ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

DetectX®

3',3'-Cyclic GAMP
Enzyme Immunoassay Kit

1 Plate Kit	Catalog Number K073-H1
5 Plate Kit	Catalog Number K073-H5

Species Independent

Sample Types Validated:

Cell Lysates, Tissue Extracts, and Tissue Culture Media.

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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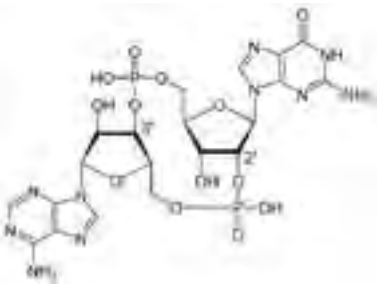
BACKGROUND

Cyclic dinucleotides are the most abundant second messenger in bacteria, modulating a variety of cellular activities in prokaryotic cells. 3'3'-cyclic GAMP (cyclic [G(3',5')pA(3',5')p], previously known as cGAMP) is a key mediator of bacterial signal transduction and regulation, controlling a range of diverse targets including transcription, enzyme activity and cell cycle progression. 3'3'-cGAMP signaling in bacteria is regulated in-part by gene regulatory RNA elements called riboswitches that bind and respond to cGAMP with high affinity and specificity[1]. The 3'3'-cGAMP riboswitches regulate genes involved in motility, biofilm formation, colonization, and virulence[2, 3]. The cyclic dinucleotides have emerged as key central regulators of bacterial physiology and inhibition studies of cGAMP signaling as an anti-microbial strategy are ongoing.

In mammalian cells, 3'3'-cGAMP and its eukaryotic analog 2'3'-cGAMP produced by cGAS, bind STING (stimulator of IFN genes) and subsequently induce TBK1-IRF3-dependent production of IFN-β[4, 5, 6]. Here, the cGAS-cGAMP-STING DNA sensing pathway is a key activator of the innate immune response to foreign or harmful native DNA. The cGAS-cGAMP-STING pathway plays a critical role in antiviral and antitumor immunity as well as mediating autoimmune responses. Dysregulation or aberrant activation of the pathway by self-DNA has emerged as an underlying cause of tumorigenesis and autoimmune disorders.



1. Serganov, A., & Nudler, E. (2013). A decade of riboswitches. *Cell, 152*(1-2), 17–24.
2. Davies, B. W., et al. (2012). Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for *V. cholerae* virulence. *Cell, 149*(2), 358–370.
3. Nelson, J. W., et al. (2015). Control of bacterial exoelectrogenesis by c-AMP-GMP. *Proceedings of the National Academy of Sciences, 112*(17), 5389–5394.
4. Sun, L., et al. (2013). Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science, 339*(6121), 786–791.
5. Wu, J., et al. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cystolic DNA. *Science, 339*(6121), 826–830.
6. Zhang, X., et al. (2013). Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. *Molecular Cell, 51*(2), 226–235.

ASSAY PRINCIPLE

The DetectX® 3',3'-Cyclic GAMP (cGAMP) Immunoassay Kit is designed to quantitatively measure 3',3'-cGAMP present in lysed cells and tissue, as well as tissue culture media samples.

Please read the complete kit insert before performing this assay. A 3',3'-cGAMP standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. A clear microtiter plate coated with an antibody to capture rabbit IgG is provided. Standards or diluted samples are pipetted into the wells. A 3',3'-cGAMP-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a rabbit polyclonal antibody to 3',3'-cGAMP to each well. After a 1 hour incubation, the plate is washed and substrate is added. The substrate reacts with the bound 3',3'-cGAMP-peroxidase conjugate and after a short incubation the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of the 3',3'-cGAMP in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

RELATED PRODUCTS

Kits	Catalog No.
2',3'-Cyclic GAMP ELISA Kits	K067-H1/H5
2',3'-Cyclic GAMP ELISA Kit (384-Well Plate)	K067-H1D
Acetylcholinesterase Fluorescent Activity Kit	K015-F1
Butyrylcholinesterase Fluorescent Activity Kit	K016-F1
Corticosterone Chemiluminescent ELISA Kits	K014-C1/C5
Corticosterone ELISA Kits	K014-H1/H5
Cortisol ELISA Kits (Strip Wells)	K003-H1/H5
Cortisol ELISA Kits (Whole Plate)	K003-H1W/H5W
Cyclic AMP Direct Chemiluminescent ELISA Kits	K019-C1/C5
Cyclic AMP Direct ELISA Kits	K019-H1/H5
Cyclic GMP Direct Chemiluminescent ELISA Kits	K020-C1/C5
Cyclic GMP Direct ELISA Kits	K065-H1/H5
DMXAA	P024-5MG/25MG
H-151	P023-10MG/50MG
IBMX	P019-100MG/1GM
Prostaglandin E_2 Multi-Format ELISA Kits	K051-H1/H5
Protein Kinase A (PKA) Colorimetric Activity Kit	K027-H1

SUPPLIED COMPONENTS

Coated Clear 96 Well Plates
A clear plastic microtiter plate(s) coated with goat anti-rabbit IgG.

Kit K073-H1 **or** -H5	1 **or** 5 Each	Catalog Number X016-1EA

3',3'-Cyclic GAMP Standard
3',3'-Cyclic GAMP at 100 nmol/mL in a special stabilizing solution.

Kit K073-H1 **or** -H5	125 µL **or** 625 µL	Catalog Number C274-125UL **or** -625UL

DetectX® 3',3'-Cyclic GAMP Antibody
A rabbit polyclonal antibody specific for 3',3'-cyclic GAMP.

Kit K073-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C272-3ML **or** -13ML

DetectX® 3',3-Cyclic GAMP Conjugate
A cyclic 3',3'-GAMP-peroxidase conjugate in a special stabilizing solution.

Kit K073-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C273-3ML **or** -13ML

Assay Buffer Concentrate
A 5X concentrate that must be diluted with deionized or distilled water. This buffer contains EDTA to chelate zinc (Zn^{+2}) ions. Zinc ions may affect 3',3'-cGAMP measurement.

Kit K073-H1 **or** -H5	28 mL **or** 55 mL	Catalog Number X065-28ML **or** -55ML

Wash Buffer Concentrate
A 20X concentrate that must be diluted with deionized or distilled water.

Kit K073-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K073-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

Stop Solution
A 1M solution of hydrochloric acid. **CAUSTIC**.

Kit K073-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K073-H1 **or** -H5	1 **or** 5 Each	Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Repeater pipet, such as an Eppendorf repeater, with disposable tips to accurately dispense 25 µL, 50 µL and 100 µL.

A microplate shaker. Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Software for carrying out four parameter logistic curve (4PLC) fitting.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers wash buffers, containing sodium azide will inhibit color production from the enzyme. Make sure all buffers used for samples are azide free and ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on page 8.

SAMPLE TYPES

This assay has been tested and validated for lysed cells, tissues, and tissue culture media samples. Samples should be stored at ≤ -70°C for long term storage. Samples containing visible particulate should be centrifuged prior to use.

3',3'-Cyclic GAMP is identical across all species. The end user should evaluate recoveries of 3',3'-cGAMP in other samples being tested.

SAMPLE PREPARATION

Samples may need to be extracted depending on source. We suggest a study of the literature to determine suitable methods of isolating cGAMP from tissues and cells. We have tested commercially available RIPA and M-PER™ (ThermoScientific) as diluents in this assay as well as a general bacterial cell lysis buffer. See page 14 for more information. It is up to the end user to determine if their lysis buffer can be used as a diluent or the necessary minimum dilution into Assay Buffer necessary.

Tissue Samples

Tissues samples should be frozen in liquid nitrogen and stored at -80°C if analysis is not to be carried out immediately. Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Lyse cells with a suitable lysis buffer on ice, and then centrifuge at ≥ 600 x g at 4°C for 15 minutes. Collect the supernatant and run in the assay directly after dilution into Assay Buffer or off a standard curve generated in lysis buffer if shown to be compatible.

TCA Protocol. For tissue that requires concentration and delipidation, a trichloroacetic acid (TCA)/ether protocol can be used. Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of ice cold 5% TCA (weight/volume) for every 100 mg of tissue and grind in a glass-Teflon mortar. Incubate in the TCA for 10 minutes on ice, and then centrifuge at ≥ 600 x g at 4°C for 15 minutes. Collect the supernatant.

For every 1 mL of TCA supernatant add 3 mL of water saturated diethyl ether* and shake in a glass vial. Allow the ether to separate as the top layer, remove it and discard the ether. Dry the aqueous layer by lyophilization or using a vacuum centrifuge. Reconstitute by adding 1 mL of 1X Assay Buffer for every mL of 5% TCA used to extract. Run in the assay immediately or store at ≤ -70°C. Samples should be diluted in Assay Buffer.
***Diethyl ether is extremely flammable and should be used in a hood.**

Cells

Cell lysis buffers containing high concentrations of SDS or other detergents require dilution. Please read Interferents section on page 14 for more information.

This kit is compatible with either adherent or non-adherent cells. The cells can be grown in any suitable sterile containers such as Petri dishes, 12-, 48- or 96-well culture plates or flasks. The cells must be isolated from the media prior to being lysed with suitable lysis buffer. Ensure that the lysis buffer contains EDTA to minimize transition metal activiated cyclic nucleotide hydrolysis. Some cell types are extremely hardy and the end user should optimize the lysis conditions, utilizing methods such as freeze-thaw cycles, and ultrasonic treatments, or alternate lysis buffers to fully lyse their cells.

For adherent cells, the media should be aspirated from the cells and the cells washed with PBS. The adherent cells should be treated directly with lysis buffer. Cells can be scraped to dislodge them from the plate surface and cells should be inspected to ensure lysis. Centrifuge the samples at ≥ 600 x g at 4°C for 15 minutes and assay the supernatant directly. Samples should be diluted in Assay Buffer or ran off a standard curve generated in lysis buffer if shown to be compatible. If required, the tissue culture media can be assayed for 3',3'-cGAMP as outlined below.

For non-adherent cells, pellet and wash the cells with PBS by centrifuging the samples at ≥ 600 x g at 4°C for 15 minutes as described above. Treat the aspirated, washed pellet directly with lysis buffer for 10 minutes at room temperature. Cells should be inspected to ensure lysis. Centrifuge the samples at ≥ 600 x g at 4 °C for 15 minutes and assay the supernatant directly. Samples should be diluted in Assay Buffer or ran off a standard curve generated in lysis buffer if shown to be compatible. If required, the culture media can be assayed for 3',3'-cGAMP as outlined below.

Tissue Culture Media

For measuring 3',3'-cGAMP in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.

Use all samples within 2 hours or store frozen at ≤ -70°C.

REAGENT PREPARATION

Allow the kit reagents to thaw and come to room temperature for 30-60 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer

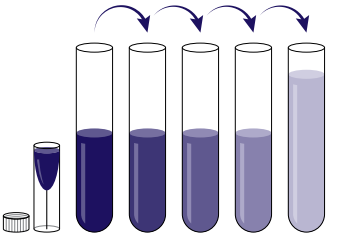
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted. this is stable at room temperature for 3 months.

Assay Buffer

Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted, this is stable for 3 months at 4°C.

Standard Preparation

Label test tubes as #1 through #7. Pipet 450 µL of Assay Buffer into tube #1 and 375 µL of Assay Buffer into tubes #2 to #7. **The 3',3'-Cyclic GAMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 50 µL of the stock solution to tube #1 and vortex completely. Take 125 µL of the solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #7. The concentration of 3',3'-Cyclic GAMP in tubes 1 through 7 will be 10,000, 2,500, 625, 156.3, 39.06, 9.77, and 2.44 pmol/mL.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer (µL)	**450**	375	375	375	375	375	375
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	**50**	125	125	125	125	125	125
Final Conc (pmol/mL)	10,000	2,500	625	156.3	39.06	9.77	2.44

Use Standards within 1 hour of preparation.

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine 3',3'-cGAMP concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.
2. Pipet 75 µL Assay Buffer (or appropriate diluent) into the non-specific binding (NSB) wells.
3. Pipet 50 µL of Assay Buffer (or appropriate diluent) into the maximum binding (B0 or Zero standard) wells.
4. Pipet 50 µL of samples or standards into wells in the plate.
5. Add 25 µL of the DetectX® 3',3'-cGAMP Conjugate to each well using a repeater pipet.
6. Add 25 µL of the DetectX® 3',3'-cGAMP Antibody to each well, **except the NSB wells**, using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour. If the plate is not shaken, signals bound will be approximately 20% lower.
8. Aspirate the plate and wash each well 4 times with 300 µL Wash Buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 50 µL of the Stop Solution to each well using a repeater pipet.
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
13. Use the plate reader's built-in 4PLC software capabilities to calculate 3',3'-cGAMP concentration for each sample.

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

CALCULATION OF RESULTS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor (if any) to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:
https://www.myassays.com/arbor-assays-detectx-3-3-cyclic-gamp-eia-kit.assay

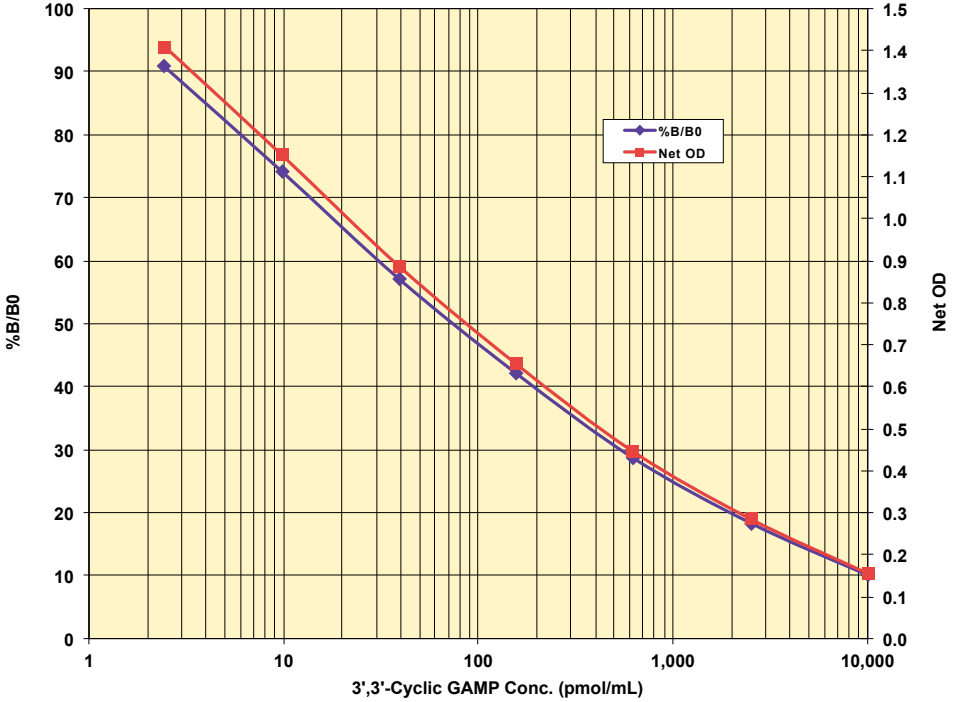
TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	3',3'-Cyclic GAMP Conc. (pmol/mL)
NSB	0.087	-	-	-
Standard 1	0.242	0.155	12.8	10,000
Standard 2	0.372	0.285	22.7	2,500
Standard 3	0.532	0.445	35.9	625
Standard 4	0.741	0.654	52.2	156.3
Standard 5	0.974	0.887	69.1	39.06
Standard 6	1.239	1.152	84.3	9.77
Standard 7	1.497	1.410	91.2	2.44
B0	1.640	1.553	-	0
Sample 1	0.559	0.472	30.39	
Sample 2	0.864	0.777	50.03	
Sample 3	1.097	1.010	65.04	

Always run your own standard curve for calculation of results. Do not use this data.

Conversion Factor: 0.1 pmol/mL of cGAMP is equivalent to 71.8 pg/mL.

Typical Standard Curve



Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #7. The sensitivity was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 0.944 pmol/mL.**

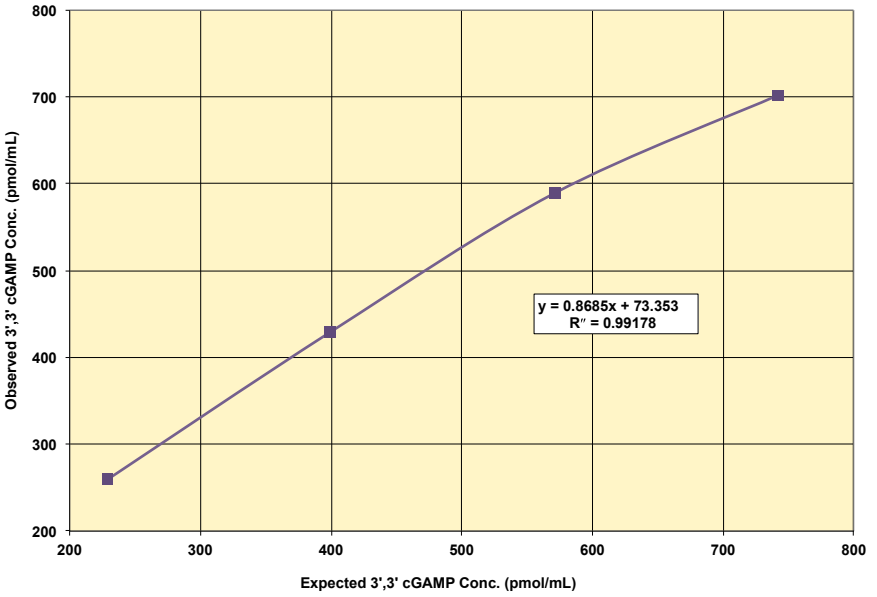
The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each the zero standard and a low concentration bacterial lysate sample. **Limit of Detection was determined as 0.610 pmol/mL.**

VALIDATION DATA

Linearity

Linearity was determined by taking two diluted cell lysate samples, one spiked with a low cGAMP level of 57.8 pmol/mL and one spiked with a higher level of 913.0 pmol/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Lysate	Low Lysate	Expected Conc. (pmol/mL)	Observed Conc. (pmol/mL)	% Recovery
80%	20%	742.0	701.4	105.7%
60%	40%	570.9	589.3	96.9%
40%	60%	399.9	429.8	93.1%
20%	80%	228.8	259.4	88.2%
			Mean Recovery	**96.0%**



Intra Assay Precision

Three samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated cGAMP concentrations were:

Sample	3',3'-Cyclic GAMP Conc. (pmol/mL)	%CV
1	528.99	6.8
2	87.68	8.2
3	29.84	12.0

Inter Assay Precision

Three samples were diluted with Assay Buffer and run in duplicates in sixteen assays run over multiple days by multiple operators. The mean and precision of the calculated cGAMP concentrations were:

Sample	3',3'-Cyclic GAMP Conc. (pmol/mL)	%CV
1	495.78	13.2
2	80.81	10.4
3	27.31	14.0

CROSS REACTIVITY

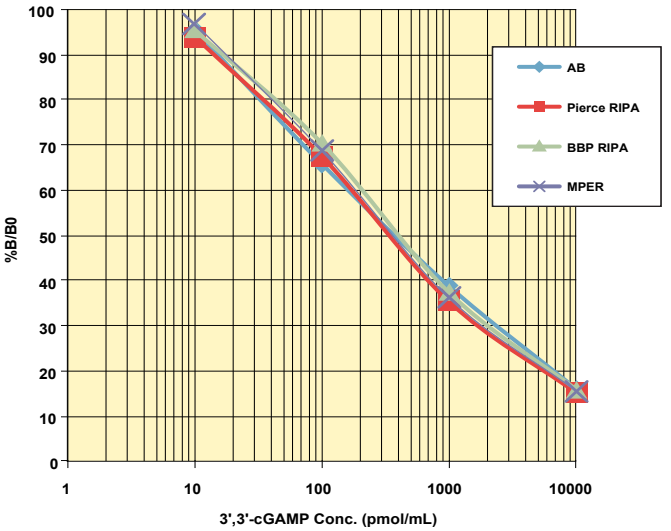
The following cross reactants were tested in the assay and calculated at the 50% binding point.

Nucleotide	Cross Reactivity (%)
3',3'-cGAMP	100%
2',3'-cGAMP (mammalian)	< 0.01%
2',2'-cGAMP (synthetic)	< 0.01%
2',3'-c-di-AMP2 (synthetic)	< 0.01%
cAMP	< 0.01%
AMP	< 0.01%
cGMP	< 0.01%
GMP	< 0.01%

SAMPLE VALUES

E.coli cell lysates were spiked with standard and diluted in Assay Buffer. Concentrations were compared to a similarly spiked control of Assay Buffer. Recovery of E.coli cell lysates diluted 1:10-1:20 averaged 96.5%.

Commercially available lysis buffers (RIPA and M-PER™) were used to generate standard curves and compared to a standard curve generated in Assay Buffer. These lysis buffers can be used to prepare standards to eliminate dilution of lysate. It is up to the end user to determine if their lysis buffer is suitable as a diluent.



INTERFERENTS

A variety of detergents at multiple dilutions were tested as possible interfering substances in the assay when comparing concentration to those generated by Assay Buffer spiked sample.

Addition	% Added	% Change in Measured 3',3'-cGAMP Conc.
Chaps	0.5%	7.7%
CTAC	0.5%	0.11%
NP-40	0.5%	8.04%
Tween 20	0.25%	- 4.89%
SDS	0.06%	- 0.29%
TritonX-100	1.0%	5.32%

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with ELISA kits for wildlife conservation research.

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FOR SERIOUS SCIENCE

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