2’3’-cGAMP ELISA Kit

Item No. 501700

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	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 cover

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
Fax: 734-971-3641
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 at 4°C 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. Orbital shaker
3. Adjustable pipettes and a repeating pipettor.
4. A source of pure water; glass distilled water or deionized water is 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 type I interferon, a potent antiviral cytokine.[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. This assay has a range from 100 ng/ml – 6.1 pg/ml with a midpoint of approximately 900 pg/ml (50% B/B_0) and a sensitivity of approximately 85 pg/ml (80% B/B_0).

Principle of the Assay

This assay is based on the competition between 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 2'3' cGAMP standards and samples vary, 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 2'3'-cGAMP in the well. This antibody 2'3'-cGAMP complex binds to a mouse anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then TMB Substrate Solution is added to the well. The concentration of the analyte is determined by measuring the enzymatic activity of HRP using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). After a sufficient period of time, the reaction is stopped with acid, forming a product with a distinct yellow color that can be measured at 450 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 2'3'-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; or

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

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

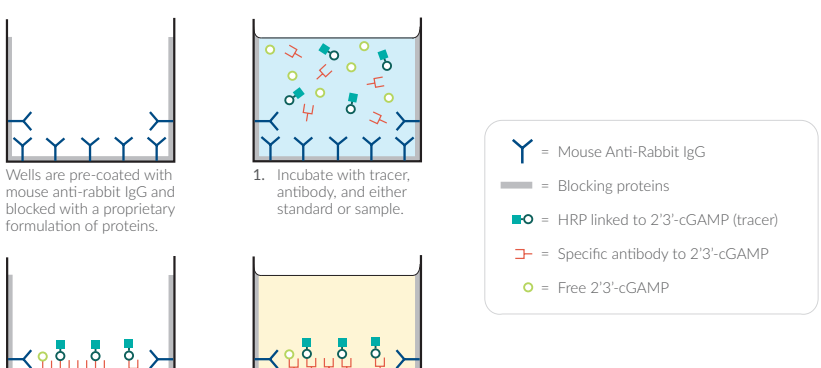


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blank: background absorbance caused by TMB Substrate Solution. The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity (TA): total enzymatic activity of the horseradish peroxidase-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 that of the maximum binding (B_0) well.

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 point, two standard deviations away from the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they should be stable for two months.

1. **Immunoassay Buffer C 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. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer 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). *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 cell lysates. Proper sample storage and preparation are essential for consistent and accurate results. 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 which 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

This assay has been tested using cell lysates. Other sample types will need to be assessed for interference by the end user. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 13 ng/ml and 85 pg/ml (i.e., between 20-80% B/B_0). 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 determine by the end user and tested for compatibility in the assay.

Sample Matrix Properties

Cell lysis buffer (mPER™ (available from ThermoFisher Scientific)) was spiked with 2'3'-cGAMP, diluted as described in the Sample Preparation section and analyzed using the 2'3'-cGAMP ELISA Kit. The results are shown below. Error bars represent standard deviations obtained from multiple dilutions of each sample.

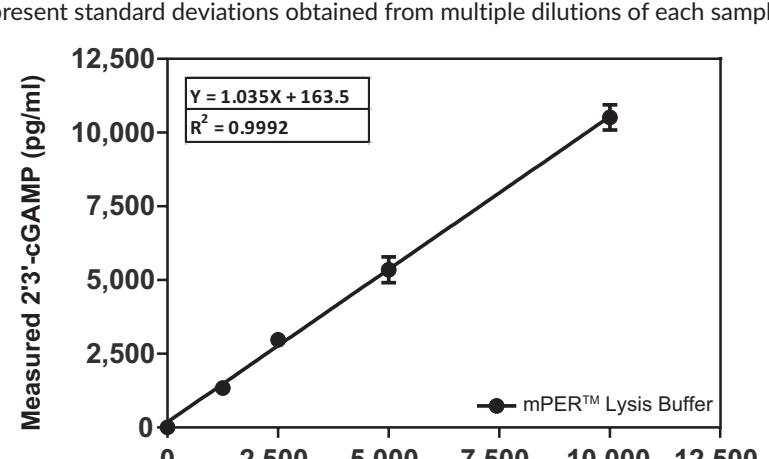


Figure 2. Spike and recovery in cell lysis buffer

Parallelism

To assess parallelism, cell lysates were checked at multiple dilutions using the 2'3'-cGAMP ELISA Kit. Concentrations were plotted as a function of the sample dilution. The results are shown below. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted cell lysates.

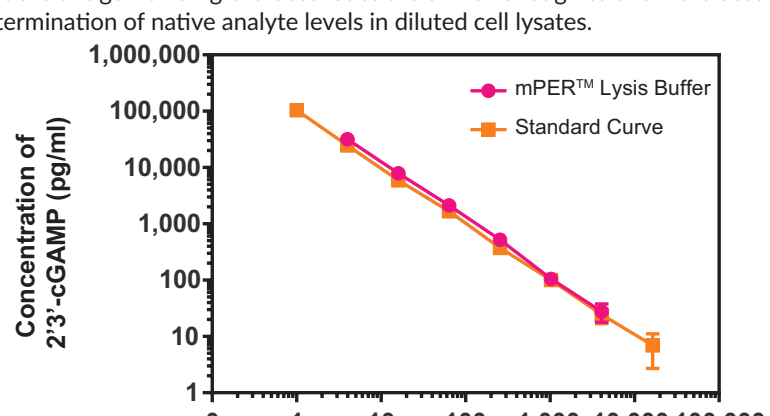


Figure 3. Parallelism of sample matrices in the 2'3'-cGAMP ELISA Kit

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 number them #1 through #8. Aliquot 900 µl 1X Immunoassay Buffer C to all tubes. Transfer 100 µl of the 10X standard (1 ug/ml) 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.

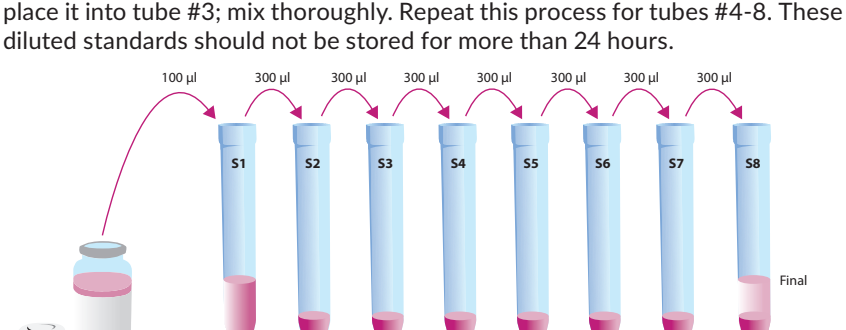


Figure 4. Preparation of 2’3’-cGAMP Standards

2’3’-cGAMP-HRP Tracer

Dilute the 2’3’-cGAMP-HRP Tracer (Item No. 401700) with 5 ml of 1X Immunoassay Buffer C. Store the diluted 2’3’-cGAMP-HRP Tracer at 4°C (*do not freeze!*) and use within 2 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 reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

2’3’-cGAMP ELISA Polyclonal Antiserum

The 2’3’-cGAMP ELISA Polyclonal Antiserum (Item No. 401702) is ready for use as provided. Store the 2’3’-cGAMP Polyclonal Antiserum at 4°C and use within 6 months. A 20% surplus of antiserum has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

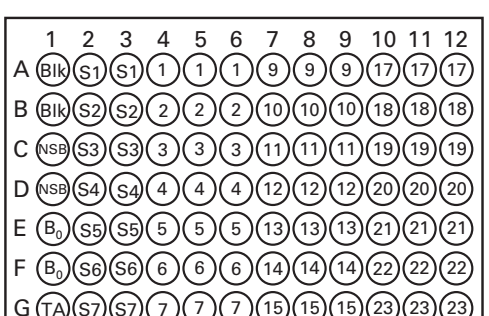
This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum).

Plate Set Up

The 96-well plate(s) included with this kit MUST be pre-washed with 5 rinses of 1X Wash Buffer (~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/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 blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (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 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, 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 19, 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 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 (1X)**

   Add 100 µl Immunoassay Buffer C to Non-Specific Binding (NSB) wells. Add 50 µl Immunoassay Buffer C to Maximum Binding (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 Total Activity (TA) and the Blank (Blk) wells

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

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

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate overnight at 4°C. Alternatively, the assay may be incubated for 2 hours at room temperature with shaking. Sensitivity and signal remain consistent between both incubation methods.

Development of the Plate

1. Empty the wells and rinse five times with ~300 µl Wash Buffer
2. Add 175 µl of TMB Substrate Solution (Item No. 400074) to each well.
3. Add 5 µl of the 1X tracer to the Total Activity (TA) wells.
4. Cover the plate with plastic film and incubate for 30 minutes at room temperature on an orbital shaker.
5. 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. *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 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.

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 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized 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 concentration or dilution of the sample prior to the 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 curves presented here are examples 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.

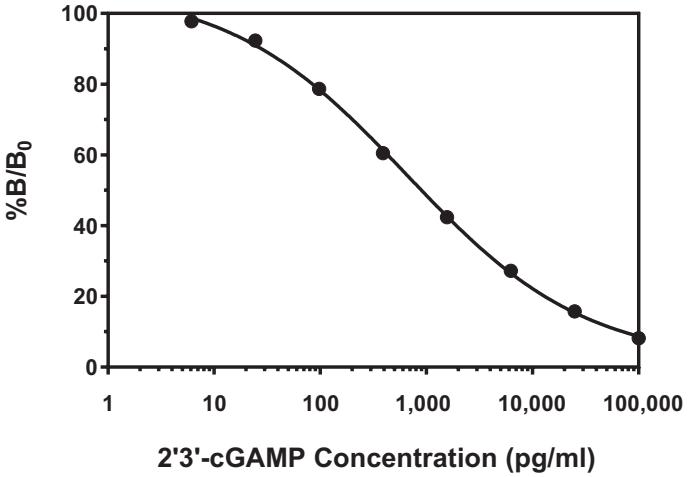
Absorbance at 450 nm - 30 minute development - Overnight Incubation Step

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
1,562.5	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 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 = 100 ng/ml - 6.1 pg/ml
Sensitivity (defined as 80% B/B_0) = 85.3 pg/ml
Mid-point (defined as 50% B/B_0) = 907.7 pg/ml
Lower Limit of Detection (LLOD) = 9.6 pg/ml

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 for 2'3'-cGAMP

Precision:

Intra-assay precision was determined by analyzing 24 replicates of three matrix controls (Spiked Mammalian Protein Extraction Reagent (mPER™) Samples) in a single assay.

Matrix Control (pg/ml)	%CV
7,165.1	8.4
828.6	14.5
102.5	21.3

Table 3. Intra-assay precision

Inter-assay precision was determined by analyzing replicates of three matrix controls (Spiked Mammalian Protein Extraction Reagent (mPER™) Samples) in separate assays spanning across several days.

Matrix Control (pg/ml)	%CV
7,530.1	9.6
811.8	9.6
72.2	18.7

Table 4. Inter-assay precision

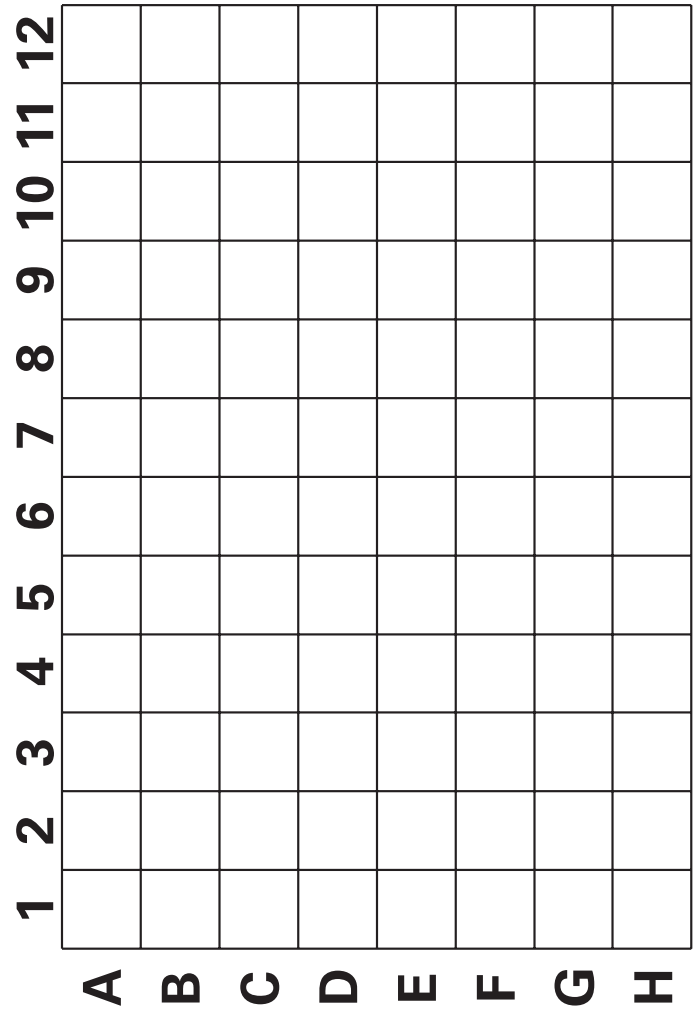
Cross Reactivity:

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 5. Cross Reactivity of the 2'3'-cGAMP ELISA

RESOURCES

2'3'-cGAMP Assay Summary					
Procedure	**Blk**	**TA**	**NSB**	**B_0**	**Standards/ Samples**
Plate Preparation	Wash strips to be used for the assay 5 times with ~300 µl Wash Buffer				
Reconstitute and Mix	Mix all reagents gently				
Immunoassay Buffer C	--	--	100 µl	50 µl	--
Standards/Samples	--	--	--	--	50 µl
2'3'-cGAMP Tracer	--	--	50 µl	50 µl	50 µl
2'3'-cGAMP Antiserum	--	--	--	50 µl	50 µl
Seal	Seal the plate and tap gently to mix				
Incubate	Incubate plate overnight at 4°C or for 2 hours at room temperature, shaking				
Aspirate and Wash	Aspirate wells and wash 5 x ~300 µl				
Apply TMB	175 µl	175 µl	175 µl	175 µl	175 µl
Total Activity (TA) - Apply Tracer	--	5 µl	--	--	--
Seal	Seal plate and incubate for 30 min. at room temperature on orbital shaker, protect from light				
Read	Carefully remove plastic seal and read absorbance at 450 nm				



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 sample purification prior to analysis.
HRP Inhibitors	Ensure that samples and all materials are free of HRP inhibitors such as azide.

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.