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ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

DetectX®

Direct Cyclic GMP
Enzyme Immunoassay Kit

1 Plate Kit Catalog Number K020-H1

5 Plate Kit Catalog Number K020-H5

Species Independent

Sample Types Validated:

Cell Lysates, Saliva, Urine, EDTA Plasma, 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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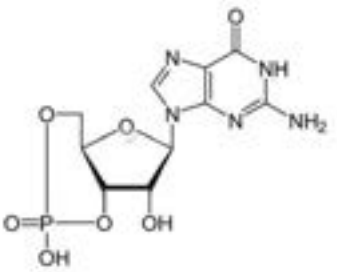
BACKGROUND

Guanosine 3', 5'-cyclic monophosphate (cyclic GMP; cGMP) is a critical and multifunctional second messenger present at levels typically 10-100 fold lower than cAMP in most tissues. Intracellular cGMP is formed by the action of the enzyme guanylate cyclase on GTP and degraded through phosphodiesterase hydrolysis[1-3]. Guanylate cyclases (GC) are either soluble or membrane bound[3,4]. Soluble GCs are nitric oxide responsive, whereas membrane bound GCs respond to hormones such as acetylcholine, insulin and oxytocin. Other chemicals like serotonin and histamine also cause an increase in cGMP levels[5,6]. Cyclic GMP regulates cellular composition through cGMP-dependent kinase, cGMP-dependent ion channels or transporters, and through its hydrolytic degradation by phosphodiesterase[1,7].



1. Domek-Lopacinska, K. and Strosznajder, JB. "Cyclic GMP metabolism and its role in brain physiology". (2005) J Physiol Pharmacol 56 Suppl 2, 15-34.
2. Lucas, K.A. et al. "Guanylyl cyclases and signaling by cyclic GMP" (2000) Pharmacol Rev 52: 375-414.
3. Ashman, DF., et al., "Isolation of adenosine 3', 5'-monophosphate and guanosine 3', 5'-monophosphate from rat urine". (1963) , Biochem Biophys Res Comm, 11: 330-4.
4. Potter LR, Abbey-Hosch S, and Dickey DM. "Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions". (2006) Endocr Rev 27: 47-72.
5. Waldman, SA and Murad, F., "Cyclic GMP synthesis and function". (1987) Pharmacol Revs, 39: 163-197.
6. Tremblay J, Gerzer R, and Hamet P., "Cyclic GMP in cell function". (1988) , Adv. 2nd Messanger & Phosphoprotein Res., 22: 319-383.
7. Matsumoto T, Kobayashi T, and Kamata K "Phosphodiesterases in the vascular system." (2003) J Smooth Muscle Res 39: 67-86.

ASSAY PRINCIPLE

The DetectX® Direct Cyclic GMP (cGMP) Immunoassay Kit is designed to quantitatively measure cGMP present in lysed cells, EDTA plasma, urine, saliva and tissue culture media samples. Please read the complete kit insert before performing this assay.

For samples where the levels of cGMP are expected to be relatively high, the regular format for the assay can be used. For samples with expected low levels of cGMP, an optional acetylation protocol can be used.

The kit is unique in that all samples and standards are diluted into an acidic Sample Diluent, which contains special additives and stabilizers, for cGMP measurement. This allows plasma, urine and saliva samples to be read in an identical manner to lysed cells. Acidified samples of cGMP are stable and endogenous phosphodiesterases are inactivated in the Sample Diluent. A cGMP 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 mouse IgG is provided and a neutralizing Plate Primer solution is added to all the used wells. Standards or diluted samples, either with or without acetylation, are pipetted into the primed wells. A cGMP-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a mouse monoclonal antibody to cGMP to each well. After a 2 hour incubation, the plate is washed and substrate is added. The substrate reacts with the bound cGMP-peroxidase conjugate. 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 cGMP 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.
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
Prostaglandin E_2 ELISA Kits	K051-H1/H5
Protein Kinase A (PKA) Colorimteric Activity Kit	K027-H1

SUPPLIED COMPONENTS

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

Kit K020-H1 **or** -H5	1 **or** 5 Each	Catalog Number X012-1EA

Cyclic GMP Standard
Cyclic GMP at 640 pmol/mL in a special stabilizing solution.

Kit K020-H1 **or** -H5	125 µL **or** 625 µL	Catalog Number C080-125UL **or** -625UL

DetectX® Cyclic GMP Antibody
A mouse monoclonal antibody specific for cyclic GMP.

Kit K020-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C078-3ML **or** -13ML

DetectX® Cyclic GMP Conjugate
A cyclic GMP-peroxidase conjugate in a special stabilizing solution.

Kit K020-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C079-3ML **or** -13ML

Sample Diluent Concentrate
Contains special stabilizers and additives. The 4X concentrate must be diluted with deionized or distilled water. **CAUSTIC**.

Kit K020-H1 **or** -H5	12 mL **or** 60 mL	Catalog Number X074-12ML **or** -60ML

Plate Primer
A neutralizing solution containing special stabilizers and additivies.

Kit K020-H1 **or** -H5	25 mL	Catalog Number X073-25ML

Acetic Anhydride
Acetic Anhydride **WARNING: Corrosive Lachrymator**

	2 mL	Catalog Number X071-2ML

Triethylamine
Triethylamine **WARNING: Corrosive Lachrymator**

	4 mL	Catalog Number X072-4ML

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

Kit K020-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K020-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

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

Kit K020-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K020-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.

Borosilicate glass test tubes.

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 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**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 9.

The Sample Diluent Concentrate is acidic. The Stop Solution is 1M HCl. These solutions should not come in contact with skin or eyes. Take appropriate precautions when handling these reagents.

The kit uses acetic anhydride and triethylamine as acetylation reagents. Triethylamine and acetic anhydride are lachrymators. **Caution - corrosive, flammable, and harmful vapor. Use in hood with proper ventilation and wear appropriate protective safety wear.**

SAMPLE TYPES

This assay has been validated for lysed cells, saliva, urine, EDTA plasma samples and for tissue culture media samples. Samples should be stored at -70°C for long term storage. 24-Hour urine samples may need to have 1 mL concentrated hydrochloric acid added for every 100 mL volume to act as a preservative. Samples containing visible particulate should be centrifuged prior to using.

Cyclic GMP is identical across all species and we expect this kit may measure cGMP from sources other than human. The end user should evaluate recoveries of cGMP in other samples being tested.

After dilution in the Sample Diluent (see page 9) there may be some precipitation of proteins and the supernatant from the centrifuged samples used. After being diluted in Sample Diluent the samples can be assayed directly within 2 hours, or frozen at ≤ -70°C for later analysis. Severely hemolyzed samples should not be used in this kit.

For samples containing low levels of cGMP, the acetylated assay protocol must be used due to its enhanced sensitivity. **All standards and samples should be diluted in glass test tubes.**

SAMPLE PREPARATION

Cells

Cell lysis buffers containing high concentrations of SDS or other detergents may not be compatible with this assay or may require extra dilution. Please read Interferents section on page 22 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 the provided Sample Diluent. The acidic Sample Diluent contains detergents to lyse the cells, inactivate endogenous phosphodiesterases and stabilize the cGMP. Some cell types are extremely hardy and the end user should optimize the lysis conditions utilizing freeze-thaw cycles and ultrasonic treatments 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 the Sample Diluent for 10 minutes at room temperature. Cells can be scraped to dislodge them from the plate surface and cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥ 600 x g at 4°C for 15 minutes and assay the supernatant directly. If required, the tissue culture media can be assayed for cGMP 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 the Sample Diluent for 10 minutes at room temperature. Cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥ 600 x g at 4 °C for 15 minutes and assay the supernatant directly. If required, the tissue culture media can be assayed for cGMP as outlined below.

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. Add 1 mL of Sample Diluent for every 100 mg of tissue. Incubate in the Sample Diluent for 10 minutes on ice, and then centrifuge at ≥ 600 x g at 4°C for 15 minutes. Collect the supernatant and run in the assay immediately or store frozen at ≤ -70°C.

For samples that require 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 Sample Diluent for every mL of 5% TCA used to extract and run in the assay immediately or store at ≤ -70°C.

***Diethyl ether is extremely flammable and should be used in a hood.**

Tissue Culture Media

For measuring cGMP 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 ≥ 1:10 with the supplied Sample Diluent and acetylated prior to running in the Acetylated Format assay (page 16).

Urine Samples

Urine samples should be diluted ≥ 1:5 with the supplied Sample Diluent prior running in the assay. Due to the high concentration of cGMP in urine, samples may need to be diluted further.

Saliva Samples

Saliva samples should be diluted ≥ 1:4 with the supplied Sample Diluent prior running in the assay. See our Saliva Sample Handling Instructions at www.ArborAssays.com/assets/saliva-sample-protocol.pdf.

Use all samples within 2 hours of dilution in Sample Diluent.

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.

Sample Diluent

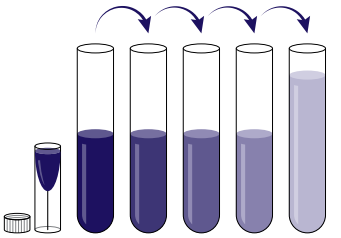
Prepare the Sample Diluent by diluting the Sample Diluent Concentrate 1:4, adding one part of the concentrate to three parts of deionized water. Once diluted this is stable at 4°C for 3 months.

REAGENT PREPARATION - REGULAR FORMAT

All standards and samples should be diluted in glass test tubes.

Standard Preparation - Regular Format

Label test tubes as #1 through #7. Pipet 380 µL of Sample Diluent into tube #1 and 200 µL into tubes #2 to #7. **The Cyclic GMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 20 µL of the cGMP stock solution to tube #1 and vortex completely. Take 200 µL of the cGMP 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 Cyclic GMP in tubes 1 through 7 will be 32, 16, 8, 4, 2, 1, and 0.5 pmol/mL.



Non-Acetylated	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Sample Diluent (µL)	**380**	200	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	**20**	200	200	200	200	200	200
Final Conc (pmol/mL)	32	16	8	4	2	1	0.5

Use Standards within 1 hour of preparation.

ASSAY PROTOCOL - REGULAR FORMAT

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cGMP 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. Add 50 µL of Plate Primer into all wells used. **FAILURE TO ADD PLATE PRIMER TO ALL WELLS FIRST WILL CAUSE ASSAY TO FAIL.**
3. Pipet 75 µL Sample Diluent into the non-specific binding (NSB) wells.
4. Pipet 50 µL of Sample Diluent into the maximum binding (B0 or Zero standard) wells.
5. Pipet 50 µL of samples or standards into wells in the plate. **NOTE: Sample Diluent will turn from orange to bright pink upon sample or standard addition to the Plate Primer in the wells.**
6. Add 25 µL of the DetectX® cGMP Conjugate to each well using a repeater pipet.
7. Add 25 µL of the DetectX® cGMP Antibody to each well, **except the NSB wells**, using a repeater pipet.
8. 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. If the plate is not shaken, signals bound will be approximately 25% lower.
9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
10. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
11. Incubate the plate at room temperature for 30 minutes without shaking.
12. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
14. Use the plate reader's built-in 4PLC software capabilities to calculate cGMP 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

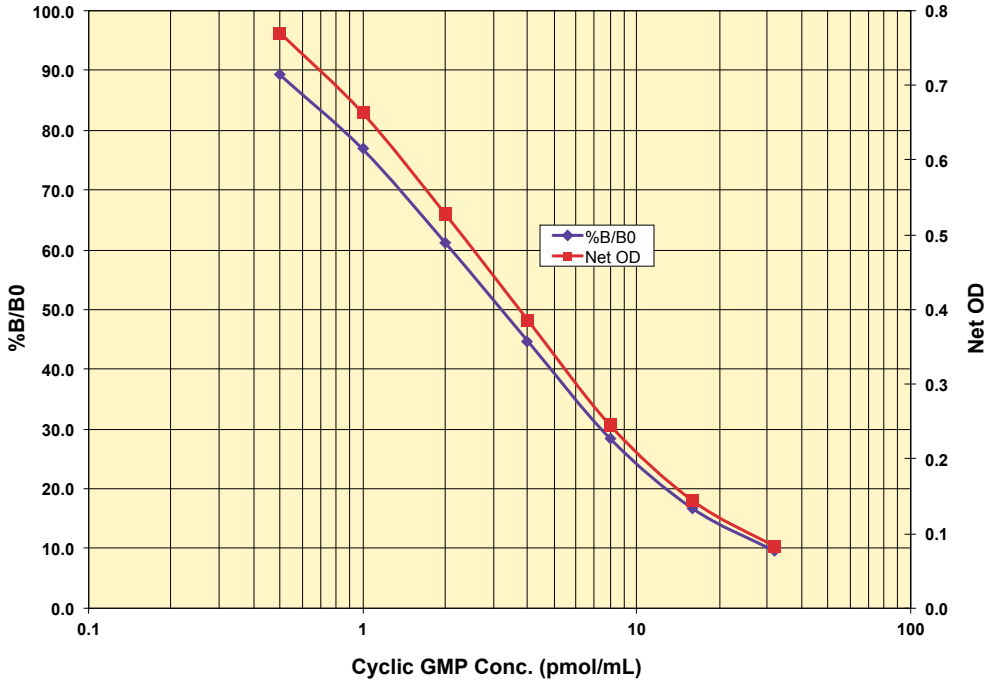
Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the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

Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-cyclic-gmp-direct-eia-kit-non-acetyl.assay

TYPICAL DATA - REGULAR FORMAT

Sample	Mean OD	Net OD	% B/B0	Cyclic GMP Conc. (pmol/mL)
NSB	0.081	0.000	-	-
Standard 1	0.163	0.082	9.5	32
Standard 2	0.225	0.144	16.7	16
Standard 3	0.326	0.245	28.4	8
Standard 4	0.467	0.386	44.7	4
Standard 5	0.609	0.528	61.2	2
Standard 6	0.744	0.663	76.8	1
Standard 7	0.851	0.770	89.2	0.5
B0	0.944	0.863	100	0
Sample 1	0.328	0.247	28.6	8.04
Sample 2	0.683	0.602	69.7	1.40

Typical Standard Curve - Regular Format



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

VALIDATION DATA - REGULAR FORMAT

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 0.28 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 of the zero standard and a low concentration human urine sample. **Limit of Detection was determined as 0.26 pmol/mL.**

ACETYLATED PROTOCOL - OVERVIEW

Use this format for any sample with low cGMP concentrations.

Prior to running the acetylated assay, all standards, samples and the Sample Diluent used for the B0 and NSB wells must be acetylated. Acetylation is carried out by adding 10 µL of the Acetylation Reagent (as prepared below) for each 200 µL of the standard, sample and Sample Diluent. Immediately vortex each treated standard, sample or Sample Diluent after addition of the Acetylation Reagent and use within 30 minutes of preparation.

Note: Upon Acetylation, all of the standards and samples diluted in the **orange** Sample Diluent will change to a pale **yellow** color.

REAGENT PREPARATION - ACETYLATED FORMAT

Acetylation Reagent

Working in a fume hood mix one part of Acetic Anhydride with 2 parts of Triethylamine in a glass test tube. Use the following table to help determine the amount of Acetylation Reagent to make.

Reagents	Number of Samples to be Tested			
	20	**40**	**100**	**200**
Acetic Anhydride Volume (µL)	200	400	1,000	2,000
Triethylamine Volume (µL)	400	800	2,000	4,000
Acetylation Reagent Vol (mL)	**0.6**	**1.2**	**3**	**6**

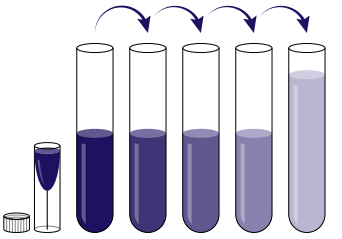
Use the Acetylation Reagent within 60 minutes of preparation.

REAGENT PREPARATION - ACETYLATED FORMAT

All standards and samples should be diluted in glass test tubes.

Standard Preparation – Acetylated Format

Label test tubes as #1 through #7. Pipet 620 µL of Sample Diluent into tube #1 and 300 µL into tubes #2 to #7. **The Cyclic GMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 20 µL of the cGMP stock solution to tube #1 and vortex completely. Take 300 µL of the cGMP 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 Cyclic GMP in tubes 1 through 7 will be 20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 pmol/mL.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Sample Diluent (µL)	620	300	300	300	300	300	300
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	20	300	300	300	300	300	300
Final Conc (pmol/mL)	20	10	5	2.5	1.25	0.625	0.313

Standard and Sample Acetylation

Pipet 300 µL of Sample Diluent into a glass tube to act as the Zero standard/NSB tube. Add 15 µL of Acetylation Reagent to this tube and vortex immediately. Proceed to assay within 30 minutes.

Pipet 200 µL of each standard or sample to be tested into fresh glass tubes. Add 10 µL of the Acetylation Reagent into each tube and vortex immediately. Proceed to assay within 30 minutes.

NOTE: Samples and Sample Diluent will turn from orange to pale yellow upon acetylation.

Use Acetylated Standards and Samples within 30 minutes of preparation.

ASSAY PROTOCOL - ACETYLATED FORMAT

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine cGMP 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. Add 50 µL of Plate Primer into all wells used. **FAILURE TO ADD PLATE PRIMER TO ALL WELLS FIRST WILL CAUSE ASSAY TO FAIL.**
3. Pipet 75 µL acetylated Sample Diluent into the non-specific binding (NSB) wells.
4. Pipet 50 µL of acetylated Sample Diluent into the maximum binding (B0 or Zero standard) wells.
5. Pipet 50 µL of acetylated samples or standards into wells in the plate.
6. Add 25 µL of the DetectX® cGMP Conjugate to each well using a repeater pipet.
7. Add 25 µL of the DetectX® cGMP Antibody to each well, **except the NSB wells**, using a repeater pipet.
8. 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. If the plate is not shaken, signals bound will be approximately 25% lower.
9. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
10. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
11. Incubate the plate at room temperature for 30 minutes without shaking.
12. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
13. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
14. Use the plate reader's built-in 4PLC software capabilities to calculate cGMP 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 to obtain neat sample values.

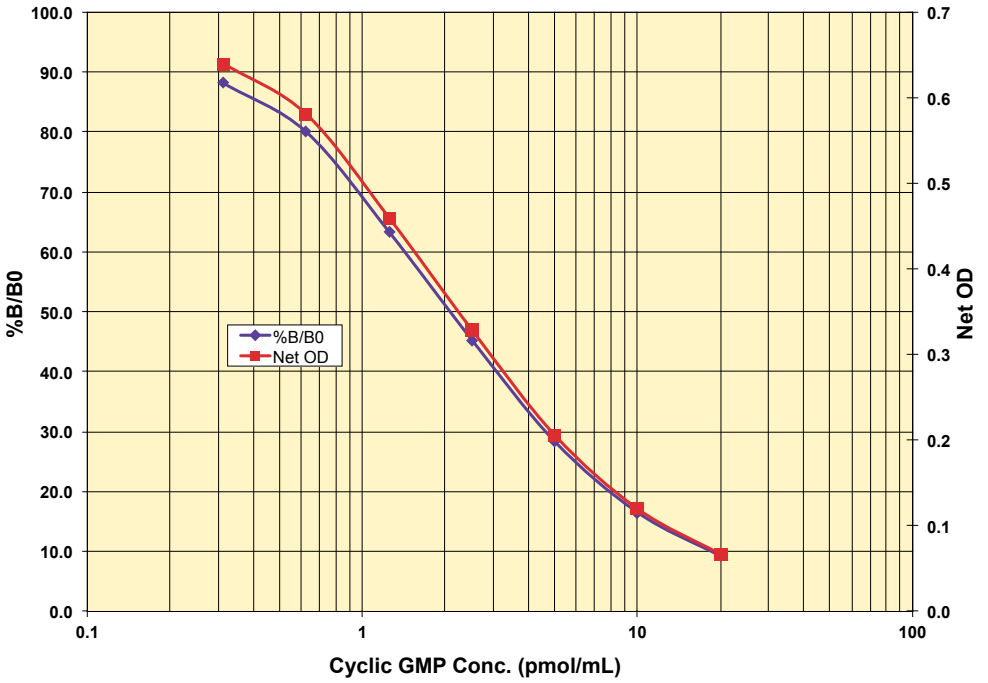
Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-cyclic-gmp-direct-eia-kit-acetyl.assay

TYPICAL DATA - ACETYLATED FORMAT

Sample	Mean OD	Net OD	% B/B0	Cyclic GMP Conc. (pmol/mL)
NSB	0.098	0.000	-	-
Standard 1	0.165	0.067	9.2	20
Standard 2	0.218	0.120	16.5	10
Standard 3	0.304	0.206	28.4	5
Standard 4	0.427	0.329	45.3	2.5
Standard 5	0.558	0.460	63.4	1.25
Standard 6	0.679	0.581	80.0	0.625
Standard 7	0.738	0.640	88.2	0.3125
B0	0.824	0.726	100.0	0
Sample 1	0.325	0.227	31.3	4.35
Sample 2	0.505	0.407	56.1	1.69

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

Typical Standard Curve - Acetylated Format



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

VALIDATION DATA - ACETYLATED FORMAT

Sensitivity and Limit of Detection - Acetylated

Sensitivity was calculated by comparing the OD's for twenty wells run for each of the acetylated B0 and standard #7. The sensitivity was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 0.188 pmol/mL.**

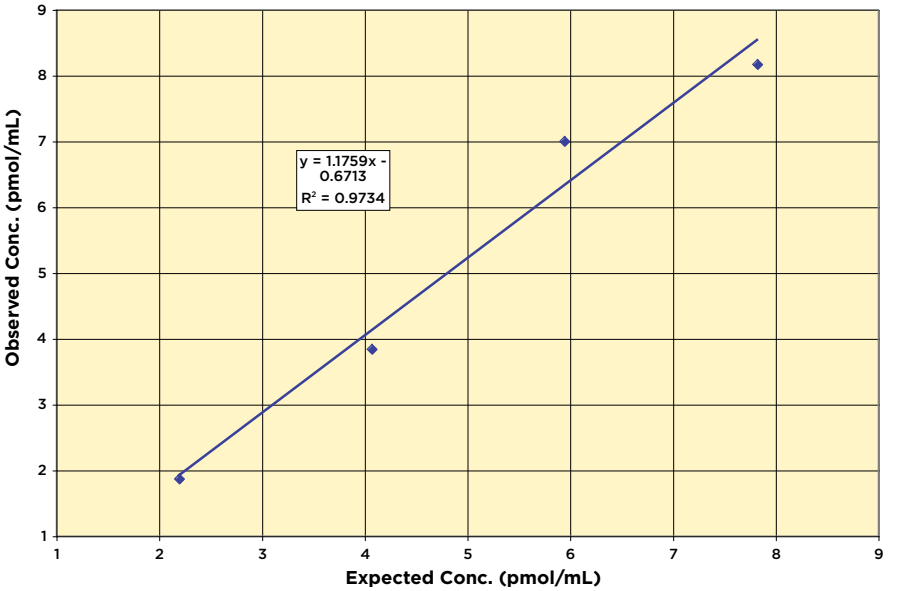
The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of acetylated zero standard and a low concentration acetylated human sample. **Limit of Detection was determined as 0.210 pmol/mL.**

VALIDATION DATA - REGULAR AND ACETYLATED

Linearity

Linearity was determined by taking two human urine samples, one with a low cGMP level of 0.3 pmol/mL and one with a higher level of 9.7 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 Urine	Low Urine	Expected Conc. (pmol/mL)	Observed Conc. (pmol/mL)	% Recovery
80%	20%	7.8	8.2	104.4
60%	40%	5.9	7.0	117.7
40%	60%	4.1	3.8	94.3
20%	80%	2.2	1.9	85.2
			Mean Recovery	**100.4%**



Intra Assay Precision - Regular

Three human urine samples were diluted with Sample Diluent and run in replicates of 20 in an assay. The mean and precision of the calculated cGMP concentrations were:

Sample	Cyclic GMP Conc. (pmol/mL)	%CV
1	8.5	6.3
2	4.0	7.8
3	1.2	13.6

Inter Assay Precision - Regular

Three human urine samples were diluted with Sample Diluent and run in duplicates in twelve assays run over multiple days by three operators. The mean and precision of the calculated cGMP concentrations were:

Sample	Cyclic GMP Conc. (pmol/mL)	%CV
1	8.5	6.5
2	4.3	7.0
3	1.5	8.3

Intra Assay Precision - Acetylated

Three human urine samples were diluted with Sample Diluent, acetylated and run in replicates of 20 in an assay. The mean and precision of the calculated cGMP concentrations were:

Sample	Cyclic GMP Conc. (pmol/mL)	%CV
1	8.1	3.8
2	3.8	5.2
3	1.2	8.2

Inter Assay Precision - Acetylated

Three human urine sample weres diluted with Sample Diluent, acetylated and run in duplicates in twelve assays run over multiple days by three operators. The mean and precision of the calculated cGMP concentrations were:

Sample	Cyclic GMP Conc. (pmol/mL)	%CV
1	8.7	12.3
2	4.3	14.3
3	1.5	14.2

SAMPLE VALUES

Four human plasma samples were tested in the assay. Samples were diluted 10-20 fold and run in the acetylated format assay. Values ranged from 5.0 to 8.6 pmol/mL with an average for the samples of 7.16 pmol/mL. Seven normal human urine samples were diluted 5-320 fold in Sample Diluent and values ranged in the neat samples from 19.9 to 3,305 pmol/mL with an average for the samples of 461.8 pmol/mL.

CROSS REACTIVITY

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

Nucleotide	Cross Reactivity (%)
Cyclic GMP	100%
Cyclic AMP	< 0.1%
GMP	< 0.1%
AMP	< 0.1%
ATP	< 0.1%

INTERFERENTS

A variety of detergents were tested as possible interfering substances in the assay. CHAPS at 0.1% increased measured cGMP by 8.6% and Tween 20 at 1.0% increased measured cGMP by 6%. Triton X-100 at 2% decreased measured cGMP by 6.1%. SDS at 0.05% decreased measured cGMP by 9%. CTAC above 0.05% should not be used in the assay.

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.

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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.

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