



# Cyclic GMP Enzyme Immunoassay Kit

Catalog No. 133-16474

## 96 Well Kit

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## **Description**

Sapphire's Cyclic GMP kit is a competitive immunoassay for the quantitative determination of cyclic GMP in biological samples. This assay is based on the competition between cyclic GMP and a cyclic GMP-alkaline phosphatase tracer for a limited amount of cyclic GMP-specific antiserum. Because of the competition between cyclic GMP in the sample and cyclic GMP tracer for the cyclic GMP antiserum, the signal obtained with the assay will be inversely proportional to the amount of cGMP in each sample. This equilibration is performed in the wells of a 96-well plate pre-coated with mouse monoclonal anti-rabbit IgG, which binds all of the cGMP antiserum added to the well. After the equilibration step, the plate is washed, and a solution of *para*-nitrophenyl phosphate (*p*NPP), a substrate for alkaline phosphatase, is added. The product of this enzymatic reaction has a distinct yellow color which absorbs at 412 nm, allowing quantification of cyclic GMP in each sample.

## **Introduction**

cGMP (guanosine 3'5' cyclic monophosphate) is synthesized from GTP by guanylate cyclase in response to cellular stimulation originating from a variety of hormones, drugs, and intracellular signaling molecules.<sup>1,2</sup> For example, binding of nanomolar concentrations of nitric oxide to soluble guanylate cyclase increases its activity several hundred fold, resulting in a pronounced increase in cGMP accumulation.<sup>3,4</sup> cGMP can also be synthesized by 6 membrane-bound, i.e. particulate, forms of the enzyme.<sup>1,2</sup> cGMP binds to and activates a variety of cellular proteins, including cGMP-gated channels, cGMP-dependent kinases (protein kinase G; PKG) and cGMP-regulated phosphodiesterases.<sup>2</sup> The vasodilator effects of nitric oxide are mediated by cGMP activation of PKG, which initiates a protein phosphorylation cascade resulting in smooth muscle relaxation.<sup>2,5</sup> Hydrolysis of cGMP to GMP by any member of a large family of phosphodiesterases results in signal termination.<sup>5</sup>

## **Precautions**

1. Please read all instructions carefully before beginning this assay.
2. Some reagents contain azide, which may react with lead or copper plumbing. If disposing of reagents in the sanitary sewer, please flush with a large volume of water to prevent azide build-up.
3. This kit is for research use only. It is not intended for human or diagnostic use.

## **Materials Supplied**

1. Cyclic GMP Antiserum	1 vial	133-64743
2. Cyclic GMP AP Tracer	1 vial	133-64742
3. Cyclic GMP Standard	1 vial	133-64741
4. Mouse Anti-rabbit IgG Coated Plate	1 plate	133-00006
5. Tris Buffer Concentrate	2 vials	133-00003
6. AP Wash Buffer Concentrate	1 vial	133-00004
7. DEA Buffer Concentrate	1 vial	133-00001
8. <i>p</i> NPP Tablets	5 tablets	133-00002
9. Acetic Anhydride	1 vial	133-00009
10. Potassium Hydroxide	1 vial	133-00010
11. Plate Cover	1 cover	133-00005

## **Storage**

This kit should be stored at -20°C and used before the expiration date printed on the box.

## **Materials Needed but Not Supplied**

1. A source of Millipore water
2. Adjustable pipettors
3. Materials used for sample preparation
4. An orbital shaker
5. A plate reader capable of measuring absorbance between 405-420 nm

### **General Precautions**

1. All samples must be free of organic solvents prior to assay.
2. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.

### **Cell Culture Samples**

1. Aspirate medium from the plate or flask.
2. Add 1 ml of 0.1 M HCl for every 35 cm<sup>2</sup> of surface area of the plate.
3. Incubate at room temperature for 20 minutes.
4. Scrape cells from flasks using a cell scraper.
5. Dissociate mixture by pipetting up and down until the suspension is homogenous and transfer to centrifuge tube.
6. Centrifuge at 1,000 x g for 10 minutes.
7. Transfer the supernatant into a clean tube.
8. The supernatants can be assayed directly following a dilution of at least 1:2. A protein concentration of at least 1 mg/ml is recommended for reproducible results.

### **Tissue Samples**

1. Cyclic nucleotides may be metabolized quickly in tissue, so it is important to rapidly freeze samples immediately following collection.
2. Weigh the frozen tissue and drop into 5-10 volumes (ml of buffer/gram of tissue) of 5% trichloroacetic acid. Homogenize the sample on ice.
3. Remove the precipitate by centrifugation at 1,500 x g for 10 minutes.
4. Transfer the supernatant to a clean test tube.
5. Extract the TCA from the sample using 5 volumes of water-saturated ether. After allowing the phases to separate, discard the upper (ether) layer. Repeat two more times.
6. Remove the residual ether from the aqueous layer by heating the sample to 70°C for five minutes.

## **Reagent Preparation**

All diluted buffers should be stored at 4°C. When stored in this manner, they will be stable for approximately two months.

### **1. Tris Buffer**

Dilute the contents of one vial of Tris Buffer Concentrate with 90 ml of Millipore water. It is common for the concentrated buffer to contain crystalline salts after thawing. It is important to rinse the vial to obtain any salts that may have precipitated.

### **2. Wash Buffer**

Dilute the 5 ml vial of Wash Buffer to a final volume of 750 ml with Millipore water.

### **3. DEA Buffer**

Dilute the 2.5 ml vial of DEA Buffer Concentrate to a final volume of 25 ml with Millipore water.

### **4. Cyclic GMP Alkaline Phosphatase Tracer**

Reconstitute the Cyclic GMP Alkaline Phosphatase Tracer with 6 ml of Tris buffer. Vortex to mix.

Store this reconstituted tracer at 4°C and use within four weeks.

### **5. Cyclic GMP Antiserum**

Reconstitute the Cyclic GMP Antiserum with 6 ml of Tris Buffer. Vortex to mix.

Store this reconstituted antibody at 4°C and use within four weeks.

## 6. Cyclic GMP Standard

*Do you need to acetylate?*

Sapphire's cGMP Assay uses an antibody that was raised against cGMP conjugated to a carrier protein through the 2' hydroxyl group of cGMP. Antibody binding is therefore increased if an acetyl group is present in this position on cGMP. The optional acetylation procedure performed on both samples and standards increases the sensitivity of the assay approximately 10 fold.

The EIA is able to detect lower concentrations of cGMP if the samples are first acetylated. If the expected concentration is less than 0.7 pmol/ml, the acetylation procedure should be performed. If the expected concentration is greater than 0.7 pmol/ml no acetylation is necessary.

*[Note: The acetylation procedure may be affected by proteins or sugars in the sample mix. In these cases, the sample should be purified before acetylation.]*

### a. Preparation of Standards and Samples - No Acetylation

#### Standard curve preparation

Reconstitute the cGMP standard with 1 ml of Tris Buffer. The concentration of this solution will be 3,000 pmol/ml. Store this solution at 4°C; it will be stable for approximately six weeks. We have included enough cGMP to run ten standard curves. This surplus should accommodate any experimental design.

*[NOTE: If the samples are prepared from TCA-extracted tissue and cannot be diluted at least 1:5 in Tris Buffer for analysis, use ether-extracted 5% TCA for preparation of the standard curve. Any dilution of samples should then be performed in this solution.]*

To prepare the standard for use in EIA: Obtain 8 clean test tubes and number them #1 through #8. Aliquot 900 µl Tris Buffer to tube #1 and 500 µl Tris Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (3,000 pmol/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 250 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 250 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

#### Sample preparation

If samples require purification, please refer to the protocols on page 3. Upon purification, no further sample preparation is necessary; however, the samples may require dilution to ensure that they will fall on the linear portion of the standard curve (20-80% B/B<sub>0</sub>). Proceed to "Performing the Assay".

### b. Preparation of Standards and Samples - Acetylation

#### Standard curve preparation

Reconstitute the cGMP standard with 1 ml of Tris Buffer (label this Standard A). Aliquot 10 µl of Standard A (3,000 pmol/ml) into 9.99 ml of Tris Buffer (label this Standard B). The concentration of this standard is 3 pmol/ml.

*[NOTE: If the samples are prepared from TCA-extracted tissue and cannot be diluted at least 1:5 in Tris Buffer for analysis, use ether-extracted 5% TCA for preparation of the standard curve. Any dilution of samples should then be performed in this solution.]*

To prepare the standard for use in EIA: Obtain 9 clean test tubes and number them #0 through #8. Aliquot 500 µl Tris Buffer to tube #0 (this tube will contain only buffer) and 500 µl Tris Buffer to tubes #2-8. Transfer 750 µl of Standard B (3 pmol/ml) to tube #1. Serially dilute the standard by removing 250 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 250 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. Discard 250 µl of the solution in tube #8 so each tube contains 500 µl. These diluted standards should not be stored for more than 24 hours.

#### Sample preparation

If samples need to be purified, do so before proceeding with the acetylation procedure (see page 3 for Purification Protocols). Although purification may not be necessary, we recommend that samples be purified to ensure assay integrity. If you are acetylating less than 500 µl of sample, you must adjust the amounts of KOH and acetic anhydride proportionally.

#### Preparation of KOH

Prepare a 4 M solution of KOH by dissolving 2.6 g of KOH (provided in this kit) in 10 ml deionized water.

### Acetylation procedure (based on 500 µl sample size)

All samples, as well as standard tubes #0-8, must be acetylated. Each sample/standard should be acetylated individually. It is important to be consistent in the acetylation technique as differences in vortex time and/or delayed addition of KOH may result in irreproducible results.

To 500 µl of sample, add 100 µl of 4 M KOH and 25 µl Acetic Anhydride in quick succession. Vortex for 15 seconds. Add an additional 25 µl of 4 M KOH and vortex. Repeat for all samples and standard tubes.

*[NOTE: If the samples contain sugars at concentration >250 mM, it may be necessary to proportionately increase the amount of KOH and acetic anhydride added to ensure complete acetylation of cGMP.]*

### Assay Procedure

*[Note: All reagents should be allowed to warm to room temperature before use.]*

#### 1. Tris Buffer

Pipet 150 µl Tris Buffer into non-specific binding (NSB) wells, and 100 µl Tris Buffer into zero standard (B<sub>0</sub>) wells. If tissue culture medium was used to dilute the standard curve, substitute 100 µl of this same medium for the Tris Buffer in the NSB and B<sub>0</sub> wells.

#### 2. cyclic GMP Standard

Pipet 100 µl of standards into the appropriate wells.

#### 3. Samples

Pipet 100 µl of sample into the appropriate wells. Each sample should be assayed in duplicate or triplicate.

#### 4. cyclic GMP Alkaline Phosphatase Tracer

Pipet 50 µl of tracer into each well except the blank wells and total activity (TA) wells.

#### 5. cyclic GMP Antiserum

Pipet 50 µl of antiserum into each well except the blank wells, TA wells and NSB wells.

Well	Tris Buffer	Std/Sample	Tracer	Antiserum
Blank				
TA			5 µl (at development)	
NSB	150 µl		50 µl	
B <sub>0</sub>	100 µl		50 µl	50 µl
Std/Sample		100 µl	50 µl	50 µl

#### Pipetting Summary

#### 6. Incubate the plate

Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.

#### 7. Prepare the pNPP Solution

Dissolve 5 pNPP tablets in 25 ml DEA Buffer (25 ml is sufficient to develop 100 wells). *[Note: Reconstituted pNPP is not stable, so we recommend that you make only the amount that you need at any one time.]*

#### 8. Wash the plate

Empty the wells and rinse five times with Wash Buffer. After the final wash, firmly tap the inverted plate on a paper towel to remove any recalcitrant drops of buffer.

#### 9. Develop the plate

Add 200 µl pNPP solution to each well including blank and TA wells. Add 5 µl of tracer to the TA wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60-90 minutes.

#### 10. Read the plate

Wipe the bottom of the plate with a paper towel to remove any finger prints, smudges or dirt which may interfere with obtaining an accurate reading of absorbance. Remove the plate cover, and read the plate at a wavelength between 405 and 420 nm.

## **Data Analysis**

Most plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as %B/B<sub>0</sub> versus log concentration of standard using either a 4-parameter logistic or a log-logit curve fit.

### 1. Prepare the Data

The following procedure is recommended to prepare the data prior to graphing (subtract the absorbance of the blank wells from all wells on the plate, if not already done).

- a. Average the absorbance readings from the NSB wells.
- b. Average the absorbance readings from the B<sub>0</sub> wells.
- c. Subtract the average NSB from the average B<sub>0</sub>. This is the corrected B<sub>0</sub>.

$$\text{Corrected } B_0 = \text{Average } B_0 - \text{Average NSB}$$

- d. Calculate the %B/B<sub>0</sub> for each standard and sample. To do this, subtract the average NSB absorbance from the absorbance and divide by the corrected B<sub>0</sub> (from step c). Multiply by 100 to obtain %B/B<sub>0</sub>. Repeat for all wells.

$$\%B/B_0 = \left[ \frac{\text{Absorbance} - \text{Average NSB}}{\text{Corrected } B_0} \right] \times 100$$

### 2. Plot the Standard Curve

Plot %B/B<sub>0</sub> for all standards *versus* cyclic GMP concentration using log (x) and linear (y) axes, and fit the data to a four parameter logistic equation. Alternatively, the data can be linearized using a logit transformation. [**Note:** Do not use %B/B<sub>0</sub> in this calculation.]

$$\text{Logit } (B/B_0) = \ln [B/B_0 / (1 - B/B_0)]$$

Plot the data as logit (B/B<sub>0</sub>) *versus* log concentration of standard and perform a linear regression fit.

### 3. Determine the Concentration of your Samples

Calculate the %B/B<sub>0</sub> for each sample. Determine the concentration of each sample using the equations obtained from the analysis of the standard curve. Remember to account for any dilutions made to the sample prior to addition to the well. %B/B<sub>0</sub> values of greater than 80% or less than 20% should be re-assayed as they generally fall outside of the linear range of the standard curve.

## **Assay Performance Characteristics**

### 1. Precision

Intra-assay precision was determined by measuring samples containing low, medium, and high concentrations of unacetylated cGMP multiple times in the same assay (eight samples per plate on a total of five plates). Inter-assay precision was determined by measuring low, medium, and high concentrations of the samples eight times using different reagents.

	cGMP (pmol/ml)	Intra-assay %CV	Inter-assay %CV
Low	1.2	7.4	5.4
Medium	3.7	5.7	7.5
High	33.3	8.6	8.6

### 2. Specificity

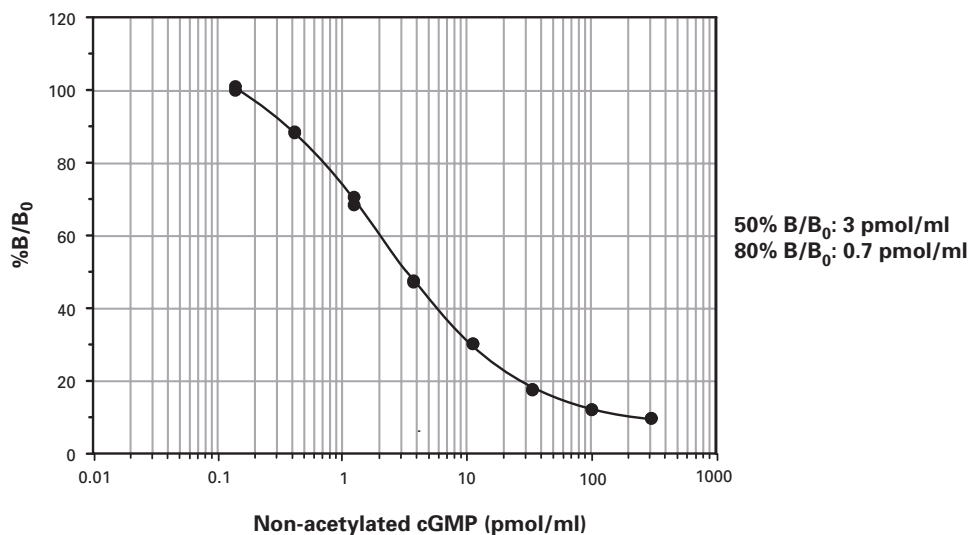
Analyte	Cross-Reactivity	Analyte	Cross-Reactivity
Acetylated cGMP	100%	ATP	<0.01%
cGMP	9%	Acetylated adenosine	<0.01%
Dibutyryl cGMP	0.8%	Acetylated AMP	<0.01%
Acetylated cAMP	0.05%	Acetylated cytidine	<0.01%
cAMP	<0.01%	Acetylated guanosine	<0.01%
AMP	<0.01%	Acetylated uridine	<0.01%

### 3. Typical Results

The standard curve shown here is an example of data typically produced by this kit. Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

	Raw Data		Average	Corrected
Total Activity	3.927	3.863	3.895	
NSB	0	0	0	
$B_0$	1.184	1.228	1.209	1.209
	1.192	1.231		

Concentration Std (pmol/ml)	Raw Data		Corrected		%B/B <sub>0</sub>	
300	0.115	0.114	0.115	0.114	9.5	9.4
100	0.144	0.143	0.144	0.143	11.9	11.8
33.3	0.209	0.211	0.209	0.211	17.3	17.5
11.1	0.363	0.363	0.363	0.363	30.0	30.0
3.7	0.573	0.568	0.573	0.568	47.4	47.0
1.2	0.851	0.825	0.851	0.825	70.4	68.3
0.4	1.064	1.068	1.064	1.068	88.0	88.4
0.1	1.219	1.206	1.219	1.206	100.9	100.0



### References

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2. Schmidt, H.H.H.W., Lohmann, S.M., and Walter, U. The nitric oxide and cGMP signal transduction system: Regulation and mechanism of action. *Biochim. Biophys. Acta* **1178**, 153-175 (1993).
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### Warranty and Limitation of Remedy

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13/12/07