ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

DetectX®

2',3'-Cyclic GAMP
Enzyme Immunoassay Kit

384-Well Plate Kit Catalog Number K067-H1D

Species Independent

Improved Sensitivity & Diluent Change

Sample Types Validated:

Cell Lysates, Tissue Extracts, and Tissue Culture Media.
Matrix tested for EDTA Plasma.

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

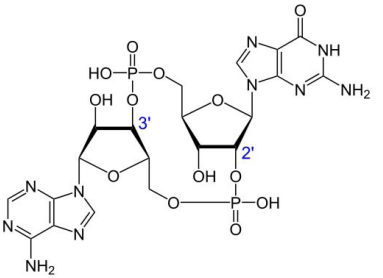
www.ArborAssays.com

TABLE OF CONTENTS

BACKGROUND

2',3'-Cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP-AMP, cGAMP, cyclic [G(2',5')pA(3',5')p]) was the first cyclic di-nucleotide found in metazoa[1]. 2',3'-cGAMP is also referred to as "noncanonical" cGAMP due to the presence of the atypical 2'-5' phosphodiester linkage between the guanosine and the adenosine. 2',3'-Cyclic GAMP is a novel second messenger in innate immunity that regulates type I interferon (IFN) production[1-6]. Produced in mammalian cells by cGAS (cGAMP synthase) in response to double-stranded DNA in the cytoplasm binding to cGAS, cGAMP binds to the stimulator of interferon genes (STING). Subsequently STING induces the TBK1-IRF3-dependent production of IFN-β. This cGAS-cGAMP-STING pathway has been shown to play a critical role in pathogen detection and physiological conditions such as metabolic dysregulation, autoimmunity, and cancer[7-10].

Cellular concentrations of cGAMP are controlled by hydrolase enzymes that cleave the phospho-nucleotide bonds. One of these, ecto-nucleotide phosphatase, ENPP1, is a zinc-stimulated hydrolase of cGAMP and is present in cells, serum, and other samples[11].



1. Wu, J., et al. (2012). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science, 339*(6121), 826–30.
2. 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.
3. Gao, P., et al. (2013). Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell, 153*(5), 1094–1107.
4. Ablasser, A., et al. (2013). cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING. *Nature, 498*(7454), 380–384.
5. Li, X-D., et al. (2013). Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science, 341*(6152), 1390–1394.
6. Paijo, J. et al. (2016). cGAS senses human cytomegalovirus and induces type I interferon responses in human monocyte-derived cells. *PLoS Pathogens, 12*(4), e1005546.
7. Cai, X., et al. (2014). The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. *Molecular Cell 54*(2), 289–296.
8. Guo, X., et al. (2017). Cyclic GMP-AMP ameliorales diet-induced metabolic dysregulation and regulates proinflammatory responses distinctly from STING activation. *Nature, 7*(6355), 1–13.
9. Gao, D., et al. (2015). Activation of cyclic GMP-AMP synthase by self-DNA causes autoimmune diseases." *Proceedings of the National Academy of Sciences, 112*(42), E5699–E5705.
10. Bose, D. (2017). cGAS/STING pathway in cancer: Jekyll and Hyde story of cancer immune response. *International Journal of Molecular Sciences, 18*(11), 2456–2466.
11. Li, L., et al. (2014). Hydrolysis of 2', 3'-cGAMP by ENPP1 and design of non-hydrolyzable analogs. *Nature Chemical Biology, 10*(12), 1043–1048.

ASSAY PRINCIPLE

The DetectX® 2',3'-Cyclic GAMP (cGAMP) Immunoassay Kit is designed to quantitatively measure 2',3'-cGAMP present in lysed cells and tissue, EDTA plasma and tissue culture media samples.

Please read the complete kit insert before performing this assay. A 2',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 primed wells. A 2',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 2',3'-cGAMP to each well. After a 2 hour incubation, the plate is washed and substrate is added. The substrate reacts with the bound 2',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 2',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
3',3'-Cyclic GAMP ELISA Kits	K073-H1/H5
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	K003-H1/H5
Cyclic AMP Direct Chemiluminescent ELISA Kits	K019-C1/C5
Cyclic AMP Direct ELISA Kits	K019-H1/H5
2',3'-Cyclic GAMP Direct ELISA	K067-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 384 Well Plate
A clear plastic microtiter plate coated with goat anti-rabbit IgG.
1 Each Catalog Number X125-1EA

2',3'-Cyclic GAMP Standard
2',3'-Cyclic GAMP at 1,000 pmol/mL in a special stabilizing solution.
125 µL Catalog Number C243-125UL

DetectX® 2',3'-Cyclic GAMP Antibody
A rabbit polyclonal antibody specific for 2',3'-cyclic GAMP.
2.3 mL Catalog Number C241-2.3ML

DetectX® 2',3-Cyclic GAMP Conjugate
A cyclic 2',3'-GAMP-peroxidase conjugate in a special stabilizing solution.
2.3 mL Catalog Number C242-2.3ML

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 2',3'-cGAMP measurement.
55 mL Catalog Number X065-55ML

Wash Buffer Concentrate
A 20X concentrate that must be diluted with deionized or distilled water.
30 mL Catalog Number X007-30ML

TMB Substrate
11 mL Catalog Number X019-11ML

Stop Solution
A 1M solution of hydrochloric acid. **CAUSTIC**.
5 mL Catalog Number X020-5ML

Plate Sealer
1 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.

Appropriate manual or automated dispensing equipment for adding 5 µL, 10 µL and 20 µL of reagents. If you are not using an automated system then a repeater pipet is needed with disposable tips.

Colorimetric reader capable of reading 384-well microplate optical density at 450 nm.

Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

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. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

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. Matrix interference has been evaluated for EDTA plasma samples, as it is unknown if 2',3'-cGAMP is present in these matrices. Samples should be stored at ≤ -70°C for long term storage. Samples containing visible particulate should be centrifuged prior to using.

2',3'-Cyclic GAMP is identical across all species. The end user should evaluate recoveries of 2',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, see pafe 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.

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 after diluting into Assay Buffer or ran 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 of a standard curve generated in lysis buffer if shown to be compatible. If required, the tissue culture media can be assayed for 2',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 of a standard curve generated in lysis buffer if shown to be compatible. If required, the culture media can be assayed for 2',3'-cGAMP as outlined below.

Tissue Culture Media

For measuring 2',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.

Plasma Samples

Plasma samples should be diluted ≥ 1:5 with Assay Buffer prior to running in the assay.

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

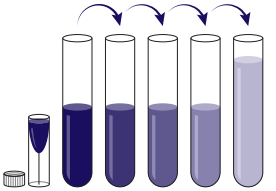
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

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 at 4°C for 3 months.

Standard Preparation

Label test tubes as #1 through #7. Pipet 490 µL of Assay Buffer into tube #1 and 120 µL into tubes #2 to #7. **The 2',3'-Cyclic GAMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 10 µL of the stock solution to tube #1 and vortex completely. Take 80 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 2',3'-Cyclic GAMP in tubes 1 through 7 will be 20, 8, 3.2, 1.28, 0.512, 0.205 and 0.082 pmol/mL.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer (µL)	**490**	120	120	120	120	120	120
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	**10**	80	80	80	80	80	80
Final Conc (pmol/mL)	20	8	3.2	1.28	0.512	0.205	0.082

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 2',3'-cGAMP concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification.
2. Pipet 15 µL Assay Buffer (or appropriate diluent) into the non-specific binding (NSB) wells.
3. Pipet 10 µL of Assay Buffer (or appropriate diluent) into the maximum binding (B0 or Zero standard) wells.
4. Pipet 10 µL of samples or standards into wells in the plate.
5. Add 5 µL of the DetectX® 2',3'-cGAMP Conjugate to each well.
6. Add 5 µL of the DetectX® 2',3'-cGAMP Antibody to each well, **except the NSB wells**.
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 2 hours. We recommend shaking at around 700–900 rpm.
8. Aspirate the plate and wash each well 4 times with 100 µL wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 20 µL of the TMB Substrate to each well.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 10 µL of the Stop Solution to each well
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 2',3'-cGAMP concentration for each sample.

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.

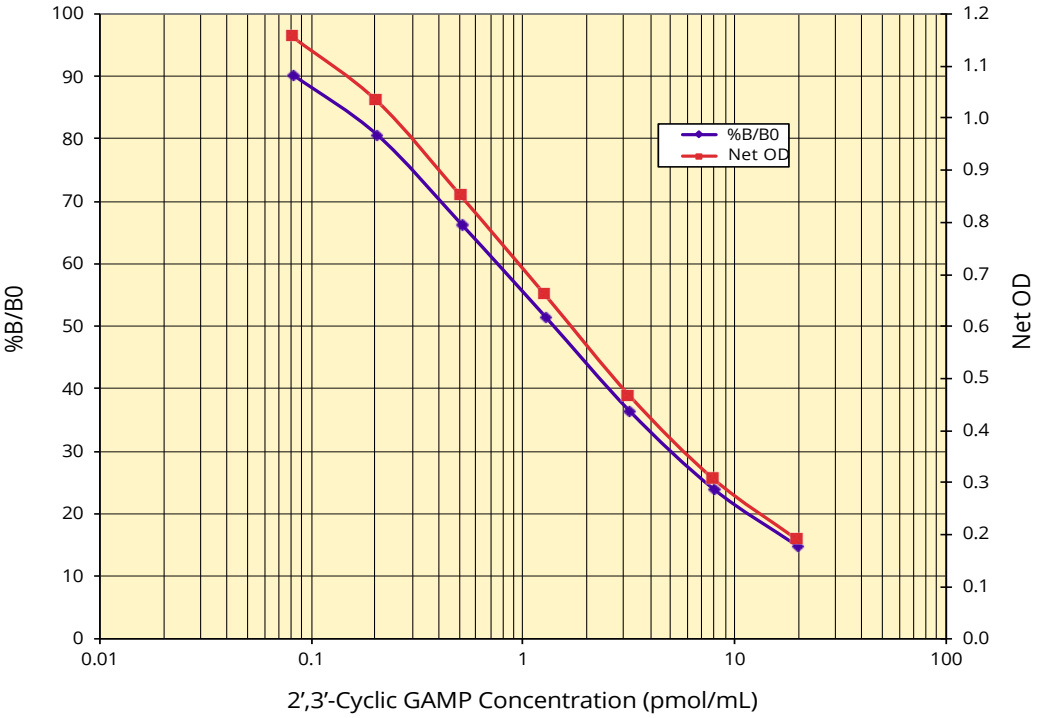
TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	2',3'-Cyclic GAMP Conc. (pmol/mL)
NSB	0.136	-	-	-
Standard 1	0.325	0.189	14.8	20
Standard 2	0.442	0.306	23.9	8
Standard 3	0.602	0.466	36.4	3.2
Standard 4	0.795	0.659	51.5	1.28
Standard 5	0.985	0.849	66.3	0.512
Standard 6	1.168	1.032	80.6	0.205
Standard 7	1.291	1.155	90.2	0.082
B0	1.416	1.280	100.0	0
Sample 1	0.860	0.724	56.5	0.95
Sample 2	0.533	0.397	31.0	4.61

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 (FROM 96-WELL KIT, K067-H)

Sensitivity

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.

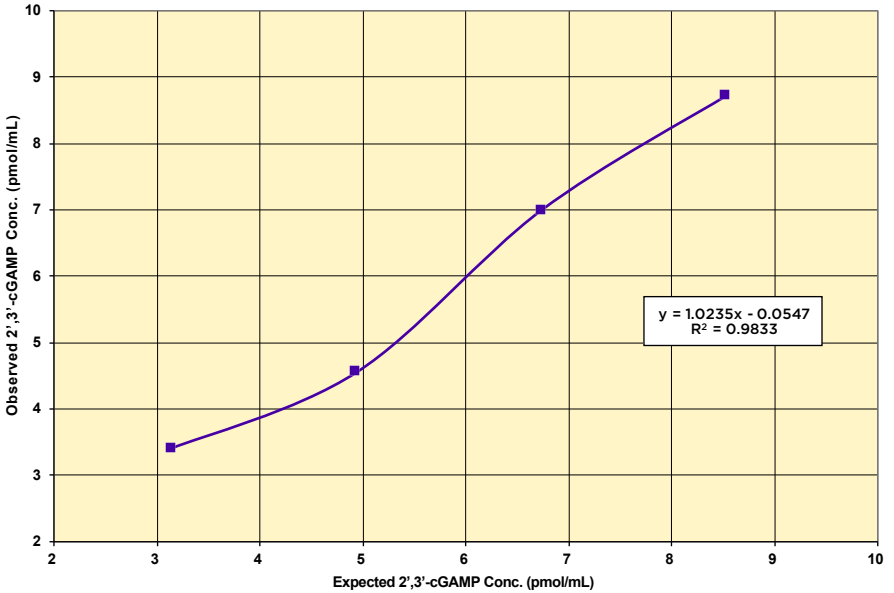
Sensitivity was determined as 0.048 pmol/mL.

VALIDATION DATA (FROM 96-WELL KIT, K067-H)

Linearity

Linearity was determined by taking two diluted cell lysate samples, one spiked with a low cGAMP level of 1.35 pmol/mL and one spiked with a higher level of 10.32 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%	8.53	8.72	102.2%
60%	40%	6.73	6.99	103.9%
40%	60%	4.94	4.55	92.2%
20%	80%	3.14	3.41	108.4%
			Mean Recovery	**101.7%**



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	2',3'-Cyclic GAMP Conc. (pmol/mL)	%CV
1	1.08	6.2
2	5.14	6.1
3	10.4	5.7

Inter Assay Precision

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

Sample	2',3'-Cyclic GAMP Conc. (pmol/mL)	%CV
1	1.00	8.5
2	4.92	6.7
3	9.97	8.0

CROSS REACTIVITY

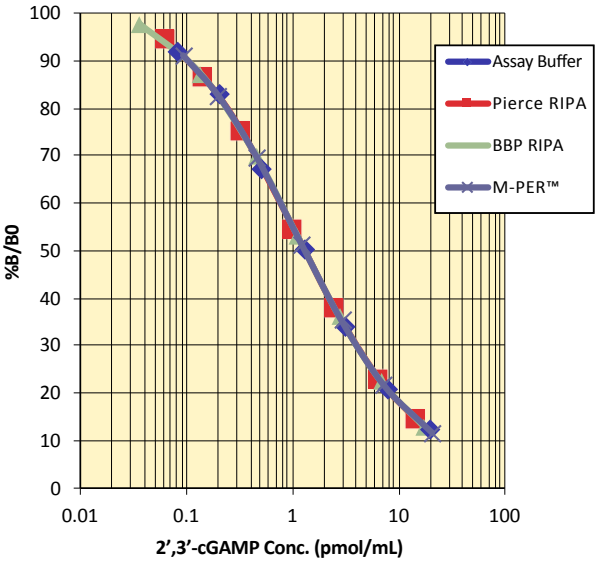
The following cross reactants were tested in the assay and calculated at the 50% binding point.

Nucleotide	Cross Reactivity (%)
2',3'-cGAMP	100%
2'2'-cGAMP (synthetic)	1.97%
3'3'-cGAMP (bacterial)	0.03%
2'3'-c-di-AMP2 (synthetic)	< 0.01%
cAMP	< 0.01%
AMP	< 0.01%
cGMP	< 0.01%
GMP	< 0.01%
ATP	< 0.01%

SAMPLE VALUES

Human EDTA plasma samples from healthy individuals were spiked with standard and diluted in Assay Buffer. Concentrations were compared to a similarly spiked control of Assay Buffer. Recovery for EDTA plasma diluted 1:5-1:40 averaged 93.7%.

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 2',3'-cGAMP Conc.
Chaps	0.5%	- 4.1%
CTAC	1.0%	- 0.1%
NP-40	1.0%	+ 3.9%
Tween 20	0.25%	- 2.2%
SDS	0.02%	+ 8.8%
TritonX-100	2.0%	+ 0.7%

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:

Arbor Assays

1514 Eisenhower Place
Ann Arbor, Michigan 48108 USA

Phone: 734-677-1774

Fax: 734-677-6860

Web: www.ArborAssays.com

Email Addresses:

Info@ArborAssays.com

Orders@ArborAssays.com

Technical@ArborAssays.com

Contracts@ArborAssays.com

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 assay kits and reagents for wildlife conservation research.

ASSAYS™
FOR SERIOUS SCIENCE

FSC Printed on Forest Stewardship Council certified paper