ab133027 – Cyclic GMP Direct ELISA Kit

Instructions for Use

For quantitative detection of Cyclic GMP in tissue culture media treated with 0.1M HCl.

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 11 January 2019

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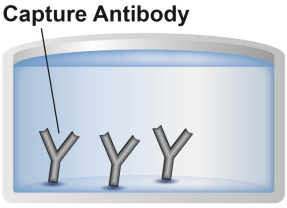
1. BACKGROUND

Abcam's Cyclic GMP (cGMP) Direct *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Cyclic GMP in tissue culture media treated with 0.1 M HCl.

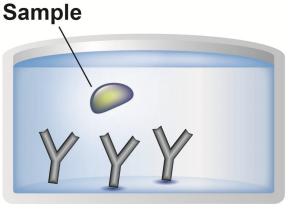
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-cGMP antigen and a polyclonal rabbit antibody specific to cGMP. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of cGMP captured in the plate.

Guanosine 3', 5'-cyclic monophosphate (cyclic GMP) has been shown to be present at levels typically 10-100 fold lower than Cyclic AMP in most tissues and is formed by the action of the enzyme guanylate cyclase on GTP. Some hormones, such as acetylcholine, insulin and oxytocin, as well as certain other chemicals like serotonin and histamine cause an increase in Cyclic GMP levels. Stimulators of guanylate cyclase such as the vasodilators nitroprusside, nitroglycerin, sodium nitrate and nitric oxide (NO) also increase Cyclic GMP levels. NO can be synthesized from L-arginine and diffuse through cell membranes. The interaction of NO with guanylate cyclase allows Cyclic GMP to act as a third messenger in some cells. Bioactive peptides like atrial natriuretic peptide (ANP) also increase Cyclic GMP concentrations.

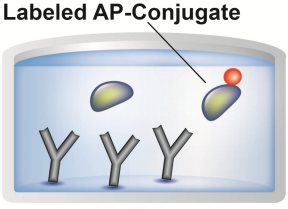
2. ASSAY SUMMARY



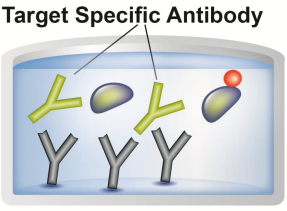
Prepare all reagents and samples as instructed.



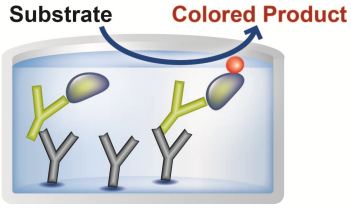
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add Cyclic GMP Direct antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance
- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up
- Some Solutions supplied in this kit are caustic; care should be taken with their use
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- The Cyclic GMP Standard provided is supplied in ethanolic buffer at a pH optimized to maintain Cyclic GMP integrity. Care should be taken handling this material because of the known and unknown effects of Cyclic GMP

4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the AP Conjugate & Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-Rabbit IgG Microplate (12 x 8 wells)	96 Wells	4ºC
Cyclic GMP Direct Alkaline Phosphatase Conjugate	5 mL	-20ºC
Cyclic GMP Direct rabbit polyclonal Antibody	5 mL	4ºC
Cyclic GMP Direct Standard	500 µL	-20ºC
20X Wash Buffer Concentrate	27 mL	4ºC
pNpp Substrate	20 mL	4ºC
Stop Solution	5 mL	4ºC
Acetylation kit - Triethylamine	2 mL	4ºC
Acetylation kit - Acetic Anhydride	1 mL	4ºC
0.1 M HCL	27 mL	4ºC
Neutralizing Reagent	5 mL	4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- Triton X-100 (optional for sample preparation)
- Optional (for tissue samples): Liquid nitrogen, mortar & pestle, and concentrated HCl

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 **Cyclic GMP Direct Alkaline Phosphatase Conjugate**

Allow the Cyclic GMP Direct Alkaline Phosphatase Conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C.

9.2 **1X Wash Buffer**

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently.

9.3 **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.

10. STANDARD PREPARATIONS – NON-ACETYLATED FORMAT

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 Allow the 5,000 pmol/mL Cyclic GMP **Stock Standard** solution to equilibrate to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

10.2 Label five tubes with numbers 1 – 5.

10.3 Add 900 µL of 0.1M HCl to tube #1.

10.4 Add 800 µL of 0.1M HCl to tubes #2 through #5

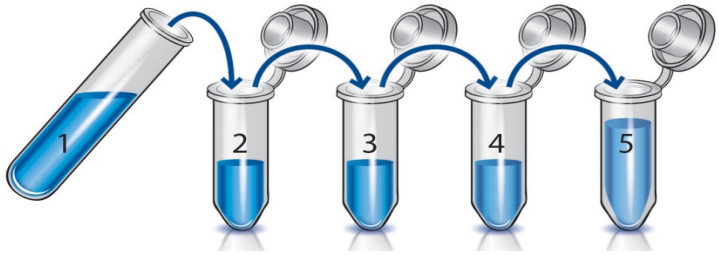
10.5 Prepare a 500 pmol/mL **Standard 1** by adding 100 µL of 5,000 pmol/mL Stock Standard to tube 1. Vortex thoroughly.

10.6 Prepare **Standard 2** by transferring 200 µL from Standard 1 to tube 2. Vortex thoroughly.

10.7 Prepare **Standard 3** by transferring 200 µL from Standard 2 to tube 3. Vortex thoroughly.

10.8 Using the table below as a guide, repeat for tubes #4 and #5.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pmol/mL)	Final Conc. (pmol/mL)
1	Standard	100	900	5,000	500
2	Standard 1	200	800	500	100
3	Standard 2	200	800	100	20
4	Standard 3	200	800	20	4
5	Standard 4	200	800	4	0.8



11. STANDARD PREPARATIONS –ACETYLATED FORMAT (optional)

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use Diluted acetylated standards should be used within 30 minutes of preparation.

11.1 Allow the 5,000 pmol/mL Cyclic GMP **Stock Standard** solution to equilibrate to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

11.2 Label five tubes with numbers #1 – #5.

11.3 Add 990 µL of 0.1M HCl to tube #1

11.4 Add 800 µL of 0.1M HCl to tubes #2-#5

11.5 Prepare a 500 pmol/mL Standard 1 by adding 10 µL of 5,000 pmol/mL Stock Standard to tube #1. Vortex thoroughly.

11.6 Prepare Standard 2 by transferring 200 µL from **Standard 1** to tube 2. Vortex thoroughly.

11.7 Prepare Standard 3 by transferring 200 µL from **Standard 2** to tube 3. Vortex thoroughly.

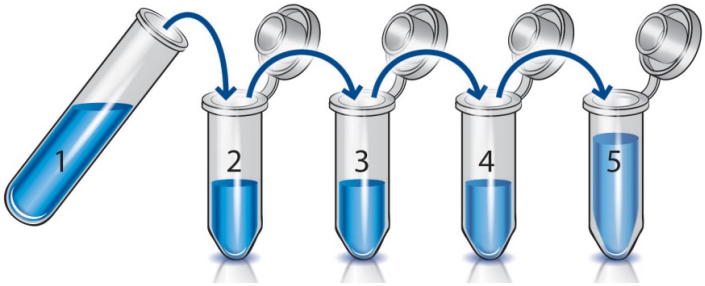
11.8 Using the table below as a guide, repeat for tubes #4 and #5.

11.9 Acetylate all standards by adding 10 µL of the Acetylating Reagent (see section 9.3) for each 200 µL of the standard. Add the Acetylating Reagent directly to the diluted standard

or sample and vortex immediately after the addition of the Acetylating Reagent.

Note: If acetylating standards, then samples must be acetylated in the same format by adding 20 µL of the Acetylating Reagent for each 100 µL of the sample.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pmol/mL)	Final Conc. (pmol/mL)
1	Standard	10	990	5,000	50
2	Standard #1	200	800	50	10
3	Standard #2	200	800	10	2
4	Standard #3	200	800	2	0.4
5	Standard #4	200	800	0.4	0.8



12. SAMPLE COLLECTION AND STORAGE

- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.
- Samples containing rabbit IgG will interfere with the assay. EDTA plasma may precipitate during acetylation.
- Culture supernatants may be run directly in the assay provided the same non-conditioned media is used as the standard diluent.
- Cells grown in tissue culture media can be treated with 0.1M HCl after first removing the media. Incubate for 10 minutes and visually inspect the cells to verify cell lysis. If adequate lysis has not occurred incubate for a further 10 minutes and inspect. Centrifuge at ≥ 600 x g at room temperature, then use the supernatant directly in the assay.
- Cell or tissue lysis can be enhanced by adding 0.1% to 1% Triton x-100 to the 0.1M HCl prior to use. When used in this concentration range, the detergent will not interfere with acetylation or the binding portion of the assay, however there will be a modest increase in the optical density.
- Samples containing Triton should be evaluated against a standard curve diluted in the same for the most accurate determination.
- Please note that some samples may contain high levels of Cyclic GMP Direct and additional dilution may be required. Samples with low levels of Cyclic GMP Direct may be assayed in the acetylated format or the samples may be concentrated.
- If acetylating standards, then samples must be acetylated in the same format by adding 10 µL of the Acetylating Reagent for each 200 µL of the sample.

13. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at +4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section

	1	2	3	4
A	B_s	Std 1	Std 5	
B	B_s	Std 1	Std 5	
C	TA	Std 2	Sample 1	
D	TA	Std 2	Sample 1	
E	NSB	Std 3	Sample 2	
F	NSB	Std 3	Sample 2	
G	B_0	Std 4	etc	
H	B_0	Std 4	etc	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

B_s = Blank; contains substrate only.

TA = Total Activity; contains conjugate (5 µL) and substrate.

NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

14. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**
- **If Acetylated Version of the kit is to be run, acetylate all standards as described in Section 11. Acetylate all samples by adding 10 µL of the Acetylating Reagent (see step 9.3) for each 200 µL of sample. Add the reagent directly to the samples and vortex for 2 seconds. Use the acetylated samples within 30 minutes.**

14.1 Add 50 µL of the Neutralizing Reagent into each well, except the TA and Blank wells.

14.2 Add 150 µL of 0.1M HCl into the NSB wells

14.3 Add 100 µL of 0.1M HCl into the B_0 wells

14.4 Add 100 µL of samples, standards and B_0 into the appropriate wells.

14.5 Add 50 µL of the Cyclic GMP Direct Alkaline Phosphatase Conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not the Total Activity (TA) and B_s wells.

14.6 Add 50 µL of the Cyclic GMP Direct antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells.

NOTE: Every well used should be green in color except the NSB wells which should be blue. The B_0 and TA wells are empty at this point and have no color.

14.7 Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided. **If using the optional Overnight Acetylated Format,incubate for 18-24 hours at 4°C tightly covered with the plate sealer provided.**

14.8 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 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.

14.9 Add 5 µL of the Cyclic GMP Direct Alkaline Phosphatase Conjugate to the TA wells.

14.10 Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.

14.11 Add 50 µL Stop Solution into each well. The plate should be read immediately.

14.12 Read the O.D. absorbance at 405 nm, preferably with correction between 570 and 590 nm.

15. CALCULATIONS

15.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

15.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

15.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of Cyclic GMP for the standards. The concentration of Cyclic GMP in the unknowns can be determined by interpolation of net OD values.

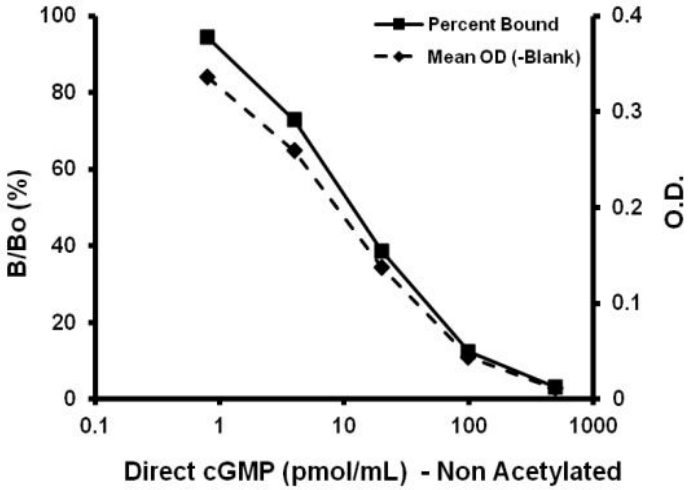
A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

16. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

Non-Acetylated Format:

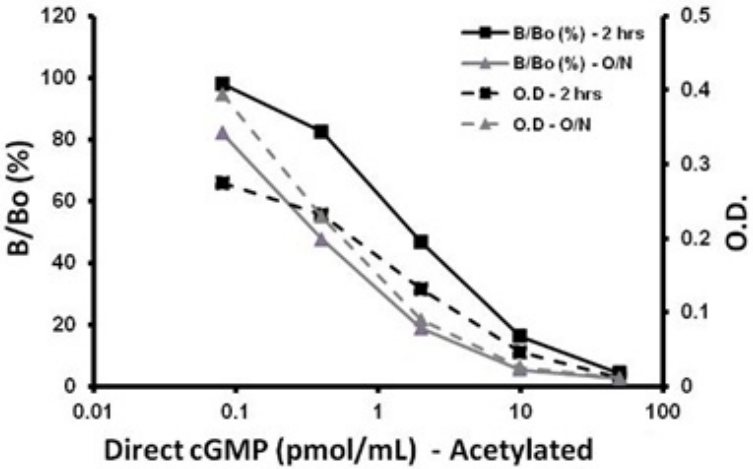


Sample	Mean OD (-Bs)	% Bound	cGMP pmol/mL
Bs	(0.229		
TA	0.389		
NSB	-0.002	0	
Standard 1	0.011	3.1	500
Standard 2	0.044	12.4	100
Standard 3	0.137	38.5	20
Standard 4	0.259	72.8	4
Standard 5	0.336	94.4	0.8
B_0	0.356	100	0
Unkonwn1	0.059	16.6	71.8
Unknown 2	0.282	79.2	2.78

Non-Acetylated Typical Quality Control Parameters

Total Activity Added	= 0.389 x 10 = 3.89
$\%B_O/TA$	= 9.2%
Quality of Fit	= 1.0000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	= 54.2 pmol/mL
50% Intercept	= 10.8 pmol/mL
80% Intercept	= 2.1 pmol/mL

Acetylated Format:



	2 hr Acetylated			Overnight Acetylated		
Sample	**Mean OD ($-B_s$)**	**% Bound**	**cGMP pmol/mL**	**Mean OD ($-B_s$)**	**% Bound**	**cGMP pmol/mL**
B_s	(0.152)			(0.113)		
TA	0.381			0.381		
NSB	-0.001	0		0.001	0	
Standard 1	0.012	4.3	50	0.012	2.5	50
Standard 2	0.046	16.2	10	0.026	5.4	10
Standard 3	0.132	46.9	2	0.09	18.8	2
Standard 4	0.232	82.5	0.4	0.229	47.7	0.4
Standard 5	0.275	98	0.08	0.94	82.1	0.08
B_0	0.281	100	0	0.48	100	0
Unknown 1	0.074	20.8	5.14	0.072	15.2	2.55
Unknown 2	0.251	70.5	0.24	0.252	52.5	0.33

Acetylated Typical Quality Control Parameters:

2 Hour Format:

Total Activity Added	= 0.381 x 10 = 3.81
$\%B_O/TA$	= 7.3 %
24	
Quality of Fit	= 1.0000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	= 7.0 pmol/mL
50% Intercept	= 1.6 pmol/mL
80% Intercept	= 300 pmol/mL

Overnight Format:

Total Activity Added	= 0.381 x 10 = 3.81
$\%B_O/TA$	= 12.6 %
Quality of Fit	= 1.0000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	= 1.5 pmol/mL
50% Intercept	= 0.3 pmol/mL

17. TYPICAL SAMPLE VALUES

SENSITIVITY –

Sensitivity was calculated by determining the average optical density bound for 16 wells run as Bo (Standard 6), and comparing to the average optical density for 16 wells run with Standard 5. The detection limit was determined as the concentration of cGMP measured at 2 standard deviations from the zero along the standard curve and was determined to be 0.604 pmol/mL in the non-acetylated assay format, 0.059 pmol/mL in the acetylated 2 hour assay format, and 0.025 pmol/mL in the acetylated overnight assay format.

SAMPLE RECOVERY –

Recovery was determined by Cyclic GMP Direct into tissue culture media. Mean recoveries are as follows:

Non-Acetylated Format:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	95.9	None

Acetylated 2 Hour Format:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	86.8	None

LINEARITY OF DILUTION –

Non-Acetylated Version

A buffer sample containing 96 pmol/mL Cyclic GMP was serially diluted 7 times 1:2 in the 0.1M HCl supplied in the kit and measured in the assay. The data was plotted graphically as actual Cyclic GMP concentration versus measured Cyclic GMP concentration.

The line obtained had a slope of 1.000 with a correlation coefficient of 0.999.

Acetylated Version

A buffer sample containing 16 pmol/mL Cyclic GMP was serially diluted 7 times 1:2 in the 0.1M HCl supplied in the kit and measured in the Acetylated 2 Hour Format of the assay. The data was plotted graphically as actual Cyclic GMP concentration versus measured Cyclic GMP concentration.

The line obtained had a slope of 1.001 with a correlation coefficient of 0.998.

PRECISION –

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Cyclic GMP Direct and running these samples multiple times (n=24) in the same assay.

Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Cyclic GMP Direct in multiple assays (n=8).

Non-Acetylated Format:

Intra-Assay

	pmol/mL	%CV
Low	1.85	4.43
Medium	9.88	7.90
High	115.3	6.57

Inter-Assay

	pmol/mL	%CV
Low	2.14	5.96
Medium	8.53	9.85
High	97.0	6.88

Acetylated 2 Hour Format:

Intra-Assay

	pmol/mL	%CV
Low	0.58	9.57
Medium	1.38	3.55
High	5.38	3.49

Inter-Assay

	pmol/mL	%CV
Low	0.349	10.89
Medium	3.51	8.35
High	10.3	4.57

18. ASSAY SPECIFICITY

CROSS REACTIVITY –

The cross reactivities for a number of related compounds were determined by using the cGMP ELISA kit, Catalog Number 900-013, which uses the same antibody and conjugate as this kit. Potential cross reactants were dissolved in the kit Assay Buffer at concentrations from 500,000 to 500 pmol/mL. These samples were then measured in the cGMP assay, and the measured cGMP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage:

Cyclic GMP	100 %
GMP	<0.001 %
GTP	<0.001 %
cAMP	<0.001 %
AMP	<0.001 %
ATP	<0.001 %
cUMP	<0.001 %
CTP	<0.001 %

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

20. NOTES

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