3’2’-cGAMP ELISA Kit

Item No. 502340

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage Temperature
400580	3'2'-cGAMP ELISA Polyclonal Antiserum	1 vial/100 dtn	4°C
400579	3'2'-cGAMP-HRP Tracer	1 vial/100 dtn	4°C
400581	3'2'-cGAMP ELISA Standard	1 vial/1 ml	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
400004/ 400006	Mouse Anti-Rabbit 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	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 3'2'-cGAMP 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 13).

INTRODUCTION

Background

3'2'-cGAMP is a second messenger found in *Drosophila* and bacteria.[1-3] It is synthesized in *Drosophila* in response to activation of cyclic GMP-AMP synthase-like receptor 1 (cGLR1), a cytosolic sensor for foreign double-stranded RNA, or cGLR2, which also induces the synthesis of 2'3'-cGAMP. 3'2'-cGAMP induces the expression of the gene encoding *Drosophila* stimulator of interferon genes (*Sting*), which activates relish, a member of the NF-κB family of proteins, to stimulate an antiviral immune response.[2] It also induces the expression of *CG13641*, *CG42825*, and *CG33926*, the *Drosophila* genes encoding Sting-related gene 1 (Srg1), Srg2, and Srg3, respectively. 3'2'-cGAMP reduces viral load and increases survival in flies infected with *Drosophila* C virus (DCV).[1] In bacteria, 3'2'-cGAMP is a second messenger in the CdnG-Cap5 antiphage defense system.[3] It is produced by the cGAS/DncV-like nucleotidyltransferase (CD-NTase) enzyme *Asticcacaulis* CdnG (AsCdnG) and induces AsCap5-mediated DNA degradation.

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About This Assay

Cayman's 3'2'-cGAMP ELISA Kit is a competitive assay that can be used for the quantification of 3'2'-cGAMP in fruit fly homogenates and bacterial lysates. The assay has a range of 7.8-1,000 pM, with a midpoint (50% B/B_0) of 50-95 pM and sensitivity (80% B/B_0) of approximately 21 pM.

To convert concentrations from pM multiply the starting concentration in pM by the preferred unit conversion factor in table below.

Starting Unit	Conversion Factor	Final Unit
pM	0.001	pmol/ml
	0.6744	pg/ml
	0.333	pM in well
	0.0337	pg/well
	0.00005	pmol/well
Example: 100 pM * 0.6744 (conversion factor) = 67.44 pg/ml		

Table 1. Unit conversion

Principle of This Assay

This assay is based on the competition between free 3'2'-cGAMP and a 3'2'-cGAMP-HRP conjugate (3'2'-cGAMP-HRP Tracer) for a limited number of 3'2'-cGAMP polyclonal antibody binding sites. Because the concentration of the 3'2'-cGAMP-HRP Tracer is held constant while the concentration of free 3'2'-cGAMP varies, the amount of 3'2'-cGAMP-HRP Tracer that is able to bind to the 3'2'-cGAMP polyclonal antibody will be inversely proportional to the concentration of free 3'2'-cGAMP in the well. This antibody-3'2'-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. 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 3'2'-cGAMP-HRP Tracer bound to the well, which is inversely proportional to the amount of free 3'2'-cGAMP present in the well during the incubation, as described in the equation:

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

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

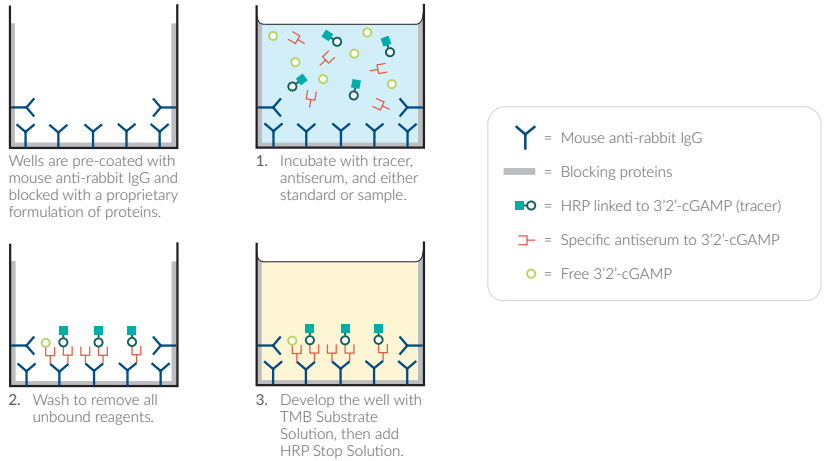


Figure 1. Schematic of the ELISA

Definition of Key Terms

Blk (Blank): background absorbance caused by TMB Substrate Solution and 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 3'2'-cGAMP 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): 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:

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

LLOD (Lower Limit of Detection): the smallest measure that can be detected with reasonable certainty for a given analytical procedure. The LLOD is defined as a concentration two standard deviations higher than the mean zero value.

PRE-ASSAY PREPARATION

Buffer Preparation

Store all diluted buffers at 4°C; they 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 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

Fruit Fly Homogenate

1. Collect fruit flies and homogenize in ultrapure water. It is recommended to homogenize between 25-400 mg of fruit flies per 1 ml of ultrapure water.
2. Heat the samples to 95°C and hold for 5 minutes.
3. Centrifuge at 21,000 x g for 5 minutes at 4°C to pellet debris.
4. Transfer supernatant to a new tube.
5. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -20°C.

Bacterial Lysate

This assay has been demonstrated to work with bacterial cell lysates prepared in B-PER™ Bacterial Protein Extraction Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Other lysis buffers or concentrated lysates may cause interference and require sample purification or a minimum dilution determined by the end user as outlined below. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -20°C.

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.

Testing for Interference

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 3'2'-cGAMP concentration, sample purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatibility in the assay.

Sample Matrix Properties

Parallelism

To assess parallelism, *Drosophila* and Ds-cGLR1-transformed *E. coli* samples were processed as described in the **Sample Preparation** section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated using the 3'2'-cGAMP ELISA Kit. Measured concentrations were plotted as a function of the sample dilution. The results are shown below.

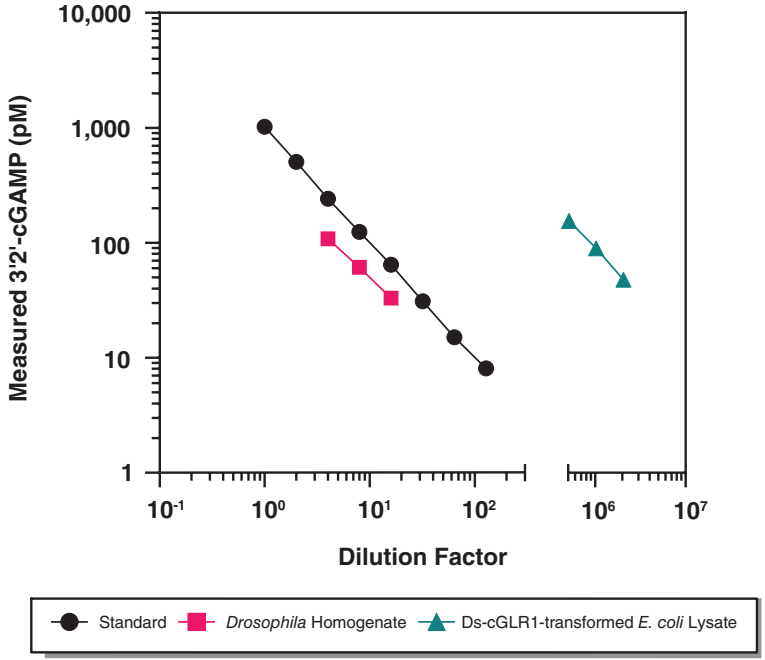


Figure 2. Parallelism of *Drosophila* homogenate and *E. coli* lysate in the 3'2'-cGAMP ELISA

Spike and Recovery

Drosophila homogenate was spiked with different amounts of 3'2'-cGAMP, processed as described in the **Sample Preparation** section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated using the 3'2'-cGAMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

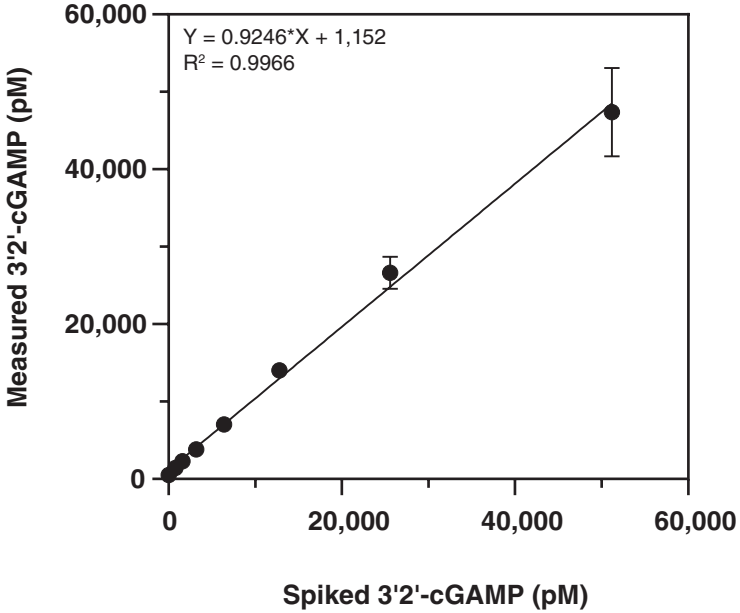


Figure 3. Spike and recovery of 3'2'-cGAMP in *Drosophila* homogenate

Ds-cGLR1-transformed *E. coli* lysate, processed as described in the **Sample Preparation** section (see page 13), was spiked into normal *E. coli* lysate prepared in the same manner and evaluated using the 3'2'-cGAMP ELISA Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

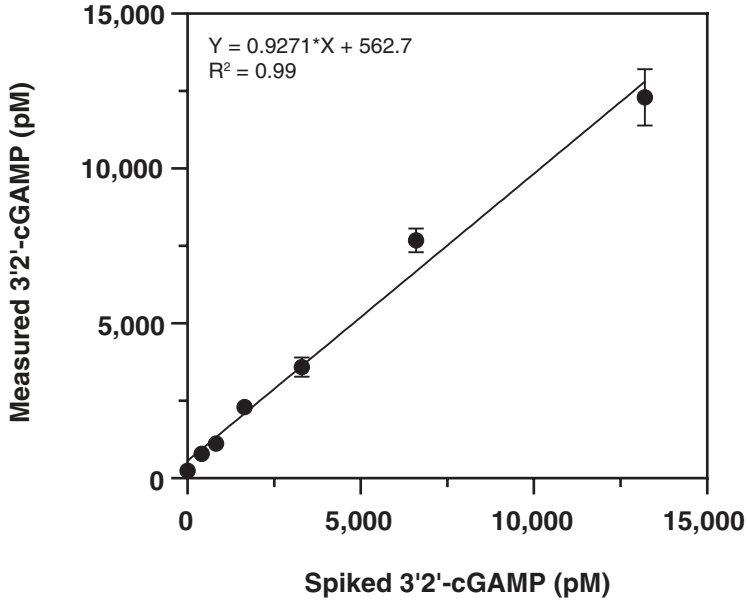


Figure 4. Spike and recovery of 3'2'-cGAMP in *E. coli* lysate

Linearity

Drosophila and *E. coli* samples were processed as described in the **Sample Preparation** section (see page 13), serially diluted with Immunoassay Buffer C (1X), and evaluated for linearity using the 3'2'-cGAMP ELISA Kit. The results are shown below.

Dilution Factor	Measured Concentration (pM)	Linearity (%)
***E. coli* Lysate (Transformed)**		
500,000	7.62×10^7	100
1,000,000	7.34×10^7	96.3
2,000,000	7.55×10^7	99.1
***E. coli* Lysate**		
4	263	100
8	226	85.8
***Drosophila* Homogenate**		
4	434	100
8	491	113
16	530	122

Table 2. Linearity in *Drosophila* homogenate and *E. coli* 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*

Preparation of Assay-Specific Reagents

3'2'-cGAMP 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 3'2'-cGAMP ELISA Standard (Item No. 400581) several times. The concentration of this solution is 10 nM. Transfer 100 µl of the 3'2'-cGAMP ELISA Standard (10 nM) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing it in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should be used within four hours.

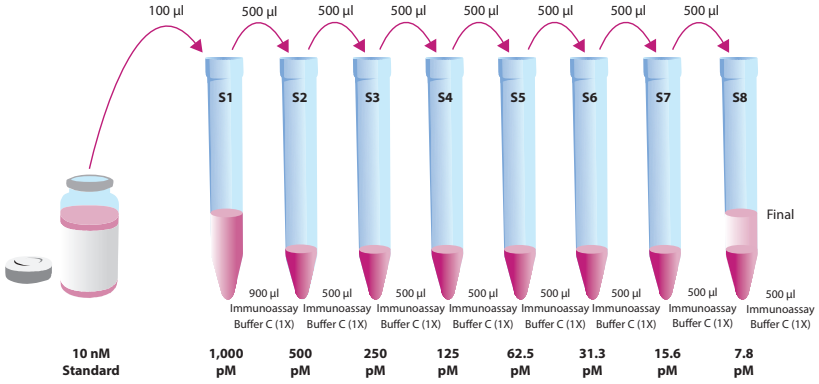


Figure 5. Preparation of the 3'2'-cGAMP standards

3'2'-cGAMP-HRP Tracer

Dilute the 3'2'-cGAMP-HRP Tracer (Item No. 400579) with 5 ml of Immunoassay Buffer C (1X). Store the 3'2'-cGAMP-HRP Tracer at 4°C (*do not freeze!*). It will be stable for at least 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.*

3'2'-cGAMP ELISA Polyclonal Antiserum

The 3'2'-cGAMP ELISA Polyclonal Antiserum (Item No. 400580) is ready to use as supplied. Store the 3'2'-cGAMP ELISA Polyclonal Antiserum at 4°C (*do not freeze!*). 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: Do not store antiserum 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: 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 wells, 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, assaying the samples in triplicate is recommended.

A suggested plate format is shown in Figure 6, 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 24 for more details). It is suggested that the contents of each well be recorded on the template sheet provided (see page 33).

	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 6. Sample plate format

Performing the Assay

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

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

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

5. **3'2'-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 the 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 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 the 96-Well Cover Sheet and protect from light. 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 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/analysistools/ELISA) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the 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* 3'2'-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.

Determine the Sample Concentration

Calculate the B/B_0 (or %B/B_0) value for each sample. Determine the concentration of each sampleby 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 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 could be an indicator of 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.

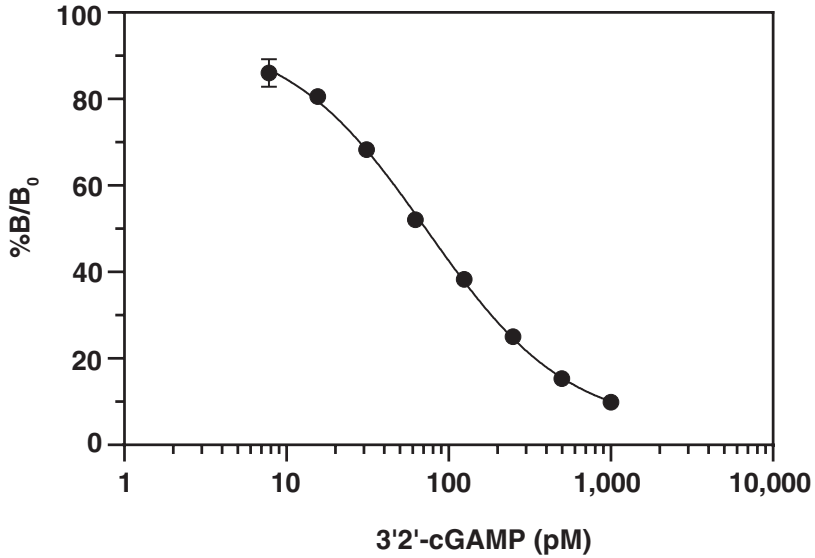
Absorbance at 450 nm

3'2'-cGAMP Standards (pM) and Controls	Absorbance	NSB-Corrected Absorbance	%B/B_0	%CV* Intra-Assay Precision	%CV* Inter-Assay Precision
TA	1.41	--	--	--	--
NSB	0.002	--	--	--	--
B_0	0.740	--	--	--	--
1,000	0.075	0.073	9.9	8.6	7.1
500	0.116	0.114	15.4	3.7	5.3
250	0.187	0.185	25.0	4.0	4.9
125	0.285	0.283	38.3	4.0	5.7
62.5	0.386	0.384	52.0	5.6	4.9
31.3	0.507	0.505	68.3	7.2	3.0
15.6	0.597	0.595	80.6	12.1	7.8
7.8	0.637	0.632	86.0	24.0**	6.2

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 = 7.8-1,000 pM
Sensitivity (defined as 80% B/B_0) = 15 pM
Mid-point (defined as 50% B/B_0) = 72 pM
Lower Limit of Detection (LLOD) = 4.0 pM

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

Figure 7. Typical standard curve

Precision:

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

Matrix Control (pM)	%CV
9.36×10^7	11.4
5.13×10^7	7.7
222	17.9

Table 4. Intra-assay precision

Inter-assay precision was determined by analyzing three *E. coli* lysates in eight separate assays on different days.

Matrix Control (pM)	%CV
6.90×10^7	11.3
3.46×10^7	6.6
174	14.0

Table 5. Inter-assay precision

Cross Reactivity:

Compound	Cross Reactivity
3'2'-cGAMP	100%
2'2'-cGAMP	0.27%
3'3'-cGAMP	0.0009%
2'3'-cGAMP	0.0006%
pApG	0.0004%
cyclic di-GMP	0.0001%
ATP	<0.0001%
GTP	<0.0001%
AMP	<0.0001%
GMP	<0.0001%
cAMP	<0.0001%
cGMP	<0.0001%
cyclic di-AMP	<0.0001%

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

RESOURCES

Troubleshooting

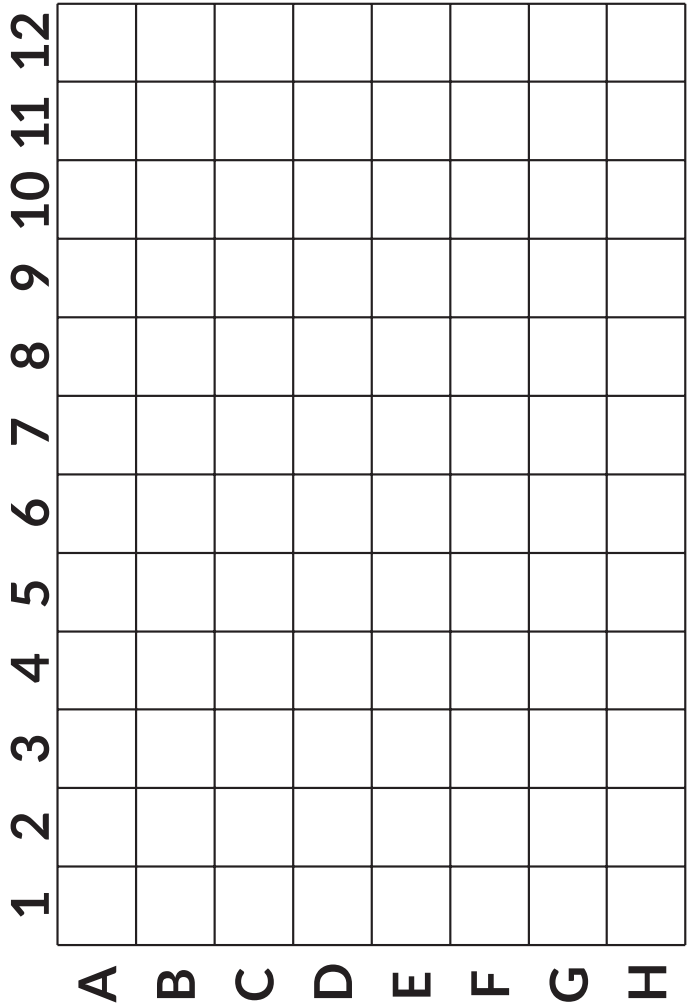
Problem	Possible Causes
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antiserum
Very low B_0	A. Trace organic contaminants in the water source B. Dilution 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: ensure 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. Slavik, K.M., Morehouse, B.R., Ragucci, A.E., *et al.* cGAS-like receptors sense RNA and control 3'2'-cGAMP signalling in *Drosophila*. *Nature* **597(7874)**, 109-113 (2021).
2. Holleufer, A., Winther, K.G., Gad, H.H., *et al.* Two cGAS-like receptors induce antiviral immunity in *Drosophila*. *Nature* **597(7874)**, 114-118 (2021).
3. Fatma, S., Chakravarti, A., Zeng, X., *et al.* Molecular mechanisms of the CdnG-Cap5 antiphage defense system employing 3',2'-cGAMP as the second messenger. *Nat. Commun.* **12(1)**, 6381 (2021).

Procedure	Blk	TA	NSB	B_0	Standards/ Samples
Dilute and Mix	Mix all reagents gently				
Plate Preparation	Wash plate or strips to be used for the assay 5 times with ~300 µl/well Wash Buffer (1X)				
Add Immunoassay Buffer C (1X)	--	--	100 µl	50 µl	--
Add Standards/Samples	--	--	--	--	50 µl
Add 3'2'-cGAMP-HRP Tracer	--	--	50 µl	50 µl	50 µl
Add 3'2'-cGAMP ELISA Polyclonal 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)				
Add TMB Substrate	175 µl				
Add 3'2'-cGAMP-HRP Tracer	--	5 µl	--	--	--
Develop	Seal the plate and incubate for 30 minutes at room temperature on an orbital shaker protected from light				
Do Not Wash. Add HRP Stop Solution	75 µl				
Read	Read absorbance at 450 nm				

Table 7. Assay summary



NOTES

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