cyclic GMP Complete ELISA Kit

Catalog Number KA0321

96 assays

Version: 06

Intended for research use only

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Table of Contents

Introduction 3

Background 3

Principle of the Assay 3

General Information 4

Materials Supplied 5

Storage Instruction 5

Materials Required but Not Supplied 5

Precautions for Use 6

Assay Protocol 7

Reagent Preparation 7

Sample Preparation 9

Assay Procedure 10

Data Analysis 12

Calculation of Results 12

Performance Characteristics 15

Resources 18

References 18

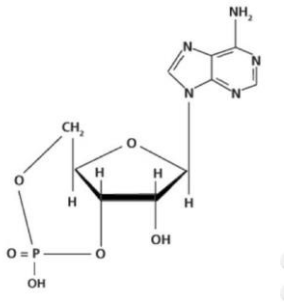
Plate Layout 19

Introduction

Background

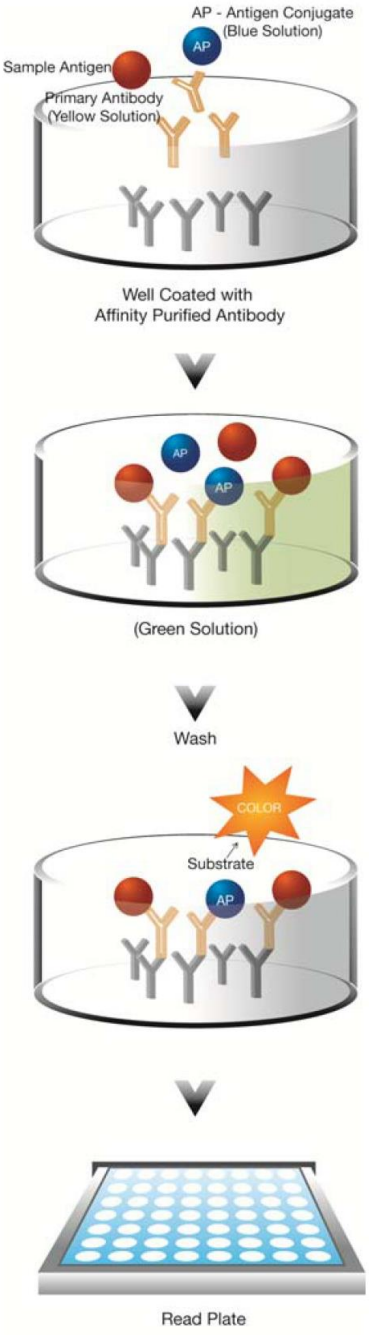
The cyclic GMP Complete ELISA Kit is a competitive immunoassay for the quantitative determination of cyclic GMP in cells and tissue treated with 0.1 M HCl, in addition to culture supernatants, saliva, and serum. The optional acetylated assay format provides an approximate 10-fold increase in sensitivity and is ideal for samples with extremely low levels of cGMP. If expected levels of cGMP are unknown, the investigator may evaluate a few samples in the non-acetylated format in order to determine if higher sensitivity is required. Guanosine 3',5'-cyclic monophosphate (cyclic GMP; cGMP) was identified in 1963[1] . It has been shown to be present at levels typically 10-100 fold lower than cGMP in most tissues and is formed by the action of the enzyme guanylate cyclase on GTP. It is involved in a number of important biological reactions. Some hormones, such as acetylcholine, insulin, and oxytocin, as well as certain other chemicals like serotonin and histamine cause an increase in cGMP levels[2,3]. Stimulators of guanylate cyclase such as the vasodilators niroprusside, nitroglycerin, sodium nitrate, and nitric oxide (NO) also stimulate cGMP levels[4] . Peptides, such as atrial natriuretic peptide (ANP), that relax smooth muscle also increase cGMP concentrations[5] . cGMP has been confirmed as a second messenger for ANP[6] . NO can be synthesized from L-arginine and diffuse through cell membranes[7,8]. The interaction of NO with guanylate cyclase allows cGMP to act as a third messenger in some cells[9].

cyclic GMP



Principle of the Assay

1. Standards and samples are added to wells coated with a GxR IgG antibody. A blue solution of cGMP conjugated to alkaline phosphatase is then added, followed by a yellow solution of rabbit polyclonal antibody to cGMP.
2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the cGMP in the sample or conjugate. The plate is washed, leaving only bound cGMP.
3. pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the cGMP conjugate.
4. Stop solution is added. The yellow color is read at 405 nm. The amount of signal is indirectly proportional to the amount of cGMP in the sample.


AP - Antigen Conjugate
(Blue Solution)
AP
Sample Antigen
Primary Antibody
(Yellow Solution)
Well Coated with
Affinity Purified Antibody
AP
AP
(Green Solution)
Wash
COLOR
Substrate
AP
Read Plate

General Information

Materials Supplied

List of component

Component	Description	Amount
Assay Buffer 2 Concentrate	Sodium acetate buffer containing proteins and sodium azide.	27 mL
0.1 M HCl	0.1 M hydrochloric acid in water.	27 mL
cyclic GMP Standard	A solution of 5,000 pmol/mL cGMP.	0.5 mL
Acetylation Kit	a. Triethylamine	2 mL
	b. Acetic Anhydride	1 mL
Goat anti-Rabbit IgG Microtiter Plate	A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody.	One plate of 96 wells
Neutralizing Reagent	-	5 mL
cGMP Antibody	A yellow solution of rabbit polyclonal antibody to cGMP.	5 mL
cGMP Conjugate	A blue solution of cGMP conjugated to alkaline phosphatase.	5 mL
Wash Buffer Concentrate	Tris buffered saline containing detergents.	27 mL
pNpp Substrate	A solution of p-nitrophenyl phosphate.	20 mL
Stop Solution	A solution of trisodium phosphate in water.	5 mL
Plate Sealer	-	1 slice

Storage Instruction

All components of this kit, except the Conjugate and Standard, are stable at 4°C until the kit's expiration date. Upon receipt, store the Conjugate and Standard at -20°C.

Materials Required but Not Supplied

- ✓ Deionized or distilled water
- ✓ Precision pipets for volumes between 5 µL and 1,000 µL
- ✓ Repeater pipet for dispensing 50 µL and 200 µL
- ✓ Disposable beakers for diluting buffer concentrates
- ✓ Graduated cylinders
- ✓ Microplate shaker
- ✓ Lint-free paper toweling for blotting
- ✓ Microplate reader capable of reading at 405 nm

Precautions for Use

- ✓ For research use only. Not for use in diagnostic procedures.
- ✓ Do not mix components from different kit lots or use reagents beyond kit expiration date.
- ✓ HCl is caustic. Keep tightly capped.
- ✓ The standard should be handled with care due to the known and unknown effects of the antigen.
- ✓ Triethylamine and acetic anhydride are lachrymators. Caution- corrosive flammable and harmful vapor.
- ✓ Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.
- ✓ Activity of conjugate is affected by concentrations of chelators > 10 mM (such as EDTA and EGTA).
- ✓ Reagents require separate storage conditions.
- ✓ Stop solution is caustic. Keep tightly capped.

Assay Protocol

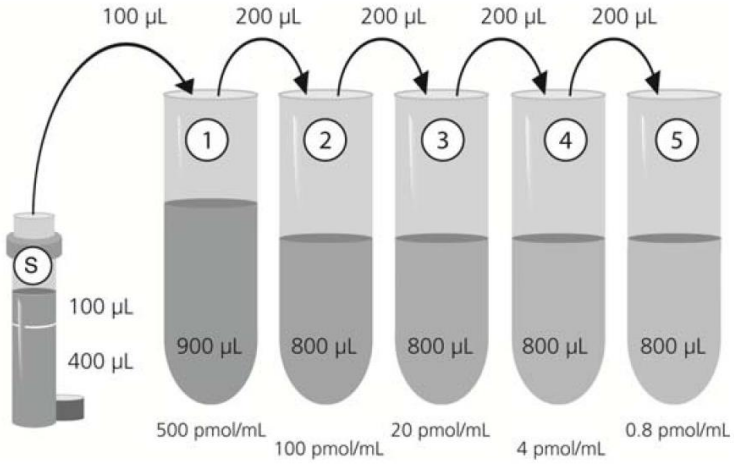
Reagent Preparation

- Wash Buffer

  Prepare the Wash Buffer by diluting 5 mL of the supplied Wash Buffer Concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

- cGMP Standard, non-acetylated format

  Three diluent options are available for the preparation of the standard curve, NSB, and Bo wells. For cell lysates and tissue samples prepared in 0.1 M HCl, use the supplied 0.1 M HCl as the standard diluent. Use Assay Buffer 2 for the standard diluent when the sample is serum, plasma, urine or saliva. For culture supernatants, use the same non-conditioned media for the standard diluent.



Allow the 5,000 pmol/mL standard stock to warm to room temperature. Label five 12 mm x 75 mm tubes #1 through #5. Pipet 900 µL of the appropriate sample diluent into tube #1. Pipet 800 µL of the appropriate sample diluent into tubes #2 through #5. Add 100 µL of the 5,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 200 µL of tube #1 to tube #2 and vortex thoroughly. Add 200 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Diluted standards should be used within 60 minutes of preparation.

The concentrations of cGMP in the tubes are labeled above.

✓ Dilution tables for making Standards 1-5 (Non-Acetylated Version)

Std.	*Std. Dil. Vol. (µL)	Vol. Added (µL)	cGMP Conc. (pmol/mL)
1	900	100, Stock	500
2	800	200, Std. 1	100
3	800	200, Std. 2	20
4	800	200, Std. 3	4
5	800	200, Std. 4	0.8

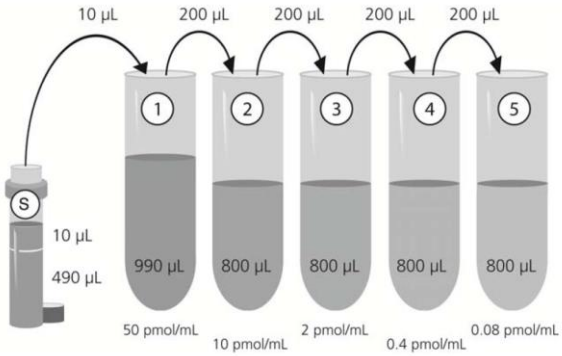
*Three options are available for the sample diluent. Assay Buffer 2, 0.1 M HCl, or non-conditioned culture media.

- Acetylation Reagent (optional)

  Prepare the Acetylating Reagent by adding 0.5 mL of Acetic Anhydride to 1 mL of Triethylamine. *Note that this volume is sufficient to add to 30 mL of diluted standards and samples.* Use the prepared reagent within 60 minutes of preparation. Discard any unused portion of the Acetylating Reagent.

- cGMP Standard, acetylated format (optional)

  Three diluent options are available for the preparation of the standard curve, NSB, and Bo wells. For cell lysates and tissue samples prepared in 0.1 M HCl, use the supplied 0.1 M HCl as the standard diluent. Use Assay Buffer 2 for the standard diluent when the sample is serum or saliva. For culture supernatants, use the same non-conditioned media for the standard diluent.



Allow the 5,000 pmol/mL standard stock to warm to room temperature. Label five 12mm x 75mm tubes #1 through #5. Pipet 990 µL of the appropriate sample diluent into tube #1. Pipet 800 µL of the appropriate sample diluent into tubes #2 through #5. Add 10 µL of the 5,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 200 µL of tube #1 to tube #2 and vortex thoroughly. Add 200 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL of the standard or sample. Add the Acetylating Reagent directly to the diluted standard or sample and vortex immediately after the addition of the Acetylating Reagent.

Label one 12 mm x 75 mm tube as the Bo/NSB tube. Pipet 1 mL of the appropriate standard diluent into this tube. Add 50 µL of the Acetylating Reagent to the Bo/NSB tube and use in Steps 2 and 3 of the Assay Procedure.

The acetylated standards should be used within 30 minutes of preparation.

The concentrations of cGMP in the tubes are labeled above.

✓ Dilution tables for making Standards 1-5 (Acetylated Version)

Std.	*Std. Dil. Vol. (µL)	Vol. Added (µL)	cGMP Conc. (pmol/mL)
1	990	10, Stock	50
2	800	200, Std. 1	10
3	800	200, Std. 2	2
4	800	200, Std. 3	0.4
5	800	200, Std. 4	0.08

*Three options are available for the sample diluent. Assay Buffer 2, 0.1 M HCl, or non-conditioned culture media.

✓ Acetylation Procedure (Optional)

1. Prepare Acetylating Reagent by mixing 0.5 mL of Acetic Anhydride with 1 mL of Triethylamine.
2. Add 10 µL of the Acetylating Reagent to 200 µL of standard (including 0 and NSB) or sample. Vortex immediately.
3. Assay the acetylated standards and samples within 30 minutes.

Note:

1. *Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.*
2. *Triethylamine and acetic anhydride are lachrymators. Caution: corrosive, flammable, and harmful vapor.*

Sample Preparation

✓ Sample Handling

Treatment of cells and tissue with the supplied 0.1 M HCl will stop endogenous phosphodiesterase activity and allow for the direct measurement of these samples in the assay without evaporation or further processing. Recommended treatment protocols follow. Samples containing rabbit IgG will interfere with the assay. EDTA plasma may precipitate during acetylation.

Biological fluids, such as serum, plasma, urine, and saliva, should be diluted in Assay Buffer 2 and run directly in the assay. Culture supernatants should also be diluted in Assay Buffer 2 and the same non-conditioned media diluted with the assay buffer used as the standard diluent.

Please note that some samples may contain high levels of cGMP and additional dilution may be required. Samples with low levels of cGMP may be assayed in the acetylated format or the samples may be concentrated.

Note:

1. *If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.*
2. *Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Repeated freeze/ thaw cycles should be avoided.*

- Protocol for Cell Lysates

The concentration of cells used must be optimized for the specific cell line and treatment conditions. Cells may be grown in typical containers such as Petri dishes, culture plates (e.g., 48-well, 12-well, or 96-well), culture flasks, etc. Some cells are particularly hardy (e.g., bacteria) and may require the addition of 0.1 to 1% Triton X-100 to the 0.1 M HCl for enhanced lysis. If Triton X-100 is added to samples it should also be added to the standard dilution as a modest increase in optical density may occur.

1. Pellet suspension cells and aspirate the media. Treat cells with 0.1 M HCl. A general starting concentration of 1 x 10^6 cells per mL of 0.1 M HCl is recommended. Remove the media from adherent cells and add enough 0.1 M HCl to cover the bottom of the plate. Avoid over-diluting the sample with an excessive volume of HCl. Please note that the culture media may be saved and

assayed separately, if desired.

2. Incubate the cells in 0.1 M HCl for 10 minutes at room temperature. Inspect the cells under a microscope to ensure uniform lysis. Continue incubating for an additional 10 minutes, if necessary.
3. Centrifuge ≥ 600 x g to pellet the cellular debris.
4. The supernatant may be assayed immediately or stored frozen for later analysis.

   Note: Standards must be diluted in 0.1 M HCl and Neutralizing Reagent used.

- Protocol for Tissue Samples

1. After collection, tissue samples should be flash frozen in liquid nitrogen. If analysis cannot be carried out immediately, store tissue at -80˚C.
2. Grind frozen tissue to a fine powder under liquid nitrogen in a stainless steel mortar.
3. When liquid nitrogen has evaporated, weigh the frozen tissue and homogenize in 10 volumes of 0.1 M HCl (e.g., 0.1 g of tissue should be homogenized in 1 mL of 0.1 M HCl).
4. Centrifuge ≥ 600 x g to pellet the debris (~10 minutes).
5. The supernatant may be further diluted in the 0.1 M HCl provided and run directly in the assay or stored frozen for later analysis.

Note: Standards must be diluted in 0.1 M HCl and Neutralizing Reagent used.

✓ Sample Recoveries

cGMP standard was spiked into the following matrices diluted with Assay Buffer 2 and measured in the kit. The results were as follows:

	Non-Acetylated Format		Acetylated Format	
Sample	% Recovery	Recommended Dilution,	% Recovery	Recommended Dilution,
Tissue Culture Media	101.7%	1:100	95.8	undiluted
Human Saliva	102.9%	1:10		
Human Serum	101.3%	≥ 1:10		Not recommended
Human Heparin Plasma	104.4%	1:10		
Human EDTA Plasma	115.0%	≥ 1:10	93.6	≥ 1:2
Human Urine	97.7%	≥ 1:100		

0.1 M HCl should not be used to dilute culture supernatant, serum, plasma, urine or saliva samples.

Assay Procedure

Refer to the Plate Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C..

Note: If the acetylated format of the assay is to be run, all standards, samples, and the diluent for the NSB and Bo wells must be acetylated as per the instructions in the Reagent Preparation section. Acetylated standards and samples must be used within 30 minutes.

- Bring all reagents to room temperature for at least 30 minutes prior to opening.
- All standards and samples should be run in duplicate.
- Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.
- Pipet the reagents to the sides of the wells to avoid possible contamination.
- Prior to the addition of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

1. If using samples prepared in 0.1 M HCl, pipet 50 µL of Neutralizing Reagent into each well except the Total Activity (TA) and Blank wells. Do not add Neutralizing Reagent for the other sample diluent options.
2. Pipet 100 µL of the appropriate standard diluent (Assay Buffer 2, 0.1 M HCl, or non-conditioned culture media) into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells.
3. Add 50 µL of the appropriate standard diluent to the NSB wells.
4. Pipet 100 µL of Standards #1 through #5 to the bottom of the appropriate wells.
5. Pipet 100 µL of the samples to the bottom of the appropriate wells.
6. Pipet 50 µL of the blue conjugate into each well except the TA and Blank wells.
7. Pipet 50 µL of the yellow antibody into each well except the Blank, TA, and NSB wells.
   Note: Every well used should be green in color except the NSB wells which should be blue. The Blank and TA wells are empty at this point and have no color.
8. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature.
9. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint-free paper towel to remove any remaining wash buffer.
10. Pipet 5 µL of the blue conjugate to the TA wells.
11. Add 200 µL of the substrate solution into each well.
12. Incubate for 1 hour at room temperature without shaking.
13. Pipet 50 µL stop solution into each well.
14. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

Data Analysis

Calculation of Results

Several options are available for the calculation of the concentration of cGMP in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.
   Average Net OD = Average OD – Average NSB OD
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Plot the Percent Bound (B/Bo) versus concentration of cGMP for the standards. Approximate a straight line through the points. The concentration of cGMP of the unknowns can be determined by interpolation.

   Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

   To normalize for protein content, divide the resulting picomole per mL determinations (pmol/mL) by the total protein concentration (mg/mL) in each sample. This is expressed as pmol cGMP per mg of total protein.

   Note: Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

✓ Typical Results

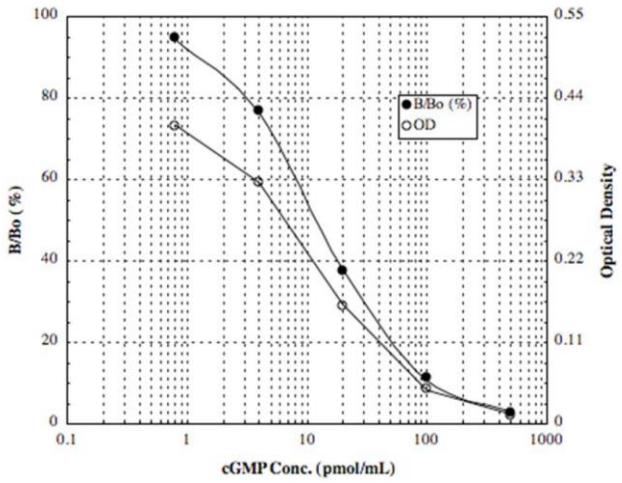
The results shown below are for illustration only and should not be used to calculate results from another assay.

	Non-acetylated assay format in Assay Buffer 2			Acetylated assay format in Assay Buffer 2		
Sample	Average Net OD	Percent Bound	cGMP (pmol/mL)	Average Net OD	Percent Bound	cGMP (pmol/mL)
Blank (mean)	(0.086)	---	---	(0.091)	---	---
TA	0.251	---	---	0.254	---	---
NSB	-0.001	---	---	-0.008	---	---
Bo	0.425	100%	0	0.290	100%	0
S1	0.012	2.8%	500	0.008	2.8%	50
S2	0.049	11.5%	100	0.026	9.0%	10
S3	0.160	37.7%	20	0.088	30.5%	2
S4	0.327	76.9%	4	0.191	65.9%	0.5
S5	0.403	94.8%	0.8	0.254	87.6%	0.08
Unknown 1	0.087	20.9%	47	0.052	17.9%	4.2
Unknown 2	0.367	86.4%	2.2	0.086	29.8%	2.1

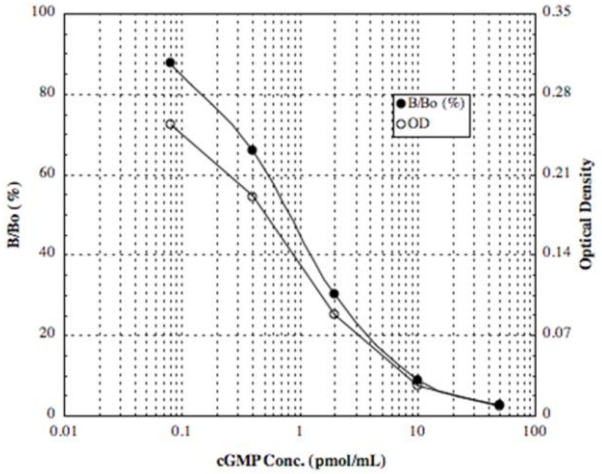
	Non-acetylated assay format in 0.1 M HCl			Acetylated assay format in 0.1 M HCl		
Sample	Average Net OD	Percent Bound	cGMP (pmol/mL)	Average Net OD	Percent Bound	cGMP (pmol/mL)
Blank (mean)	(0.094)	---	---	(0.123)	---	---
TA	0.298	---	---	0.335	---	---
NSB	0.000	---	---	-0.002	---	---
Bo	0.478	100%	0	0.281	100%	0
S1	0.015	3.1%	500	0.008	2.8%	50
S2	0.062	13.0%	100	0.032	11.4%	10
S3	0.190	39.7%	20	0.098	34.9%	2
S4	0.354	74.1%	4	0.220	78.3%	0.4
S5	0.451	94.4%	0.8	0.279	99.3%	0.08
Unknown 1	0.145	30.5%	31	0.023	8.2%	14
Unknown 2	0.344	71.9%	4.5	0.055	19.9%	4.4

✓ Typical Standard Curve

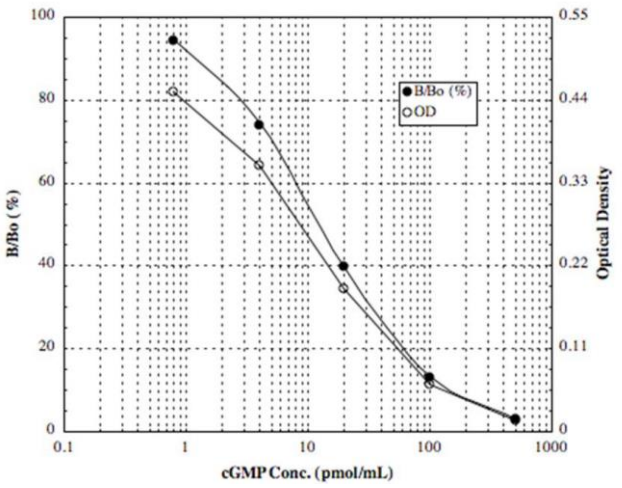
Non-acetylated assay format in Assay Buffer 2



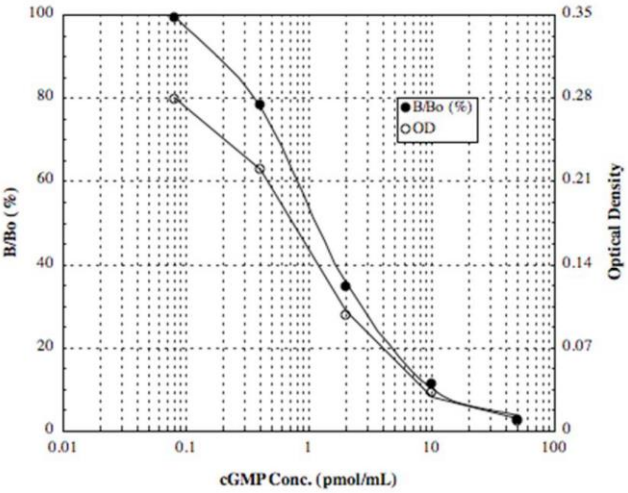
Acetylated assay format in Assay Buffer 2



Non-acetylated assay format in 0.1 M HCl



Acetylated assay format in 0.1 M HCl



Performance Characteristics

- Specificity

  The cross reactivities for a number of related compounds were determined by diluting the cross reactants in the kit assay buffer at a concentration of ten times the high standard. These samples were then measured in the assay.

Compound	Cross Reactivity
cGMP	100%
GMP	<0.001%
GTP	<0.001%
cAMP	<0.001%
AMP	<0.001%
ATP	<0.001%
cUMP	<0.001%
CTP	<0.001%

- Sensitivity

✓ Assay Buffer 2

The sensitivity of the assay, defined as the concentration of cGMP measured at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 0.420 pmol/mL in the non-acetylated assay format and 0.043 pmol/mL in the acetylated assay format.

✓ 0.1 M HCl

The sensitivity of the assay, defined as the concentration of cGMP measured at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 0.604 pmol/mL in the non-acetylated assay format and 0.059 pmol/mL in the acetylated assay format.

- Linearity

  A buffer sample containing cGMP was serially diluted 1:2 in the kit assay buffer and measured in the assay. The results are shown in the table below.

	Non-acetylated			Acetylated		
Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	45.6	---	---	3.4	---
1:2	22.8	24.6	108%	1.7	1.7	100%
1:4	11.4	13.5	118%	0.85	0.89	105%
1:8	5.7	6.3	111%	0.43	0.42	98%
1:16	2.9	3.0	103%	0.21	0.19	90%
1:32	1.4	1.9	135%	---	---	---
1:64	0.71	0.96	135%	---	---	---
1:128	0.36	0.38	106%	---	---	---

A 0.1M HCl sample containing cGMP was serially diluted 1:2 in the 0.1M HCl diluent and measured in the assay. The results are shown in the table below.

	Non-acetylated			Acetylated		
Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	87.4	---	---	14.4	---
1:2	43.7	48.8	112%	7.2	9.4	130%
1:4	21.9	25.4	116%	3.6	4.4	122%
1:8	10.9	10.1	93%	1.8	2.2	122%
1:16	5.5	6.3	115%	0.90	1.3	144%
1:32	2.7	3.3	122%	0.45	0.63	140%
1:64	---	---	---	0.23	0.22	96%

- Precision

✓ Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing cGMP in a single assay.

Non-Acetylated Format				Acetylated Format			
In Assay Buffer 2		In 0.1 M HCl		In Assay Buffer 2		In 0.1 M HCl	
pmol/mL	%CV	pmol/mL	%CV	pmol/mL	%CV	pmol/mL	%CV
1.5	5.2	1.6	4.4	0.54	6.5	0.58	9.6
16.6	4.0	9.9	7.9	1.5	4.6	1.4	3.6
481	7.6	115	6.6	6.8	4.5	5.4	3.5

✓ Inter-assay precision was determined by measuring buffer controls of varying cGMP concentrations in multiple assays over several days.

Non-Acetylated Format				Acetylated Format			
In Assay Buffer 2		In 0.1 M HCl		In Assay Buffer 2		In 0.1 M HCl	
pmol/mL	%CV	pmol/mL	%CV	pmol/mL	%CV	pmol/mL	%CV
1.8	13.7	2.1	6.0	0.70	5.9	0.35	11
16.9	3.5	8.5	9.9	2.0	6.2	3.6	8.4
359	5.0	92	6.9	8.6	6.8	10	4.6

Resources

References

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Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 1	Std 5									
B	Blank	Std 1	Std 5									
C	TA	Std 2										
D	TA	Std 2										
E	NSB	Std 3										
F	NSB	Std 3										
G	B0	Std 4										
H	B0	Std 4										