



cGMP HTS Immunoassay Kit (Chemiluminescent)

Cat. No. 17-419

192 Wells

**FOR RESEARCH USE ONLY
Not for use in diagnostic procedures**

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Introduction

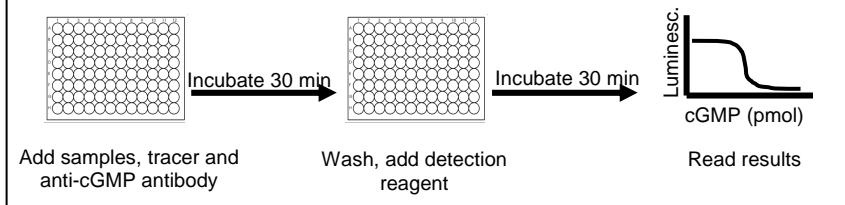
Cyclic GMP (cGMP, Guanosine 3', 5'-cyclic monophosphate) is a nucleotide which acts as a key second messenger in multiple signal transduction pathways, and is found in most tissues at levels 10 to 100 times lower than cAMP [1]. cGMP is synthesized from guanosine triphosphate (GTP) by the action of guanylate cyclase, degraded by cyclic nucleotide phosphodiesterases, which hydrolyze cGMP into 5'-GMP. Levels of cGMP are increased by the action of certain hormones, such as acetylcholine, insulin and oxytocin, as well as by chemicals such as serotonin and histamine. cGMP can also be elevated by agents which stimulate guanylate cyclase, such as nitroprusside, nitroglycerin, sodium nitrate and nitric oxide [2].

Downstream effects of cGMP are mediated primarily by cGMP-gated ion channels, cGMP-dependent protein kinases and cGMP-regulated phosphodiesterases [3]. cGMP has become an important target for drug discovery because it regulates many cellular functions, including ion channel conductance, glycogenolysis, and cellular apoptosis [4, 5]. Significantly, it also relaxes smooth muscle tissues. In blood vessels, relaxation of vascular smooth muscles leads to vasodilation and increased blood flow. This effect of cGMP has led to the notable clinical success of Sildenafil (Viagra), which enhances the vasodilatory effects of cGMP within the corpus cavernosum by inhibiting a phosphodiesterase, and is widely used as a treatment for erectile dysfunction [6].

Test Principle

Competitive ELISAs differ from traditional sandwich ELISAs in that a competition between labeled and unlabeled antigen for available antibody binding sites occurs. In order to utilize a competitive ELISA, one reagent must be conjugated to a detection enzyme, such as alkaline phosphatase. In Millipore[®]'s cGMP HTS Immunoassay kit, standards or unknown samples are mixed with an alkaline phosphatase (AP)-labeled cGMP conjugate and a highly specific anti-cGMP antibody, and incubated in wells of a 96 well microtiter plate pre-coated with a capture antibody. The AP-labeled cGMP conjugate will bind to the anti-cGMP antibody wherever its binding sites are not already occupied by unlabeled cGMP. Thus, the more cGMP in the sample or standard, the lower the amount of AP-labeled cGMP-conjugate bound. Upon plate development, the chemiluminescence intensity is inversely proportional to the amount of cGMP in a sample or standard.

Figure 1: Assay Overview



Application

Millipore[®]'s cGMP HTS Immunoassay kit is a competitive immunoassay for highly sensitive and rapid chemiluminescent quantitation of cyclic GMP (cGMP, Guanosine 3', 5'-cyclic monophosphate) from extracts, lysates or supernatants of cells of any species. The kit comprises a specific anti-cGMP antibody which recognizes all species, an alkaline phosphatase (AP)-labeled cGMP conjugate, two 96 well microplates pre-coated with an anti-Rabbit antibody, cGMP standard, and an Alkaline Phosphate chemiluminescent substrate. This kit contains antibody coated plates and all reagents required to perform 192 assays.

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Kit Components

1. White 96-Well anti-Rabbit Coated Plate: (Part No. 2004070) Two 96-well immuno-plates pre-coated with anti-Rabbit polyclonal antibody, sealed in a foil pouch.
2. Rabbit anti-cGMP polyclonal Antibody: (Part No. 2004761) One 25 μ L vial.
3. cGMP Standard: (Part No. 2004762) One 2 mL bottle.
4. Alkaline Phosphatase cGMP Tracer Conjugate: (Part No. 2004760) One 20 μ L vial.
5. 2x cGMP Assay Diluent: (Part No. 2004811) One 25 mL bottle.
6. 5x Wash Buffer: (Part No. 2004075) One 125 mL bottle.
7. Lysis Buffer: (Part No. 2004076) One 60 mL bottle.
8. 6x Alkaline Phosphatase Substrate: (Part No. 2004077) One 5 mL bottle.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Pipettors & tips capable of accurately measuring 10-1000 μL
3. Plate shaker (optional)
4. Graduated serological pipettes
5. Luminometer capable of reading 96-well microplates.
6. Polystyrene test tubes for standard and sample dilutions
7. Mechanical vortex
8. Containers for dilutions

Storage of Kit Components

Maintain the unopened kit at 2-8°C until the expiration date indicated on the label. After opening the kit, maintain the White 96-Well anti-Rabbit Coated Plates, Alkaline Phosphatase cGMP Tracer Conjugate, cGMP Standard, 5x Wash Buffer, 2x Assay Diluent, Lysis Buffer, and Alkaline Phosphatase Substrate at 2-8°C until the expiration date indicated on the label. For long-term storage, maintain the Rabbit anti-cGMP Polyclonal Antibody at -20°C.

Precautions

- The White 96-Well anti-Rabbit Coated Plates, 5x Wash Buffer, 2x Assay Diluent, and Lysis Buffer contain thimerosal. Thimerosal is highly toxic by inhalation, contact with skin, or if swallowed. Thimerosal is a possible mutagen and should be handled accordingly.
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.

Technical Hints

- Allow samples and all assay reagents to reach room temperature before use.
- Standards should be made in either glass, or polypropylene tubes; avoid polystyrene.

- Mix samples thoroughly before use; avoid excessive foaming.
- Pipet the sample / standard to the bottom of the well.
- To avoid contamination, add additional assay reagents to the side of the well.
- The use of plate sealers is recommended to reduce the possibility of well-to-well contamination.
- During the incubation time, the use of a plate shaker may improve assay sensitivity.
- Minimize contamination by endogenous alkaline phosphatase. Care should be taken to not touch pipet tips or other reagents with bare hands.
- Ensure that no residual wash buffer remains in the wells. Be sure to completely pat dry the plate on paper towels following the last wash step.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values.
- **Recommended Method for Plate Washing:**
 1. Remove existing fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
 2. Pipet 250 μ L of diluted 1x Wash Buffer into each well with a multi-channel pipet.
 3. Remove the Wash Buffer from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels to remove excess fluid.
 4. Repeat washing and flicking 5 times.

Preparation of Reagents

1. Wash Buffer

Add the entire contents of the 5x Wash Buffer to an appropriate container, and adjust volume to 300 mL with deionized water. Stir to homogeneity.

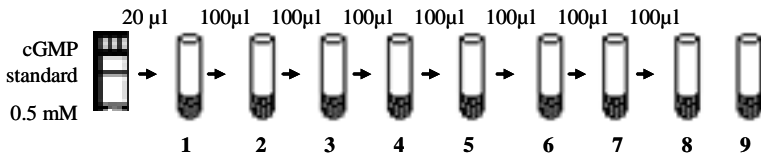
2. Assay Diluent

Add the entire contents of the 2x Assay Diluent to an appropriate container, and adjust volume to 50 mL with deionized water. Vortex or stir thoroughly. Use this 1x solution for diluting the Rabbit anti-cGMP Antibody and cGMP Alkaline Phosphatase Conjugate Tracer.

3. cGMP Standard

The cGMP Standard is provided at a concentration of 0.5 mM (500,000 pmol/mL). This stock material is then used to generate a standard curve. Use Lysis Buffer to make cGMP Standard dilutions. A suggested dilution scheme is described and illustrated below:

- Label 9 glass, or polypropylene tubes #1 – 9. Add 900 μ L Lysis Buffer to tubes #2 – 9.
- Add 980 μ L Lysis Buffer to tube #1. Remove 20 μ L of the cGMP Standard solution from the stock bottle and add to tube #1, giving rise to 10,000 pmol/mL solution. Vortex for approximately 2 seconds.
- Standards #2 – 8 are then prepared by performing 1:10 dilutions from the preceding standard. For example, to make standard #2, remove 100 μ L from Standard #1 and add to Tube #2. Vortex for approximately 2 seconds. Repeat for Standards #3 – 8. Do not add any cGMP Standard to the #9 tube.
- The concentration of cGMP in tubes #1 – 9 will be 10,000, 1,000, 100, 10, 1, 0.1, 0.01, 0.001 and 0 pmol/mL respectively.



<u>Standard Number:</u>	#1	#2	#3	#4	#5	#6	#7	#8	#9
<u>Lysis Buffer (μL):</u>	980	900	900	900	900	900	900	900	900
<u>Standard (μL):</u>	20	100	100	100	100	100	100	100	0
<u>cGMP (pmol/mL):</u>	10,000	1000	100	10	1	0.1	0.01	0.001	0

Figure 2: Serial Dilution of cGMP Standard

Note: The cGMP Standard curve can be set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern.

4. Rabbit anti-cGMP Antibody

Immediately before use, dilute a sufficient amount of the Rabbit anti-cGMP antibody 1:1,000 with 1x Assay Diluent. Mix thoroughly.

5. Alkaline Phosphatase cGMP Tracer Conjugate

Immediately before use, dilute a sufficient amount of the cGMP Alkaline Phosphatase – Conjugated Tracer 1:3000 with 1x Assay Diluent.

6. Alkaline Phosphate Substrate

Immediately before use, dilute a sufficient amount of the Alkaline Phosphate Substrate 1:6 with 1x Wash Buffer. Mix thoroughly.

Preparation of Samples

It is recommended that you test each sample in triplicate. Prepare cell lysates by incubating the cells with Lysis Buffer. If using 96-well cell culture plated samples, add 50 μ L of Lysis Buffer to each well and incubate 10 minutes. Samples can be further diluted with 1x Assay Diluent or Lysis Buffer.

Notes:

1. *Sample dilution may vary between different cells. Appropriate sample dilutions should be established by each investigator.*
2. *Always use freshly prepared samples.*

Assay Instructions

1. Remove the White 96-Well anti-Rabbit Coated Plate from its foil pouch.
2. Add 100 μ L of cGMP Standards 1 through 8 or prepared samples to wells. It is recommended that standards and samples be run in triplicate.
Note: A standard curve must be performed for each assay.
3. Add 50 μ L of the diluted Alkaline Phosphatase cGMP Tracer Conjugate to all wells being tested.
4. Add 50 μ L of the Rabbit anti-cGMP Antibody dilution to all wells being tested.
5. Cover or seal the plate with a plate sealer. Incubate the plate for 30 minutes at room temperature (the use of a plate shaker during this step may improve assay sensitivity).
6. Remove the fluid from the wells with an automated plate washer or by inverting the plate over a sink.
7. Carefully wash the wells at least 5 times with 1x Wash Buffer.

For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. This may result in poor precision and an unsuitable standard curve. For best results, we recommend at least 5 wash cycles.

8. Following the last wash, completely flick and pat dry the plate on a stack of paper towels. This will ensure that remaining wash buffer residue does not interfere with the assay results.
9. Add 100 μ L of the diluted Alkaline Phosphatase Substrate. Cover or seal the plate and incubate at room temperature for 30 minutes.
10. Read the plate for 1.0 second per well on a luminometer device.

CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.*

Calculation of Results

Manual Plotting: Plot the standard curve on semi-log graph paper. Known concentrations of cGMP are plotted on the X-axis and the corresponding RLUs (Relative Light Units) on the Y-axis. The standard curve should result in a graph that shows an inverse relationship between cGMP concentrations and the corresponding luminescence. Therefore, the greater the concentration of unconjugated, or “free” cGMP in the sample, the lower the Relative Luminescence Units.

Plate Reader/PC Interface: An alternative approach is to enter the data into a computer program curve fitting software. A good fit can be obtained with a log regression analysis. Some data points at the top or bottom of the range tested may need to be dropped to get a good fit. Currently existing spreadsheet software can perform such plotting.

Sample Results

The Millipore® cGMP HTS Immunoassay is a competitive ELISA, thus low levels of endogenous cGMP are indicated by a high signal, while high levels of endogenous cGMP are indicated by a low signal. An example of a typical standard curve is shown in Figure 4 below. This data is presented for reference only and should not be used to analyze assay results. We strongly recommend that a standard curve be generated for each set of samples assayed.

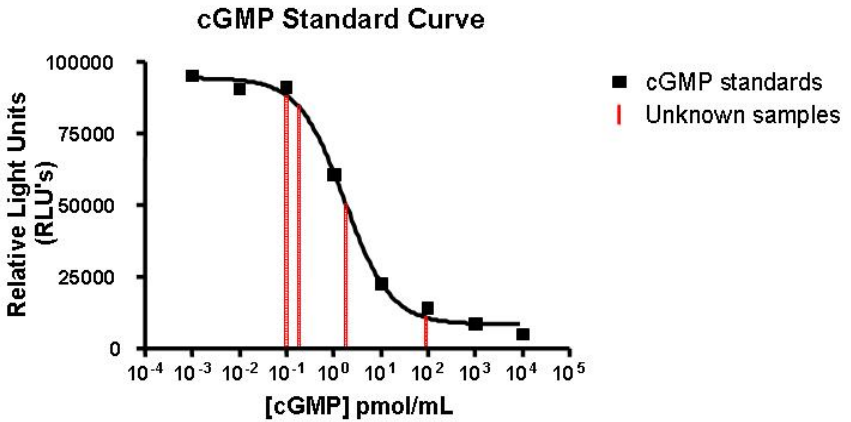


Figure 4. The provided cGMP standard containing 0.5 mM cGMP (500,000 pmol/ml) was used in this experiment. Eight serial dilutions were prepared in Lysis buffer. 100 μ L of each standard was used to generate the standard curve in a range from 0.001 to 10,000 pmol cGMP/well. The unknown samples represent cGMP synthesis in P19 cell lysates treated with a range of sodium nitroprusside concentrations.

Cross-reactivity of rabbit anti-cGMP polyclonal antibody

cGMP	100%	ADP	<0.01%
cAMP	<0.1%	ATP	<0.01%
GTP	<0.01%		
GMP	<0.01%		
CTP	<0.01%		
AMP	<0.01%		

References:

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