2’3’-cGAMP ELISA Kit

Item No. 501700

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 wells Quantity/Size
401702	2'3'-cGAMP ELISA Polyclonal Antiserum	1 vial/100 dtn
401700	2'3'-cGAMP-HRP Tracer	1 vial/100 dtn
401704	2'3'-cGAMP ELISA Standard	1 vial
401703	Immunoassay Buffer C Concentrate (10X)	1 vial/10 ml
400062	Wash Buffer Concentrate (400X)	1 vial/5 ml
400035	Polysorbate 20	1 vial/3 ml
400004/400006	Mouse Anti-Rabbit IgG-Coated Plate	1 plate
400074	TMB Substrate Solution	2 vials/12 ml
10011355	HRP Stop Solution	1 vial/12 ml
400040	ELISA Tracer Dye	1 ea
400042	ELISA Antiserum Dye	1 ea
400012	96-Well Cover Sheet	1 ea

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. An orbital plate 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

Cyclic GMP-AMP synthase (cGAS) is a cytosolic DNA sensor that detects the presence of nucleic acids in the cytosol of mammalian cells as an indicator of bacterial or viral infection.[1] cGAS catalyzes the synthesis of a second messenger, 2'3'-cGAMP, from cytosolic ATP and GTP in response to dsDNA binding. 2'3'-cGAMP then binds tightly to the adaptor protein stimulator of interferon genes (STING), resulting in the recruitment of TBK1 and subsequent IRF3 phosphorylation.[2] IRF3 induces the transcription and translation of type I interferons.[3] Activation of cGAS and the production of 2'3'-cGAMP are important in host defense but also may play role in autoimmune or inflammatory diseases. Modulation of cGAS activity, with subsequent inhibition or induction of 2'3'-cGAMP formation, is an active target of pharmacological intervention.[4]

About This Assay

Cayman's 2'3'-cGAMP ELISA Kit is a competitive assay that can be used for quantification of 2'3'-cGAMP in cell lysates, plasma, serum, and tissue samples. This assay has a range of (6.1 pg/ml - 100 ng/ml (9 pM - 148.3 nM), with a midpoint (50% B/B_0) of approximately 900 pg/ml (1,335 pM), and a sensitivity (80% B/B_0) of approximately 85 pg/ml (126 pM).

To convert concentrations from pg/ml, multiply starting concentration in pg/ml by the preferred unit conversion factor in Table 1 below.

Starting Unit	Conversion Factor	Final Unit
pg/ml	1.483	fmol/ml
	1.483	pM
	0.05	pg/well
	0.0742	fmol/well
	0.494	pM (in well)
Example: 100 pg/ml * 1.483 (conversion factor) = 148.3 fmol/ml		

Table 1. Unit conversion

Principle of the Assay

This assay is based on the competition between native 2'3'-cGAMP and a 2'3'-cGAMP-horseradish peroxidase (HRP) conjugate (2'3'-cGAMP-HRP Tracer) for a limited amount of 2'3'-cGAMP Polyclonal Antiserum. Because the concentration of the 2'3'-cGAMP-HRP Tracer is held constant while the concentration of native 2'3'-cGAMP varies, the amount of 2'3'-cGAMP-HRP Tracer that is able to bind to the 2'3'-cGAMP Polyclonal Antiserum will be inversely proportional to the concentration of native 2'3'-cGAMP in the well. This antibody-2'3'-cGAMP 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 (which contains the substrate to HRP) is added to the well, followed by the HRP Stop Solution. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 2'3'-cGAMP-HRP Tracer bound to the well, which is inversely proportional to the amount of free 2'3'-cGAMP present in the well during the incubation as described in the equation:

$$\text{Absorbance} \propto [\text{Bound 2'3'-cGAMP-HRP Tracer}] \propto 1/[\text{2'3'-cGAMP}]$$

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

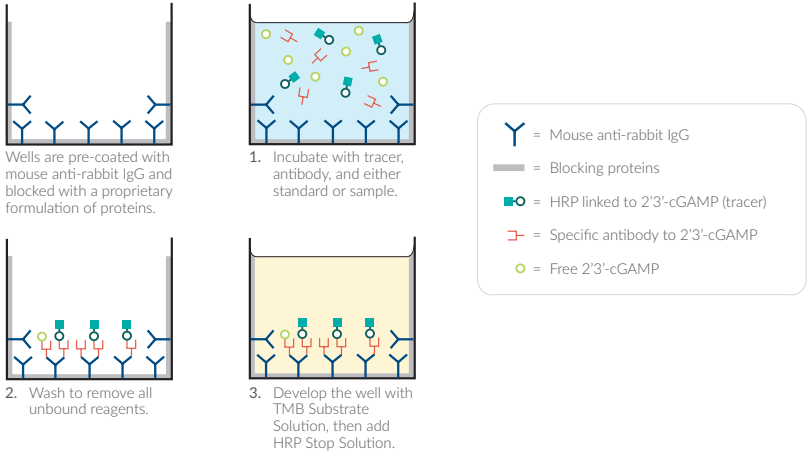


Figure 1. Schematic of the ELISA

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 HRP-linked tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antiserum 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 antiserum 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, 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:

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

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 should be stable for approximately two months. NOTE: It is normal for the concentrated buffers to contain crystalline salts. 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 the vial of Wash Buffer Concentrate (400X) (Item No. 400062) to a total volume of 2 L with ultrapure water 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: 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 human plasma and serum, tissues prepared in Tissue Protein Extraction Reagent (T-PER™), and cell lysates prepared in Mammalian Protein Extraction Reagent (M-PER™). Both T-PER™ and M-PER™ are available from ThermoFisher Scientific. Other lysis buffers or concentrated lysates may cause interference and require sample purification or 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 assayed immediately should be stored at -80°C.
- Samples of rabbit origin may contain antibodies that interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.

Testing for Interference

To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample within the linear portion of the standard curve. If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 2'3'-cGAMP 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 compatibility in the assay.

Sample Matrix Properties

Linearity

To assess dilutional linearity, THP-1 cell lysate prepared in M-PER™ was spiked with 12,500 and 50,000 pg/ml of 2'3'-cGAMP and analyzed at multiple dilutions using the 2'3'-cGAMP ELISA Kit. The results are shown in the table below.

Dilution	Concentration (pg/ml)	Dilutional Linearity (%)
Spike: 12,500 pg/ml		
10	15,887	100
20	13,837	87.1
40	13,832	87.1
80	15,636	98.4
Spike: 50,000 pg/ml		
50	58,921	100
100	57,119	96.9
200	48,995	83.2
400	47,868	81.2

Table 2. Dilutional linearity of THP-1 cell lysate

Spike and Recovery

Human plasma (EDTA) and serum, mouse lung, brain, and liver tissue (prepared in T-PER™), and THP-1 cell lysate (prepared in M-PER™) were spiked with different concentrations of 2'3'-cGAMP, serially diluted with Immunoassay Buffer C (1X), and analyzed using the 2'3'-cGAMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

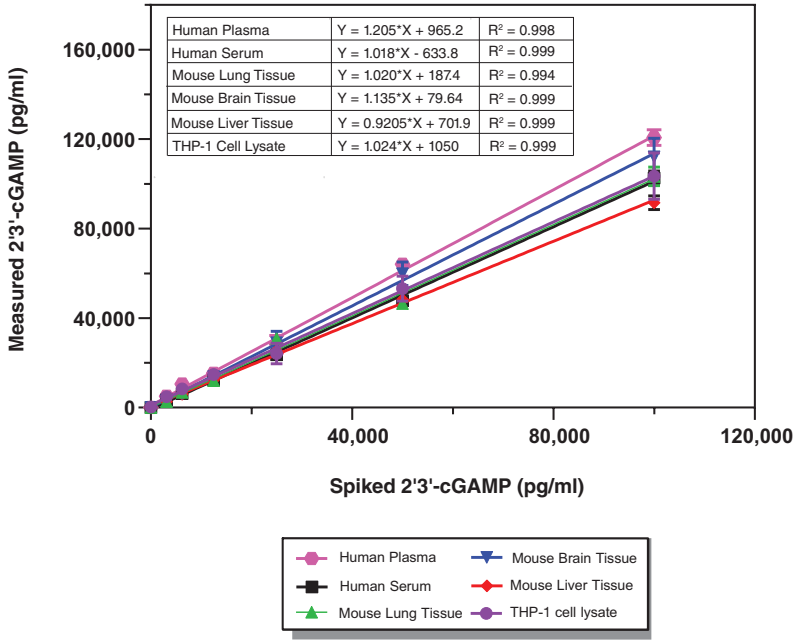


Figure 2. Spike and recovery of 2'3'-cGAMP

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

2’3’-cGAMP ELISA Standard

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 all of the tubes. Transfer 100 µl of the 2’3’-cGAMP ELISA Standard (Item No. 401704) to tube #1 and mix thoroughly. Serially dilute the standard by removing 300 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 300 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours. *NOTE: If other units are preferred, please see Table 1 on page 7 for unit conversion.*

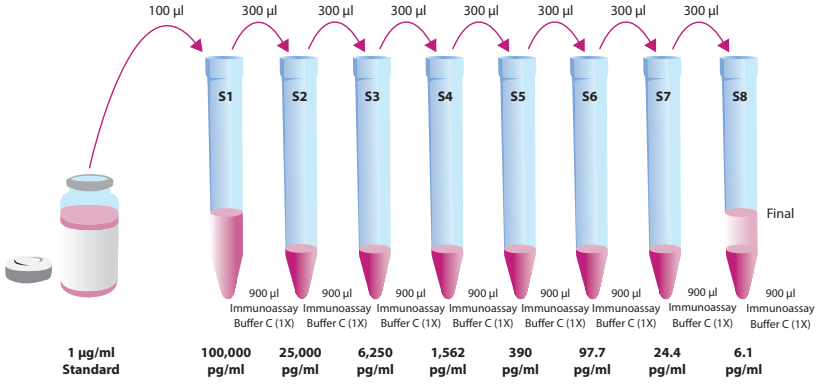


Figure 3. Preparation of the 2’3’-cGAMP Standards

2’3’-cGAMP-HRP Tracer (1X)

Dilute the 2’3’-cGAMP-HRP Tracer (Item No. 401700) with 5 ml of Immunoassay Buffer C (1X). Store the 2’3’-cGAMP-HRP Tracer (1X) at 4°C (*do not freeze!*) and use within two weeks. A 20% surplus of tracer has been included to account for any incidental losses.

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: Store tracer with dye at 4°C where it will be stable for at least once week.*

2’3’-cGAMP ELISA Polyclonal Antiserum (1X)

The 2’3’-cGAMP ELISA Polyclonal Antiserum (Item No. 401702) is ready to use as supplied. Store the 2’3’-cGAMP ELISA Polyclonal Antiserum at 4°C and use within six months. A 20% surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

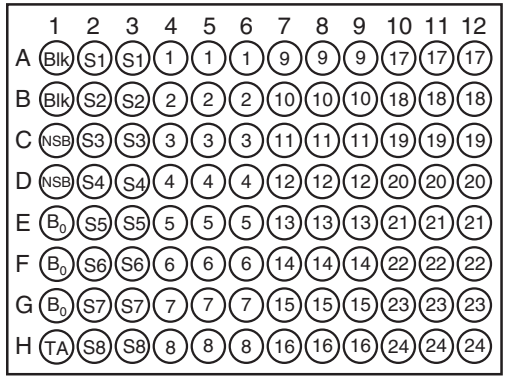
This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum). *NOTE: Store antiserum with dye at 4°C where it will be stable for at least once week.*

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: Do not store strips after pre-washing. If you do not need to use all the strips at once, place the unwashed/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, and an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration 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 4, on page 19. 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 22 for more details). We suggest you record the contents of each well on the template sheet provided (see page 29).



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

Figure 4. 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.

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

2. **2'3'-cGAMP ELISA Standard**

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

3. **Samples**

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

4. **2'3'-cGAMP-HRP Tracer**

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

5. **2'3'-cGAMP ELISA Polyclonal Antiserum**

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

Incubation of the Plate

Cover each plate with the 96-Well Cover Sheet (Item No. 400012) and incubate overnight at 4°C. Alternatively, the assay may be incubated for two hours at room temperature with shaking. The sensitivity and signal are consistent for both incubation methods.

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 2'3'-cGAMP-HRP Tracer (1X) 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 plate cover being careful to keep TMB Substrate Solution from spashing 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'-cGAMP 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.

NOTE: If other units are preferred, please see Table 1 on page 7 for unit conversion.

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 it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

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.

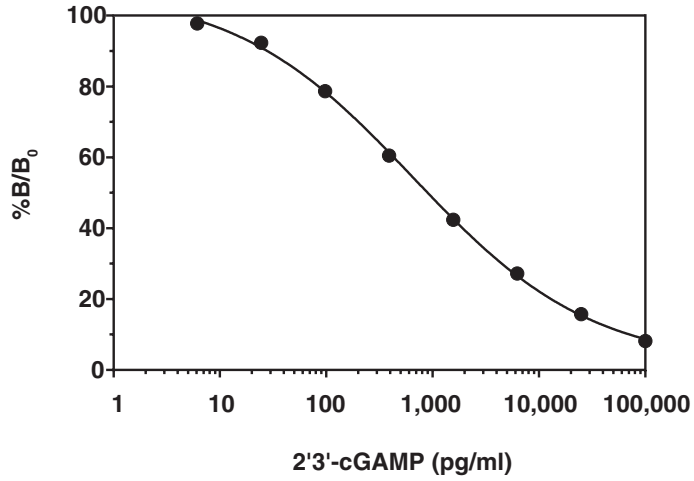
Absorbance at 450 nm - 30 minute development - overnight incubation

2'3'-cGAMP Standards (pg/ml)	Blank-Subtracted Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
NSB	0.004				
B_0	1.196	1.192			
100,000	0.102	0.098	8.2	7.7	10.5
25,000	0.193	0.189	15.8	8.1	5.1
6,250	0.330	0.326	27.2	11.9	7.0
1,562	0.509	0.505	42.4	13.5	5.7
390.6	0.725	0.721	60.5	18.6	7.3
97.7	0.942	0.938	78.7	16.5	14.2
24.4	1.103	1.099	92.3	30.3**	30.5**
6.1	1.170	1.166	97.8	62.9**	35.2**
TA	1.104				

Table 3. 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 = 6.1 pg/ml-100 ng/ml (9 pM-148.3 nM)
Sensitivity (defined as 80% B/B_0) = 85.3 pg/ml (126.5 pM)
Mid-point (defined as 50% B/B_0) = 907.7 pg/ml (1,346 pM)
Lower Limit of Detection (LLOD) = 9.6 pg/ml (14.2 pM)

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

Figure 5. Typical standard curve

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (spiked M-PER™) in a single assay.

Matrix Control (pg/ml)	%CV
7,165	8.4
828	14.5
102	21.3

Table 4. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (spiked M-PER™) in 11 separate assays on different days.

Matrix Control (pg/ml)	%CV
7,530	9.6
811	9.6
72.2	18.7

Table 5. Inter-assay precision

Cross Reactivity:

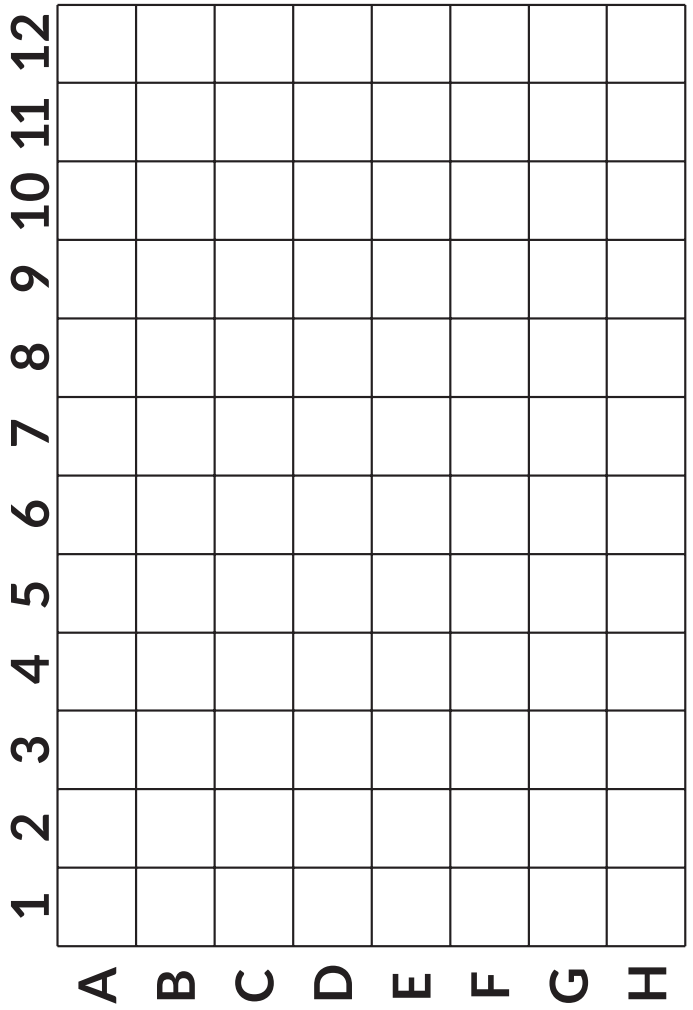
Compound	Cross Reactivity
2'3'-cGAMP	100%
2'2'-cGAMP	0.8%
3'3'-cGAMP	<0.01%
cyclic di-AMP	<0.01%
cyclic di-GMP	<0.01%
cGMP	<0.01%
cAMP	<0.01%
ATP	<0.01%
GTP	<0.01%

Table 6. Cross reactivity of the 2'3'-cGAMP ELISA

RESOURCES

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Plate Preparation	Wash plate or strips to be used for the assay 5 x 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'-cGAMP-HRP Tracer	--	--	50 µl	50 µl	50 µl
2'3'-cGAMP Antiserum	--	--	--	50 µl	50 µl
Seal	Seal the plate				
Incubate	Incubate plate overnight at 4°C or for two 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 Solution	Apply 175 µl TMB Substrate Solution				
HRP Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 30 minutes at room temperature on an orbital shaker protected from light				
HRP Stop Solution	Apply 75 µl HRP Stop Solution				
Read	Read optical density at 450 nm				

Table 7. 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 (>0.100 O.D.)	A. Poor washing B. Exposure of NSB wells to specific antiserum
Very low B_0	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
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 was not added to the wells

References

1. Sun, L., Wu, J., Du, F., *et al.* Science **339(6121)**, 786-791 (2013).
2. Hall, J., Brault, A., Vincent, F., *et al. PLoS One* **12(9)**, e0184843 (2017).
3. O'Neill, L.A.J. *Science* **339(6121)**, 763-764 (2013).
4. Cai, X., Chiu, Y.-H., and Chen, Z.J. *Mol. Cell* **54(2)**, 289-296 (2014).

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.