pM).

To convert concentrations from pg/ml, multiply starting concentration in pg/ml by the preferred unit conversion factor in table below.

Starting Unit	Conversion Factor	Final Unit
pg/ml	1.519	fmol/ml
	1.519	pM
	0.05	pg/well
	0.076	fmol/well
	0.506	pM in well
Example: 100 pg/ml * 1.519 (conversion factor) = 151.9 pM		

Table 1. Unit conversion

Principle Of This Assay

This assay is based on the competition between native cyclic di-AMP and a cyclic di-AMP-horseradish peroxidase conjugate (Cyclic di-AMP-HRP Tracer) for a limited amount of Cyclic di-AMP Monoclonal Antibody. Because the concentration of the Cyclic di-AMP-HRP Tracer is held constant while the concentration of native cyclic di-AMP varies, the amount of Cyclic di-AMP-HRP Tracer that is able to bind to the Cyclic di-AMP Monoclonal Antibody will be inversely proportional to the concentration of native cyclic di-AMP in the well. This antibody-cyclic di-AMP 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 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution is added to the well, followed by the HRP Stop Solution. The product of this enzymatic reaction has a distinct yellow color that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of Cyclic di-AMP-HRP Tracer bound to the well, which is inversely proportional to the amount of free cyclic di-AMP present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto [\text{Bound Cyclic di-AMP-HRP Tracer}] \propto 1/[\text{cyclic di-AMP}]$$

A schematic of this process is shown in Figure 1, on page 8.

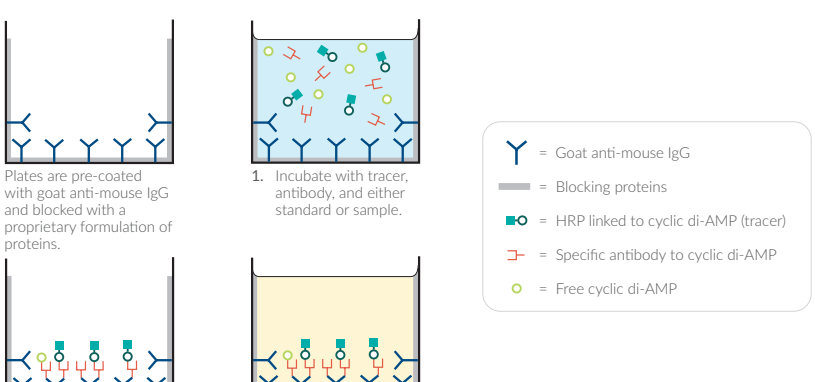


Figure 1. Schematic of the Cyclic di-AMP ELISA Kit

Definition of Key Terms

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 NSB wells.

Total Activity: total enzymatic activity of the cyclic di-AMP-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 particular 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, 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:

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

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 about two months.

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

   Dilute the contents of one vial of Immunoassay Buffer C (10X) (Item No. 401703 with 90 ml of pure 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. These will completely dissolve upon dilution with pure water.*

2. **Wash Buffer Preparation**

   Dilute the contents of one vial of Wash Buffer Concentrate (400X) (Item No. 400062) with pure 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 Polysorbate 20 to an end concentration of 0.5 ml/L. *NOTE: It is normal for the concentrated buffer to contain crystalline salts. These will completely dissolve upon dilution with pure water. 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

This assay has been demonstrated to work with bacterial cell lysates prepared in Bacterial Protein Extraction Reagent (B-PER™) (available from ThermoFisher Scientific) without causing interference in the assay. Other lysis buffers or concentrated lysates may cause interference and require sample purification or a minimum dilution determined by the end user outlined below. Please read this section thoroughly before beginning the assay.

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples should be assayed immediately after collection; samples that cannot be asayed immediately should be stored at -80°C.

Testing for Interference

To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 90 pg/ml and 400 pg/ml (*i.e.*, between 25-70% B/B_0, which is 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 cyclic di-AMP concentration, 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 end user and tested for compatability in the assay.

Sample Matrix Properties

Linearity

To assess dilutional linearity, *E. coli* was lysed in B-PER™, spiked with cyclic di-AMP, serially diluted with Immunoassay Buffer C (1X), and evaluated for linearity using the Cyclic di-AMP ELISA Kit. The results are shown in the table below.

Dilution Factor	Concentration (pg/ml)	Dilutional Linearity (%)
60	11,001	100
120	11,124	101
240	11,920	108

Table 2. Dilutional linearity of *E. coli* lysates

Spike and Recovery

E. coli was lysed in B-PER™, spiked with different amounts of cyclic di-AMP, serially diluted in Immunoassay Buffer C (1X), and analyzed using the Cyclic di-AMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

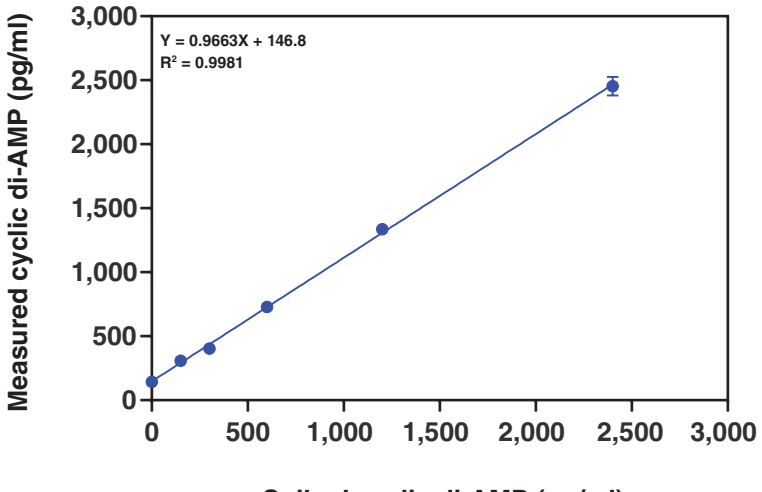


Figure 2. Spike and recovery in *E. coli* lysates

Spike Concentration (pg/ml)	Measured Concentration (pg/ml)	% Recovery
0	142.9	
150	308.6	110
300	403.9	87.1
600	728.9	97.7
1,200	1,345	100
2,400	2,453	96.3

Table 3. Spike and recovery in *E. coli* lysates

Parallelism

To assess parallelism, *E. coli* was lysed in B-PER™ and assayed at multiple dilutions using the Cyclic di-AMP ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below.

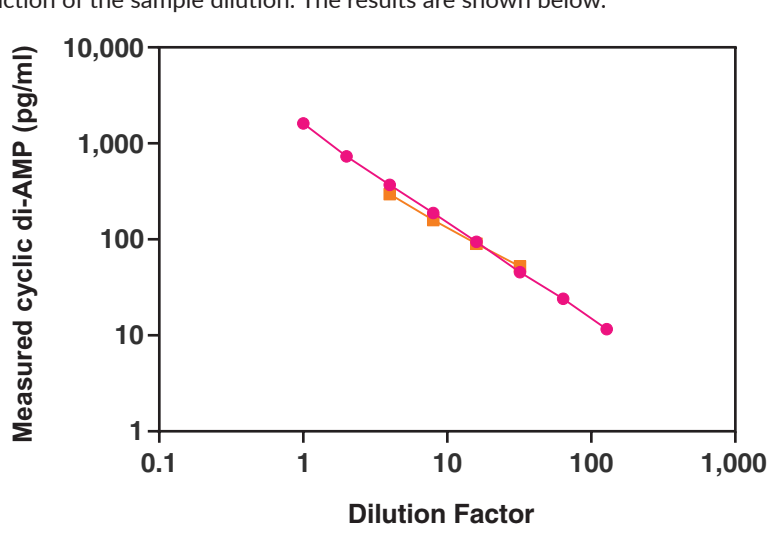


Figure 3. Parallelism in the Cyclic di-AMP ELISA Kit

Preparation of Assay-Specific Reagents

Cyclic di-AMP ELISA Standard

Resconstitute the lyophilized Cyclic di-AMP ELISA Standard (Item No. 401964) in 0.75 ml of Immunoassay Buffer C (1X). The concentration of this solution (the bulk standard) is 20 ng/ml. It will be stable for at least 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 900 µl Immunoassay Buffer C (1X) to tube #1 and 500 µl Immunoassay Buffer C (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (20 ng/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 two hours.

Figure 4. Preparation of the cyclic di-AMP standards

Cyclic di-AMP-HRP Tracer

Dilute the Cyclic di-AMP-HRP Tracer (Item No. 401960) with 5 ml of Immunoassay Buffer C (1X). Store the diluted Cyclic di-AMP-HRP Tracer at 4°C (*do not freeze!*) and use within 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 four weeks at 4°C.*

Cyclic di-AMP Monoclonal Antibody

The Cyclic di-AMP Monoclonal Antibody (Item No. 401962) is ready to use as supplied. Store the Cyclic di-AMP Monoclonal Antibody at 4°C (*do not freeze!*). A 20% surplus of antibody has been included to account for any incidental losses.

Antibody Dye Instructions (optional)

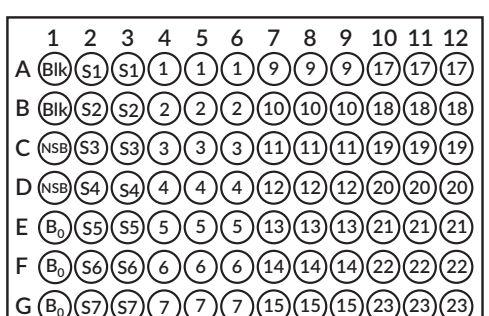
This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the supplied antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). *NOTE: Do not store antibody with dye for more than four weeks at 4°C.*

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 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 two 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, 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 32 for more details). We suggest recording the contents of each well on the template sheet provided (see page 33).



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. **Immunoassay Buffer C**

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

2. **Cyclic di-AMP 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 sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Cyclic di-AMP-HRP Tracer**

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

5. **Cyclic di-AMP 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 Plate Cover Sheet (Item No. 400012) and incubate 2 hours at room temperature on an orbital shaker.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Add 5 µl of the diluted tracer to the TA wells.
4. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes.
5. Remove the plate cover 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 appropiate 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 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. Erratic absorbance values could indicate the presence of organic solvents in the buffer or other technical problems (see page 30 for Troubleshooting). 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* cyclic di-AMP 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 greater than 70% or less than 25% 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 it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

Representative Data

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.

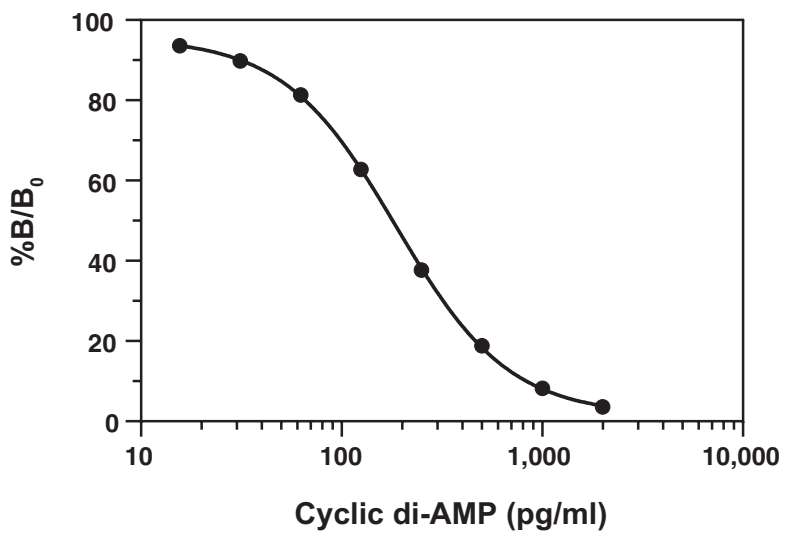
Absorbance at 450 nm (30 minutes)

Analyte Standards (pg/ml)	Blank-subtracted Absorbance	NSB-corrected Absorbance	%B/B_0	%CV* Intra-assay Precision	%CV* Inter-assay Precision
NSB	0.002				
B_0	1.166	1.164			
2,000.0	0.044	0.042	3.6	13.0	7.6
1,000.0	0.097	0.095	8.2	2.7	1.6
500.0	0.221	0.219	18.8	2.0	1.8
250.0	0.440	0.438	37.7	2.0	1.1
125.0	0.732	0.730	62.8	4.7	1.4
62.5	0.948	0.946	81.3	9.6	2.4
31.3	1.046	1.044	89.8	22.7**	5.3
15.6	1.090	1.088	93.6	30.0**	14.1
TA	1.252				

Table 4. 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 = 15.6-2,000 pg/ml (23.7-3,038 pM)
Sensitivity (defined as 80% B/B_0) = 65.5 pg/ml (99.5 pM)
Mid-point (defined as 50% B/B_0) = 180.2 pg/ml (273.7 pM)
Lower Limit of Detection (LLOD) = 20.7 pg/ml (31.4 pM)

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

Figure 6. Typical standard curve

Precision:

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

Matrix Control (pg/ml)	%CV
35,167	2.3
4,355	2.9
448	3.6

Table 5. Intra-assay Precision

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

Matrix Control (pg/ml)	%CV
36,153	4.1
5,233	4.4
464	4.9

Table 6. Inter-assay Precision

Cross Reactivity:

Compound	Cross Reactivity
Cyclic di-AMP	100%
c[A(3',5')pA(3',5')pG(3',5')p]	0.015%
pApA	0.013%
pG(2',5')pA	<0.01%
pApG	<0.01%
c-hexa-AMP	<0.01%
c-ApUp	<0.01%
c-tetra-AMP	<0.01%
3'3'-cGAMP	<0.01%
Cyclic di-GMP	<0.01%

Table 7. Cross reactivity of the Cyclic di-AMP ELISA Kit

RESOURCES

Troubleshooting

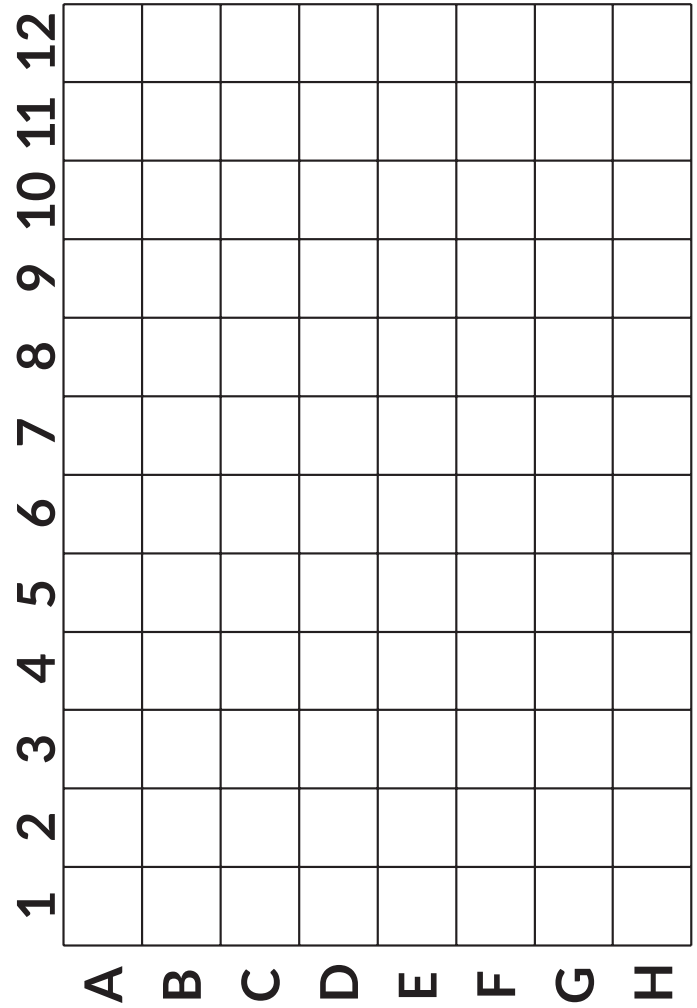
Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>0.100 O.D.)	A. Poor washing; ensure proper washing is used B. Exposure of NSB wells to specific antibody
Very low B_0	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response curve)	Standard is degraded or contaminated
Analysis of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present; consider an alternative sample preparation

References

1. Römling, U., Galperin, M.Y., and Gomelsky, M. Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* **77(1)**, 1-52 (2013).
2. Jenal, U., Reinders, A., and Lori, C. Cyclic di-GMP: Second messenger extraordinaire. *Nat. Rev. Microbiol.* **15(5)**, 271-284 (2017).
3. Commichau, F.M., Heidemann, J.L., Ficner, R., *et al.* Making and breaking of an essential poison: The cyclases and phosphodiesterases that produce and degrade the essential second messenger cyclic di-AMP in bacteria. *J. Bacteriol.* **201(1)**, e00462-18 (2019).
4. Fahmi, T., Faozia, S., Port, G.C., *et al.* The second messenger c-di-AMP regulates diverse cellular pathways involved in stress response, biofilm formation, cell wall homeostasis, SpeB expression, and virulence in *Streptococcus pyogenes*. *Infect. Immun.* **87(6)**, e00147-19 (2019).
5. Sauer, J.D., Sotelo-Troha, K., von Moltke, J., *et al.* The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the *in vivo* interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect. Immun.* **79(2)**, 688-694 (2011).
6. Parvatiyar, K., Zhang, Z., Teles, R.M., *et al.* DDX41 recognizes bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nat. Immunol.* **13(12)**, 1155-1161 (2012).

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Plate Preparation	Wash 5 x ~300 µl with Wash Buffer (1X)				
Reconstitute and Mix	Mix all reagents gently				
Immunoassay Buffer C (1X)	--	--	100 µl	50 µl	--
Standards/Sample	--	--	--	--	50 µl
Cyclic di-AMP-HRP Tracer	--	--	50 µl	50 µl	50 µl
Cyclic di-AMP ELISA Monoclonal Antibody	--	--	--	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate for 2 hours at room temperature on an orbital shaker				
Aspirate and Wash	Aspirate wells and wash 5 x ~300 µl with Wash Buffer (1X)				
Apply TMB Substrate	175 µl	175 µl	175 µl	175 µl	175 µl
TA - Apply Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 30 minutes at room temperature on orbital shaker, protected from light				
Apply HRP Stop Solution	Apply HRP Stop Solution				
Read	Read optical density at 450 nm				

Table 6. 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.