



## **Direct cGMP ELISA kit**

Catalog #: ADI-901-014

480 Well (5 x 96 Well) Kit

For use with cells and tissue samples

For the latest product information, including support documentation, visit us online:

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# Product Manual

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Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



Check our website for additional protocols, technical notes and FAQs.



Please contact Enzo Life Sciences Technical Support if necessary.

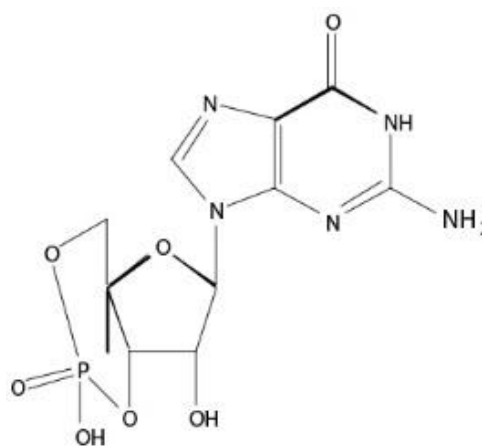
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## INTRODUCTION

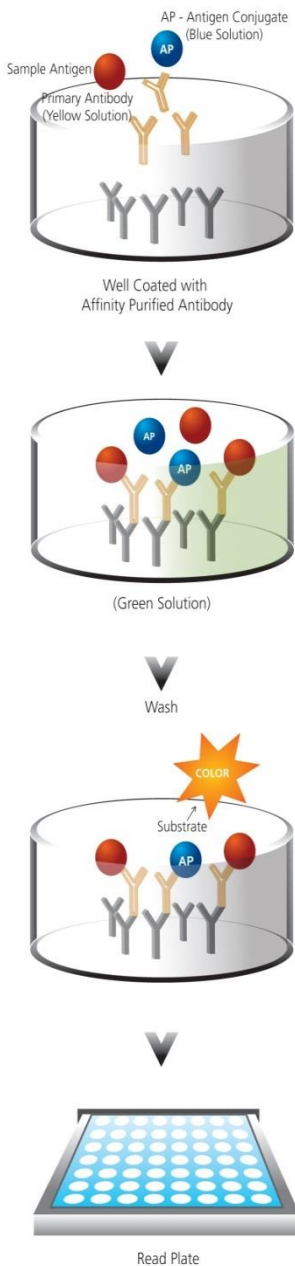
The cyclic GMP Enzyme-Linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of cyclic GMP in samples treated with 0.1 M HCl. The acetylated assay format provides an increase in sensitivity and is ideal for samples with extremely low levels of cGMP. The optional overnight acetylated assay format allows super-sensitive detection of cGMP. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard<sup>1</sup> or Tijssen<sup>2</sup>.

Guanosine 3', 5'-cyclic monophosphate (cyclic GMP; cGMP) was identified in 1963. It has been shown to be present at levels typically 10-100 fold lower than cAMP in most tissues and is formed by the action of the enzyme guanylate cyclase on GTP<sup>3</sup>. 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<sup>4,5</sup>. Stimulators of guanylate cyclase such as the vasodilators nitroprusside, nitroglycerin, sodium nitrate and nitric oxide (NO) also stimulate cGMP levels<sup>6</sup>. Peptides, such as atrial natriuretic peptide (ANP) that relax smooth muscle also increase cGMP concentrations<sup>7</sup>. cGMP has been confirmed as a second messenger for ANP<sup>8</sup>. NO can be synthesized from L-arginine and diffuse through cell membranes<sup>9,10</sup>. The interaction of NO with guanylate cyclase allows cGMP to act as a third messenger in some cells<sup>11</sup>.

### Cyclic GMP



## PRINCIPLE



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 405nm. The amount of signal is indirectly proportional to the amount of cGMP in the sample.

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The standard should be handled with care due to the known and unknown effects of the antigen.



Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.



Activity of conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).



Triethylamine and acetic anhydride are lachrymators. **Caution:** corrosive, flammable, and harmful vapor.



Protect substrate from prolonged exposure to light.



HCl and stop solution are caustic. Keep tightly capped.

## MATERIALS SUPPLIED

1. **Goat anti-Rabbit IgG Microtiter Plate, 5 Plates of 96 Wells, Catalog No. 80-0060**  
Plates using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **Cyclic GMP Standard, 3 x 0.5 mL, Catalog No. 80-0153**  
A solution of 5,000 pmol/mL cGMP.
3. **cGMP ELISA Antibody, 25 mL, Catalog No. 80-0161**  
A yellow solution of a rabbit polyclonal antibody to cGMP.
4. **cGMP Conjugate, 25 mL, Catalog No. 80-0159**  
A blue solution of alkaline phosphatase conjugated with cGMP.
5. **0.1 M HCl, 100 mL, Catalog No. 80-0082**  
0.1 M hydrochloric acid in water.
6. **Acetylation Kit, 2 x 2 vials, Catalog No. 950-001**
  1. **Triethylamine, 2 x 2 mL, Catalog No. 80-0063**
  2. **Acetic Anhydride, 2 x 1 mL, Catalog No. 80-0064**
7. **Neutralizing Reagent, 28 mL, Catalog No. 80-1476**
8. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**  
Tris buffered saline containing detergents.
9. **pNpp Substrate, 100 mL, Catalog No. 80-0076**  
A solution of p-nitrophenyl phosphate in buffer. Ready to use.
10. **Stop Solution, 30 mL, Catalog No. 80-0248**  
A solution of trisodium phosphate in water. Keep tightly capped.
11. **Direct cGMP Assay Layout Sheet, 1 each, Catalog No. 30-0033**
12. **Plate Sealers, 5 each, Catalog No. 30-0012**



Reagents require separate storage conditions.

## STORAGE

All components of this kit, except the standard and conjugate, are stable at 4°C until the kit's expiration date. Upon receipt, store the standard and conjugate at -20°C. Recommended storage temperatures do not necessarily reflect shipping conditions.



Avoid repeated freeze/ thaw cycles of the conjugate.

## OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
9. A centrifuge (for sample preparation).
10. Triton X-100 or equivalent (optional for sample preparation).
11. Liquid nitrogen, stainless steel mortar & pestle (optional – for tissue samples).



Sample handling procedures should be completed prior to reagent 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. EDTA plasma may precipitate during acetylation. **Samples containing rabbit IgG will interfere with the assay.**



Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

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 or overnight acetylated format, or the samples may be concentrated. The end user **must verify** that the recommended dilutions are appropriate for their samples.

## 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 at the same percentage, 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  $1 \times 10^6$  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.
2. Incubate the cells in 0.1 M HCl for 10 minutes at room temperature.
3. Inspect the cells under a microscope to ensure uniform lysis. Continue incubating for an additional 10 minutes, if necessary.
4. Centrifuge  $\geq 600 \times g$  to pellet the cellular debris.
5. The supernatant may be assayed immediately or stored frozen, ideally at  $-70^\circ\text{C}$  or below, for later analysis.



If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

## 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  $-70^\circ\text{C}$  or below.
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 0.1M HCl). Centrifuge  $\geq 600 \times g$  to pellet the debris (~10 minutes).
4. The supernatant may be further diluted in the 0.1 M HCl provided and run directly in the assay or stored frozen, ideally at  $-70^\circ\text{C}$  or below, for later analysis.

Biological fluids (e.g. serum, plasma, saliva) and culture supernates should be used in the cyclic GMP ELISA Kit (Cat. #ADI-900-013, ADI-901-013) or the cyclic GMP Complete ELISA Kit (Cat. #ADI-900-164, ADI-901-164).



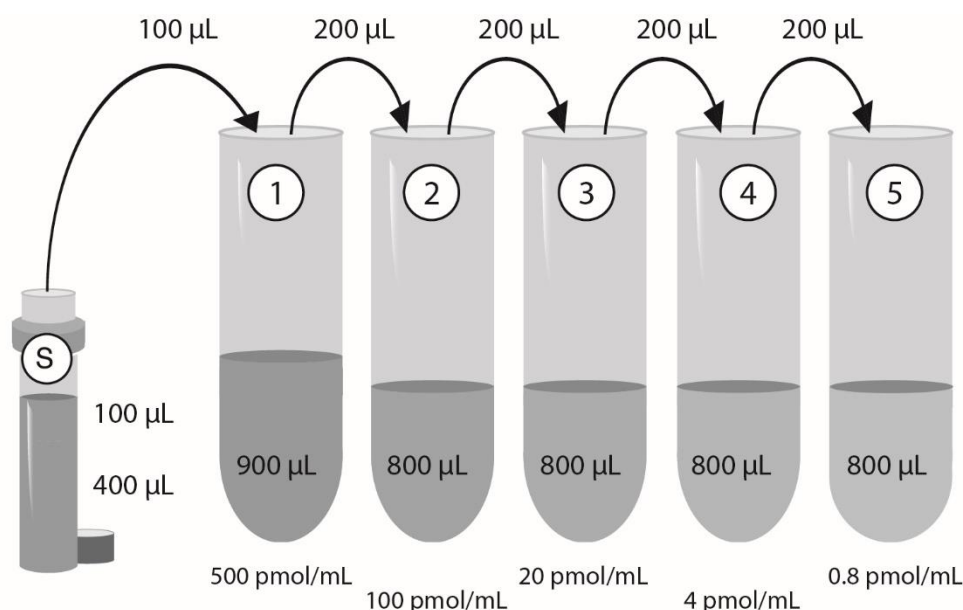
## REAGENT PREPARATION



Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

### 1. cGMP Standard - Non-Acetylated Format

Allow the 5,000 pmol/mL cGMP standard stock to warm to room temperature. Label five 12 x 75mm tubes #1 through #5. Pipet 900  $\mu$ L of the 0.1 M HCl into tube #1. Pipet 800  $\mu$ L of the 0.1 M HCl into tubes #2 through #5. Add 100  $\mu$ L of the 5,000 pmol/mL standard stock to tube #1. Vortex thoroughly. Add 200  $\mu$ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.



Bring all reagents to room temperature for at least 30 minutes prior to opening.

The concentrations of cGMP in the tubes are labeled above.

**Diluted standards should be used within 60 minutes of preparation.**



Triethylamine and acetic anhydride are lachrymators. **Caution:** corrosive, flammable, and harmful vapor.

### 2. Acetylation Reagent (optional)

Prepare the Acetylation Reagent by adding 0.5 mL of acetic anhydride to 1 mL triethylamine and vortex thoroughly. Note that this volume is sufficient to add to up to 30 mL of diluted standard and samples. Use the prepared reagent within 60 minutes of preparation. Discard any unused portion of the Acetylation Reagent.

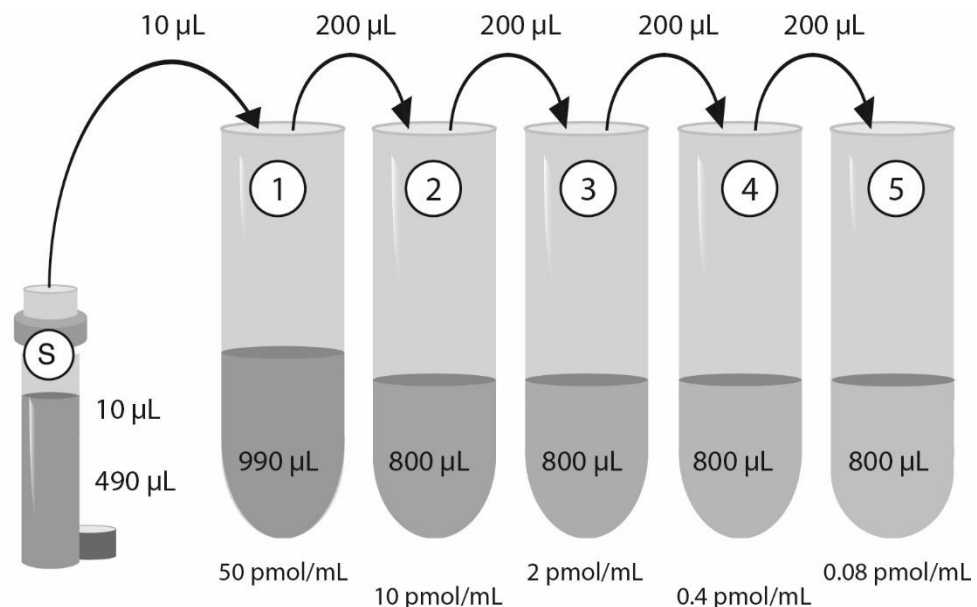


Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

### 3. cGMP Standard - Acetylated Format

Allow the 5,000 pmol/mL cGMP standard stock to warm to room temperature. Label five 12 x 75 mm tubes #1 through #5. Pipet 990  $\mu$ L 0.1 M HCl into tube #1 and 800  $\mu$ L 0.1 M HCl into tubes #2-5. Add 10  $\mu$ L of the 5,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 200  $\mu$ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.

**Acetylate all standards and samples by adding 10  $\mu\text{L}$  of the Acetylation Reagent for each 200  $\mu\text{L}$  of standard or sample. Add the reagent directly to the samples and vortex thoroughly for 2 seconds immediately after the addition of the Acetylation Reagent.**



The concentrations of cGMP in the tubes are labeled above.

Label one 12 x 17 mm tube as the Zero Standard/NSB tube. Pipet 1 mL of the 0.1 M HCl into this tube. Add 50  $\mu\text{L}$  of the Acetylating Reagent to the Zero Standard/NSB tube, vortex immediately, and use this for Steps 2 and 5, on Page 9. **Failure to acetylate the NSB and Zero standard will result in inaccurate B/Bo values.**

**Use the acetylated standards and samples within 30 minutes of preparation.**

#### 4. Wash Buffer

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



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 the substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.



Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.

## ASSAY PROCEDURE

Refer to the Assay 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.

1. Pipet 50  $\mu$ L of the Neutralizing Reagent into each well, except the Total Activity (TA) and Blank wells.
2. Pipet 100  $\mu$ L of the 0.1 M HCl into the NSB (non-specific binding) and the Bo (0 pmol/mL Standard) wells.
3. Pipet an additional 50  $\mu$ L of 0.1M HCl into the NSB wells.
4. Pipet 100  $\mu$ L of Standards into the appropriate wells.
5. Pipet 100  $\mu$ L of the Samples into the appropriate wells. Pipet 50  $\mu$ L of blue Conjugate into each well except the TA and Blank wells.
6. Pipet 50  $\mu$ L of 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.

7. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired. If using the optional Overnight Acetylated Format, incubate for 18-24 hours at 4°C tightly covered with the plate sealer provided.
8. Empty the contents of the wells and wash by adding 400  $\mu$ L of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
9. 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. Add 5  $\mu$ L of the blue Conjugate to the TA wells.
11. Add 200  $\mu$ L of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.
12. Add 50  $\mu$ L of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

13. After blanking the plate reader against the substrate blank, read the optical density at 405 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean optical density of the substrate blank from all readings.

## CALCULATION OF RESULTS

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



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

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

2. Plot the Net OD versus Concentration of cGMP for the standards. Sample concentrations of cGMP may be calculated by interpolation off of the standard curve using Net OD values.
3. Alternatively, calculate the binding of each standard and sample as a percentage of the maximum binding wells (Bo), using the formula below:

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

4. Plot the Percent Bound (B/Bo) versus Concentration of cGMP for the standards. Sample concentrations of cGMP may be calculated by interpolation off of the standard curve using Percent Bound values.

To normalize samples 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 total protein.

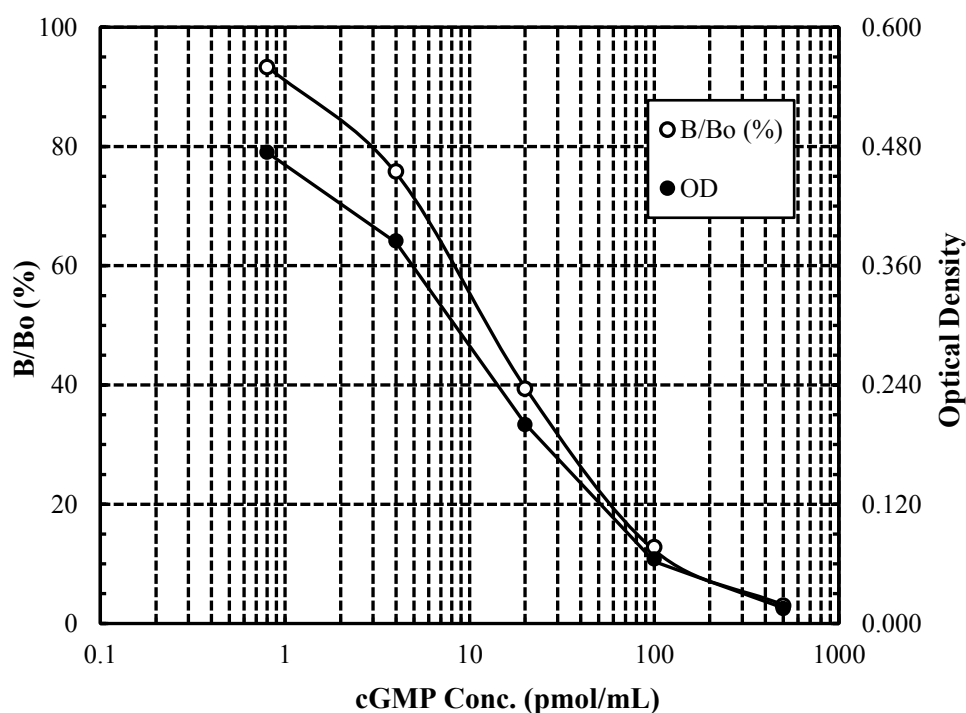


Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

## TYPICAL RESULTS

The results shown below are for illustration only and **should not** be used to calculate results from another assay; each user must run a standard curve for each assay and version used.

Non-Acetylated Format			
Sample	Ave Net OD	% B/Bo	cGMP (pmol/mL)
Blank	(0.083)	---	---
TA	0.326	---	---
NSB	-0.001	-0.3%	---
Bo	0.508	100%	<b>0</b>
S1	0.015	2.9%	<b>500</b>
S2	0.065	12.7%	<b>100</b>
S3	0.200	39.3%	<b>20</b>
S4	0.385	75.8%	<b>4</b>
S5	0.474	93.3%	<b>0.8</b>
Control1	0.070	13.7%	<b>87.9</b>
Control2	0.173	34.0%	<b>25.7</b>



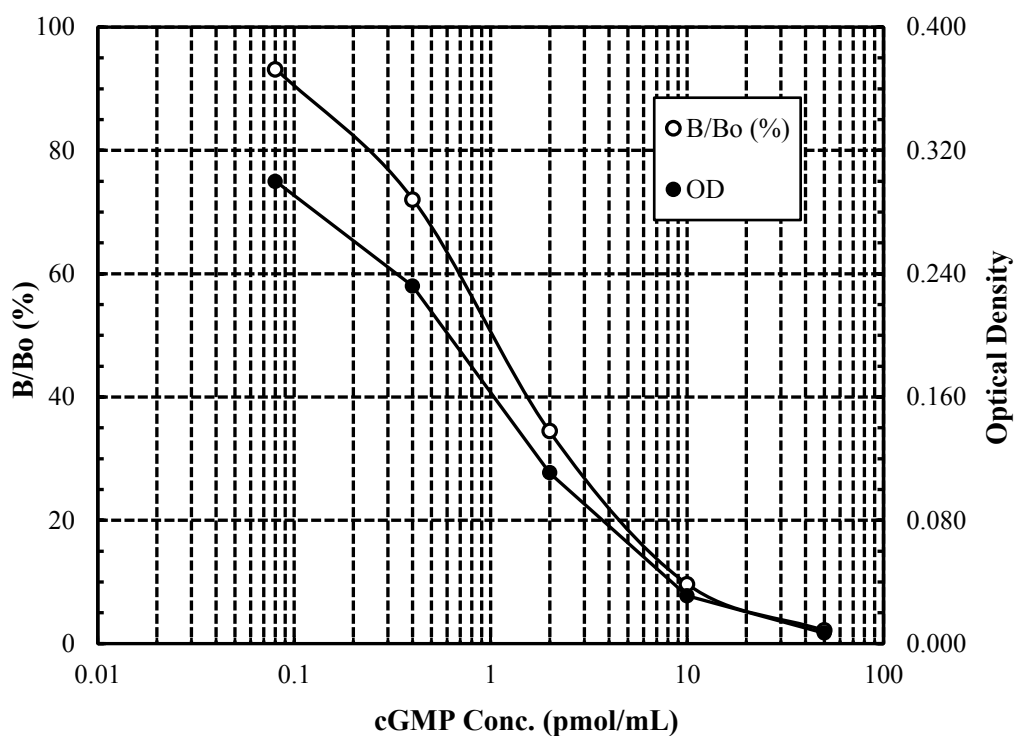
Quality of Fit = 1.000 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 54.6 pmol/mL

50% Intercept = 12.9 pmol/mL

80% Intercept = 3.1 pmol/mL

Acetylated 2 Hour Format			
Sample	Ave Net OD	% B/Bo	cGMP (pmol/mL)
Blank	(0.086)	---	---
TA	0.322	---	---
NSB	-0.005	-1.6%	---
Bo	0.322	100%	<b>0</b>
S1	0.007	2.2%	<b>50</b>
S2	0.031	9.5%	<b>10</b>
S3	0.111	34.3%	<b>2</b>
S4	0.232	71.9%	<b>0.4</b>
S5	0.300	93.2%	<b>0.08</b>
Control3	0.038	11.8%	<b>7.93</b>
Control4	0.093	28.9%	<b>2.57</b>



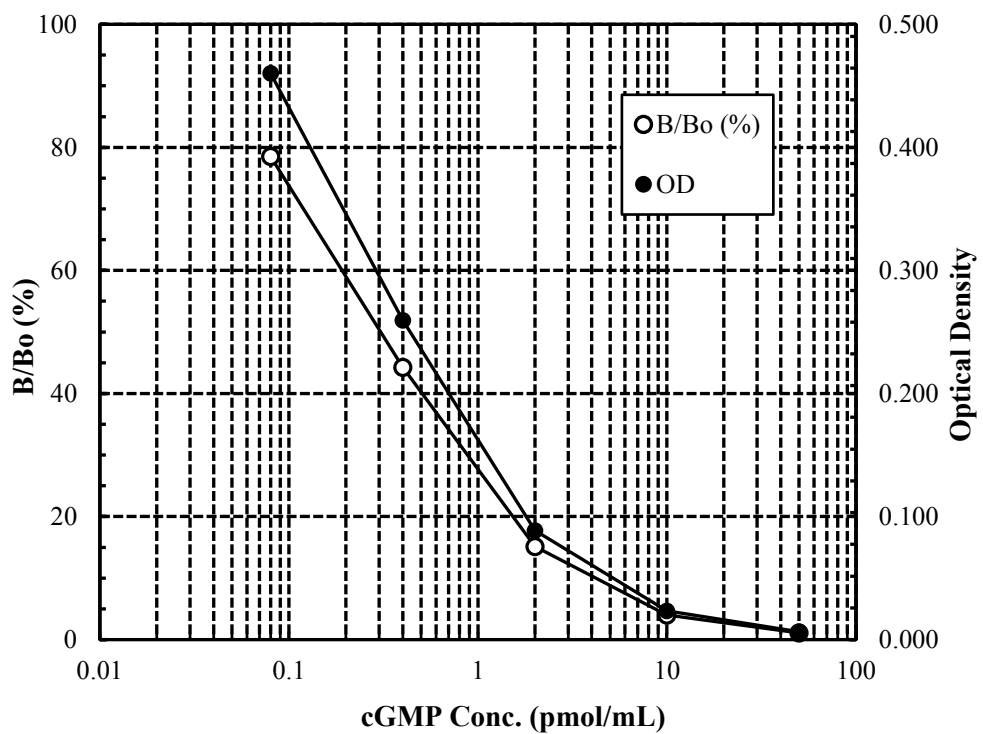
Quality of Fit = 1.000 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 4.21 pmol/mL

50% Intercept = 1.03 pmol/mL

80% Intercept = 0.26 pmol/mL

Acetylated Overnight Format			
Sample	Ave Net OD	% B/Bo	cGMP (pmol/mL)
Blank	(0.084)	---	---
TA	0.342	---	---
NSB	-0.005	-0.8%	---
Bo	0.584	100%	<b>0</b>
S1	0.005	0.9%	<b>50</b>
S2	0.026	4.4%	<b>10</b>
S3	0.088	15.0%	<b>2</b>
S4	0.260	44.5%	<b>0.4</b>
S5	0.461	78.9%	<b>0.08</b>
Control3	0.022	3.8%	<b>10.71</b>
Control4	0.071	12.1%	<b>2.66</b>



Quality of Fit = 1.000 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 1.40 pmol/mL

50% Intercept = 0.32 pmol/mL

80% Intercept = 0.08 pmol/mL

## SAMPLE RECOVERIES

Please refer to pages 6-8 for Sample Handling recommendations and Standard preparation.

cGMP concentrations were measured in 0.1 M HCl, which is reflective of the lysate or tissue supernatant after sample treatment. cGMP was spiked into the undiluted sample, which was then serially diluted with the kit 0.1 M HCl and then tested in the kit. The following results were obtained:

Sample	Non-Acetylated Format		Acetylated 2-Hr Format		Acetylated Overnight Format	
	% Recovery	Recommended Dilution*	% Recovery	Recommended Dilution*	% Recovery	Recommended Dilution*
0.1M HCl	101.7	None	115.6	None	108.1	None

\* See Sample Handling instructions on page 6 for details.

## PERFORMANCE CHARACTERISTICS SENSITIVITY

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 format, 0.059 pmol/mL in the acetylated 2 hour format, and 0.025 pmol/mL in the acetylated overnight format.

### Linearity

A 0.1 M HCl sample containing cGMP was serially diluted 1:2 in the 0.1 M HCl kit diluent and measured in the non-acetylated assay format. A separate 0.1 M HCl sample was serially diluted and measured in both acetylated assay formats. The results are shown below.



<b>Non-Acetylated Format in 0.1 M HCl</b>			
<b>Dilution</b>	<b>Expected (pmol/mL)</b>	<b>Observed (pmol/mL)</b>	<b>Recovery (%)</b>
Neat	---	240.6	---
1:2	120.3	115.5	96%
1:4	60.1	61.0	101%
1:8	30.1	29.7	99%
1:16	15.0	15.3	102%
1:32	7.5	7.3	97%
1:64	3.8	4.0	107%
1:128	1.9	2.0	108%
1:256	0.94	0.98	104%

<b>Acetylated 2 Hour Format in 0.1 M HCl</b>			
<b>Dilution</b>	<b>Expected (pmol/mL)</b>	<b>Observed (pmol/mL)</b>	<b>Recovery (%)</b>
Neat	---	27.6	---
1:2	13.8	14.7	106%
1:4	6.9	7.3	106%
1:8	3.4	3.7	106%
1:16	1.7	2.0	117%
1:32	0.86	1.1	122%
1:64	0.43	0.56	130%
1:128	0.22	0.27	127%
1:256	0.11	0.12	110%

Acetylated Overnight Format in 0.1 M HCl			
Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	30.3	---
1:2	15.2	19.3	128%
1:4	7.6	7.4	97%
1:8	3.8	3.8	101%
1:16	1.9	2.0	106%
1:32	0.95	1.0	108%
1:64	0.47	0.53	113%
1:128	0.24	0.27	114%
1:256	0.12	0.12	98%

## Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of cGMP 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 cGMP in multiple assays (n=8). Different sample concentrations were used for the non-acetylated vs. acetylated format, to fall within the different standard curve ranges.

The precision numbers listed below represent the percent coefficient of variation for the concentrations of cGMP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Non-Acetylated Format			Acetylated 2 hr Format		
	cGMP (pmol/ml)	Intra-assay (%CV)	Inter-assay (%CV)	cGMP (pmol/ml)	Intra-assay (%CV)	Inter-assay (%CV)
Low	1.9	4.4		0.58	9.6	
Medium	9.9	7.9		1.38	3.6	
High	115	6.6		5.38	3.5	
Low	2.1		6.0	0.35		10.9
Medium	8.5		9.9	3.51		8.4
High	97		6.9	10.3		4.5

## Cross Reactivities

The cross reactivities for a number of related compounds were determined by using the cGMP ELISA kit, Catalog Number ADI-900-013, which uses the same antibody and conjugate as this kit. Potential cross reactants (purity checked by N.M.R. and other analytical methods) were dissolved in the kit Assay Buffer at a concentration of ten times the high standard, and 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%

## REFERENCES

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# Product Manual

## **GLOBAL HEADQUARTERS**

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