Cyclic tetra-AMP ELISA Kit

Item No. 502350

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size	Storage Temperature
400769	Cyclic tetra-AMP-HRP Tracer	1 vial/100 dtn	-20°C
400770	Cyclic tetra-AMP ELISA Antiserum	1 vial/100 dtn	-20°C
400771	Cyclic tetra-AMP ELISA Standard	1 vial/0.5 ml	-20°C
400004/ 400006	Mouse Anti-Rabbit IgG-Coated Strip Plate/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 Cyclic tetra-AMP 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 11).

INTRODUCTION

Background

Cyclic tetra-adenosine monophosphate (cyclic tetra-AMP) is a second messenger found in bacteria and archaea that is involved in type III CRISPR immunity against invasive genetic elements from viruses or bacteriophages.[1,2] It is synthesized by the Palm domain of Cas10 from ATP following binding of target nucleic acids. Cyclic tetra-AMP then binds to proteins containing CRISPR-associated Rossman fold (CARF) domains to activate type III-A (Csm) or type III-B (Csr) effector complexes with ribonuclease and DNase activities.[1-3] It also binds to the CalpL, a protease that contains a SMODS-associated and fused to various effector domain (SAVED) instead of a CARF domain and is involved in transcription regulation.[4] Cyclic tetra-AMP is degraded by host and viral ring nucleases to prevent cell dormancy and death or neutralize the defense system, respectively.[2,5,6] Mutations in the cyclic tetra-AMP-binding pocket of cyclic-oligoadenylate-activated single-stranded ribonuclease and single-stranded deoxyribonuclease 1 (Card1) prevent immunity development in dsDNA phage-infected *S. aureus*.[3]

About This Assay

Cayman's Cyclic tetra-AMP ELISA Kit is a competitive assay that can be used for the quantification of cyclic tetra-AMP in bacterial cell lysates. The assay has a range of 0.080-500 nM, an average sensitivity (80% B/B_0) of 0.86 nM, and a lower limit of detection (LLOD) of 0.11 nM.

Principle of the Assay

This assay is based on the competition between free cyclic tetra-AMP and a cyclic tetra-AMP-HRP conjugate (cyclic tetra-AMP-HRP Tracer) for a limited number of cyclic tetra-AMP polyclonal antibody binding sites. Because the concentration of the cyclic tetra-AMP-HRP Tracer is held constant while the concentration of free cyclic tetra-AMP varies, the amount of cyclic tetra-AMP-HRP Tracer that is able to bind to the cyclic tetra-AMP polyclonal antibody will be inversely proportional to the concentration of free cyclic tetra-AMP in the well. This antibody-cyclic tetra-AMP complex binds to mouse anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and TMB Substrate Solution (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 cyclic tetra-AMP-HRP Tracer bound to the well, which is inversely proportional to the amount of free cyclic tetra-AMP present in the well during the incubation, as described in the equation:

$$\text{Absorbance} \propto \text{[bound cyclic tetra-AMP-HRP tracer]} \propto 1/\text{[cyclic tetra-AMP]}$$

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

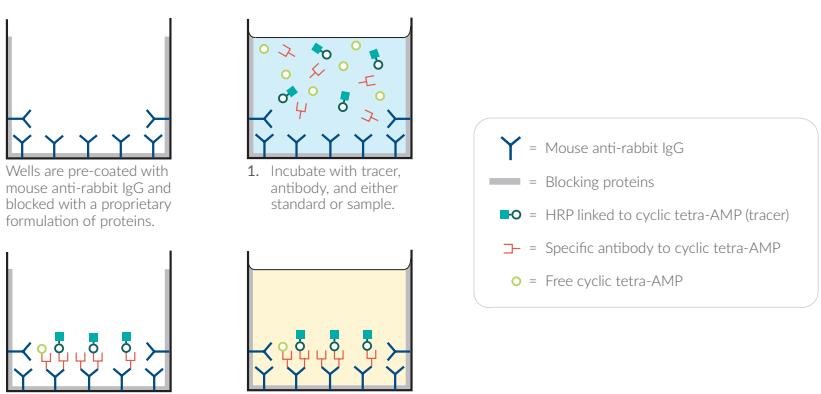


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and 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 cyclic tetra-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 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 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:

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

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 at least 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 Concentrate (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 purified bacterial cell lysates prepared in ultrapure water or B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific). Other sample types, lysis buffers, or concentrated lysates should be tested for interference to evaluate the need for sample purification or a minimum dilution determined by the user 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 cyclic tetra-AMP concentration, no interfering substances are present. If interference is seen, alternative methods for sample preparation, purification, or required minimum dilution should be tested by the user for compatibility in the assay.

Bacterial Cell Lysates

Lysates Prepared in B-PER™ Bacterial Protein Extraction Reagent

Weigh and resuspend pelleted bacterial cells in B-PER™ Bacterial Protein Extraction Reagent following the manufacturer's protocol. Proceed to the purification section for best results. Lysates should be purified immediately or stored at -20°C.

Lysates Prepared 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 with liquid nitrogen and 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 5 minutes at 4°C and transfer the supernatant to a clean tube. Proceed to the purification section for best results. Lysates should be purified immediately or stored at -20°C.

Bacterial Cell Lysate Purification

It is recommended that bacterial cell lysates be purified prior to use in the assay using the protocol below. Alternative protocols may be used based on the experimental requirements, sample type, and the user's expertise.

1. Incubate the lysates at 95°C for 15 minutes.
2. Centrifuge at 21,000 x g for 5 minutes at 4°C to pellet debris.
3. Transfer the supernatant to a new tube.
4. Samples that cannot be assayed immediately should be stored at -20°C.

NOTE: Different species of bacteria will have various amounts of interfering substances. The minimum dilution for each purified bacteria cell lysate must be determined by the user.

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

Sample Matrix Properties

Parallelism

To assess parallelism, various bacterial cell lysates were processed as described in the **Sample Preparation** section (see page 11), serially diluted with Immunoassay Buffer C (1X), and evaluated using the Cyclic tetra-AMP ELISA Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

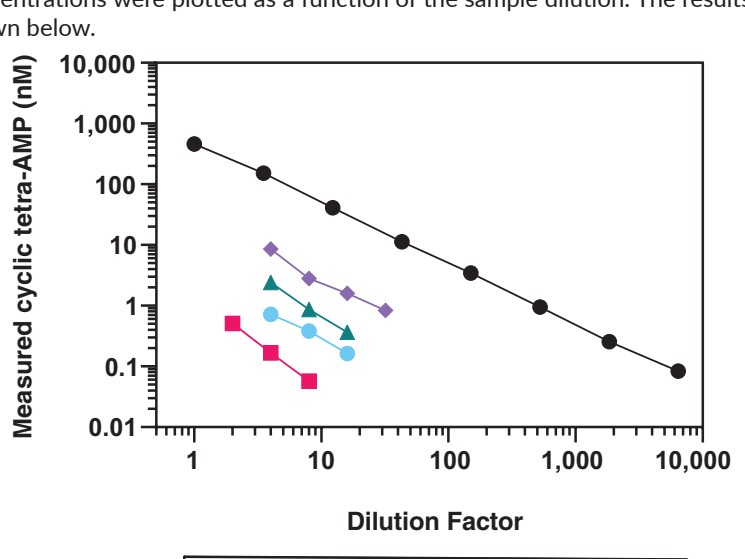


Figure 2. Parallelism of various bacterial cell lysates in the Cyclic tetra-AMP ELISA

Spike and Recovery

E. coli cell lysates prepared in B-PER™ were spiked with cyclic tetra-AMP, purified as described in the Sample Preparation section, serially diluted with Immunoassay Buffer C (1X), and evaluated using the Cyclic tetra-AMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

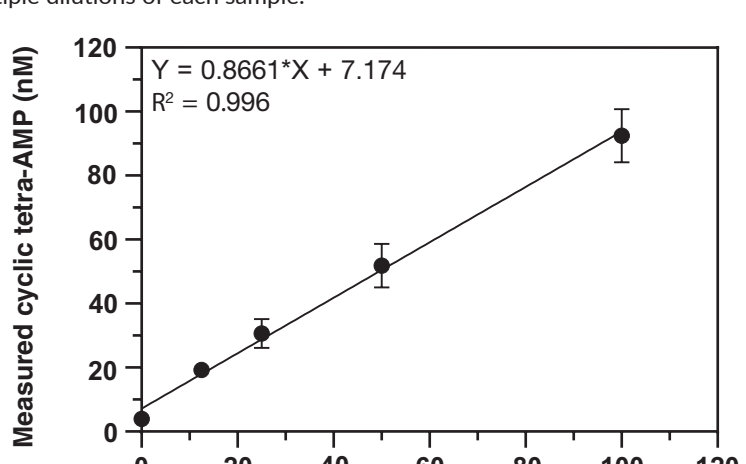


Figure 3. Spike and recovery of cyclic tetra-AMP in *E. coli* cell lysates

Linearity

E. coli cell lysates prepared in B-PER™ were spiked with cyclic tetra-AMP, purified as described in the Sample Preparation section, serially diluted with Immunoassay Buffer C (1X), and evaluated for linearity using the Cyclic tetra-AMP ELISA Kit.

Dilution Factor	Measured Concentration (nM)	Linearity (%)
Spiked with 10,000 nM cyclic tetra-AMP		
700	8,444	100
1,400	8,251	97.7
2,800	8,532	101
5,600	9,346	111
Spiked with 1,250 nM cyclic tetra-AMP		
50	1,371	100
100	1,244	90.7
200	1,252	91.3
400	1,261	92.0

Table 1. Linearity in *E. coli* cell lysates

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

Cyclic tetra-AMP ELISA Standard

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl of Immunoassay Buffer C (1X) to tube #1 and 500 µl of Immunoassay Buffer C (1X) to tubes #2-8. Equilibrate a pipette tip by repeatedly filling and expelling the tip with the Cyclic tetra-AMP ELISA Standard (Item No. 400771) several times. Using the equilibrated pipette tip, transfer 100 µl of the Cyclic tetra-AMP ELISA Standard to tube #1 and mix thoroughly. Serially dilute the standard by removing 200 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 200 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. The unused and undiluted cyclic tetra-AMP standard will be stable for at least 3 weeks if stored at 4°C.

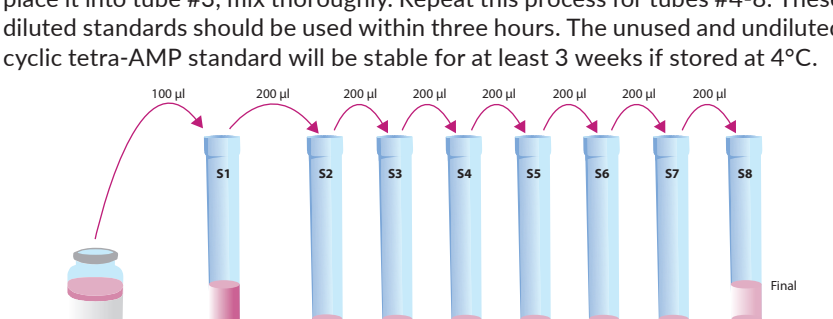


Figure 4. Preparation of the cyclic tetra-AMP standards

Cyclic tetra-AMP-HRP Tracer

Dilute the Cyclic tetra-AMP-HRP Tracer (Item No. 400769) with 5 ml of Immunoassay Buffer C (1X). Store the diluted Cyclic tetra-AMP-HRP Tracer at 4°C (*do not freeze!*). It will be stable for 4 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 two weeks at 4°C.*

Cyclic tetra-AMP ELISA Antiserum

Dilute the Cyclic tetra-AMP ELISA Antiserum (Item No. 400770) with 5 ml of Immunoassay Buffer C (1X). Store the diluted Cyclic tetra-AMP ELISA Antiserum at 4°C (*do not freeze!*). It will be stable for at least 4 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 antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the diluted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum). *NOTE: Do not store antiserum with dye for more than two 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 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 21 for more details). We suggest you record the contents of each well on the template sheet provided (see page 29).

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

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.

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

Pre-wash the plate.

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. **Cyclic tetra-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 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. **Cyclic tetra-AMP-HRP Tracer**

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

5. **Cyclic tetra-AMP ELISA Antiserum**

   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 20 µl of the previously diluted tracer with 20 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.
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* cyclic tetra-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 using the equation obtained from the standard curve plot. *NOTE: Remember to account for any dilution of the sample concentration 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

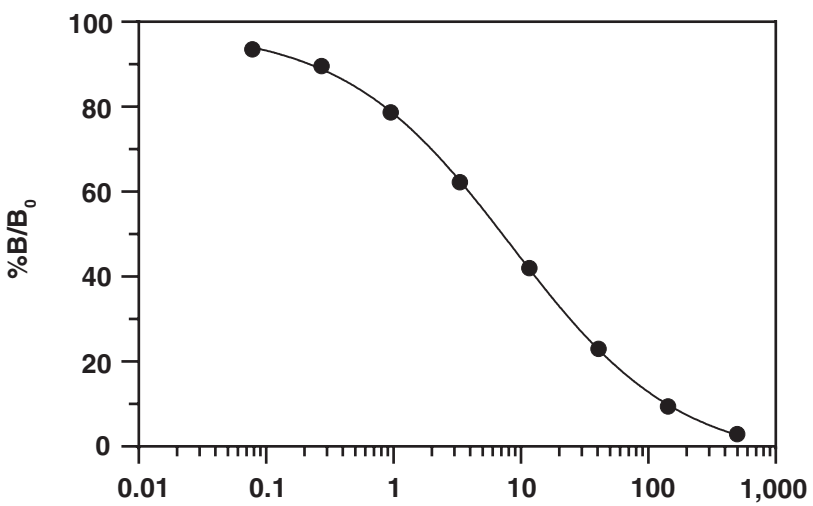
The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples.

Absorbance at 450 nm (30 minutes)

Cyclic tetra-AMP Standards (nM) and Controls	Blk-Subtracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
NSB	0.001	--	--	--	--
B_0	1.092	1.091	--	--	--
TA	1.184	--	--	--	--
500	0.032	0.031	2.8	3.8	2.2
142.9	0.104	0.103	9.4	3.1	1.3
40.8	0.252	0.251	23.0	3.9	1.1
11.7	0.459	0.458	42.0	3.0	1.0
3.3	0.679	0.678	62.1	4.6	1.0
0.95	0.859	0.858	78.6	10.4	3.2
0.27	0.978	0.977	89.6	14.5	9.5
0.08	1.021	1.020	93.5	17.2	9.9

Table 2. Typical results

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



Assay Range = 0.080-500 nM
Sensitivity (defined as 80% B/B_0) = 0.853 nM
Mid-point (defined as 50% B/B_0) = 7.168 nM
Lower Limit of Detection (LLOD) = 0.112 nM

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

Figure 6. 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 cyclic tetra-AMP (nM)	%CV
Control 1	4,480	6.9
Control 2	433	10.4
Control 3	3.6	11.3

Table 3. Intra-assay precision

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

Matrix Control	Measured cyclic tetra-AMP (nM)	%CV
Control 1	4,120	5.9
Control 2	380	6.4
Control 3	3.9	12.3

Table 4. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity	Compound	Cross Reactivity
Cyclic tetra-AMP	100%	3'2'-cGAMP	<0.01%
Cyclic hexa-AMP	0.562%	2'3'-cGAMP	<0.01%
AMP	<0.01%	pApA	<0.01%
ADP	<0.01%	pG(2'5')pA	<0.01%
ATP	<0.01%	pApG	<0.01%
Adenine	<0.01%	c[A(3'5')pA(3'5')pG(3'5')p] (cAAG)	<0.01%
Adenosine	<0.01%	Cyclic ApUp	<0.01%
cAMP	<0.01%	cGMP	<0.01%
Cyclic di-AMP	<0.01%	pGpG	<0.01%
Cyclic di-GMP	<0.01%	GDP	<0.01%
2'3'-cAMP	<0.01%	Guanosine	<0.01%
2'3'-cGMP	<0.01%	GTP	<0.01%
2'2'-cGAMP	<0.01%	GMP	<0.01%
3'3'-cGAMP	<0.01%		

Table 5. Cross reactivity of the Cyclic tetra-AMP ELISA

RESOURCES

References

1. Kazlauskiene, M., Kostiuk, G., Venclovas, Č., *et al.* A cyclic oligonucleotide signaling pathway in type III CRISPR-Cas systems. *Science* **357(6351)**, 605-609 (2017).
2. Athukoralage, J.S., Rouillon, C., Graham, S., *et al.* Ring nucleases deactivate type III CRISPR ribonucleases by degrading cyclic oligoadenylate. *Nature* **562(7762)**, 277-280 (2018).
3. Rostøl, J.T., Xie, W., Kuryavyi, V., *et al.* The Card1 nuclease provides defence during type III CRISPR immunity. *Nature* **590(7847)**, 624-629 (2021).
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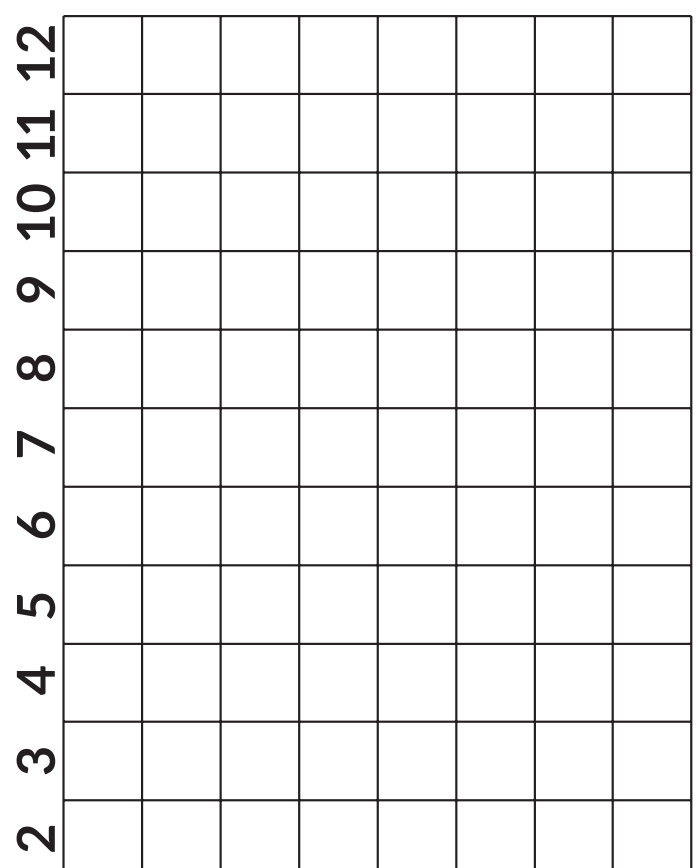
Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Wash	Pre-wash the plate				
Dilute and Mix	Mix all reagents gently				
Immunoassay Buffer C (1X)	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--	--	50 µl
Cyclic tetra-AMP-HRP Tracer	--	--	50 µl	50 µl	50 µl
Cyclic tetra-AMP ELISA Antiserum	--	--	--	50 µl	50 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 diluted 1:2	--	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 replicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0) after blank substraction	A. Poor washing B. Exposure of NSB wells to specific antiserum
Very low B_0, with signal <0.3	A. Trace organic contaminants in the water source B. Dilution error in preparing reagents
Low sensitivity (shift in dose-response)	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; purification is needed
Low signal in sample wells (below range of standard curve)	A. HRP inhibitors present: insure that 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

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.