Cyclic GMP Competitive ELISA Kit

For lysate and homogenate samples

Catalog Number: EMSCGMPL

Product description

A competitive immunoassay for the quantitative determination of cyclic GMP (cGMP) in samples treated with 0.1 M HCl. The assay is based on the competition between cGMP in the standard or sample and Alkaline Phosphatase conjugated cGMP (cGMP-AP) for a limited amount of cGMP monoclonal antibody bound to an Anti-Rabbit IgG precoated 96-well plate. As the concentration of cGMP in the sample increases, the amount of cGMP-AP captured by the coating antibody decreases. Thus, there is an inverse relationship between optical density (OD) and the amount of analyte in the sample.

Contents and storage

Upon receipt, store the kit at ≤ –20°C.

Description	Size
Antibody coated 96-welll plate	1 plate
cGMP Antibody	5 mL
cGMP-AP Conjugate	5 mL
0.1 M HCl	27 mL
Neutralizing Reagent	5 mL
Triethylamine*	2 mL
Acetic Anhydride*	1 mL
cGMP Standard (5,000 pmol/mL)	0.5 mL
20X Wash Buffer	27 mL
pNpp Substrate Solution	20 mL
Stop Solution	5 mL
Plate Sealer	1 each

CAUTION! * Components that are flammable, corrosive or produce harmful vapors. Keep away from open flames, hot surfaces, and sources of ignition. Avoid exposure and use only under a chemical fume hood.

Additional required materials

- Deionized or distilled water
- Precision pipettes (for volumes between 5 µL and 1,000 µL)
- Repeater pipettes (for dispensing 50 µL and 200 µL)
- Disposable beaker for diluting buffer concentrates
- 12 × 75 mm glass tubes
- Graduated cylinders
- Triton™ X-100 (optional for sample preparation)
- Microplate shaker
- Absorbent lint-free paper for blotting
- Microplate reader capable of reading at 405 nm.

General guidelines

- Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Care must be taken to minimize contamination by endogenous alkaline phosphatase (AP). Contaminating AP activity, especially in the substrate solution, may lead to high blanks.
- Care should be taken not to touch pipette tips and other items that are used in the assay with bare hands.
- Allow kit components to come to room temperature for at least 30 minutes before use.
- 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.

Assay compatibility

The cGMP Competitive ELISA for lysate and homogenate samples is compatible with cGMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity.

Prepare 1X Wash Buffer

1. Allow the 20X Wash Buffer to reach room temperature and mix to redissolve any precipitated salts.
2. Dilute 5 mL of 20X Wash Buffer with 95 mL of deionized or distilled water. Label as 1X Wash Buffer.

   The diluted buffer is stable for up to 3 months at room temperature.

Prepare frozen tissue

1. Grind tissue samples frozen in liquid nitrogen to a fine powder under liquid nitrogen in a stainless steel mortar.
2. Allow the liquid nitrogen to evaporate, then weigh the frozen tissue and homogenize in 10 volumes of 0.1 M HCl.
3. Centrifuge at ≥600 × *g* at room temperature for 10 minutes.
4. Dilute the sample in the 0.1 M HCl (provided with kit).

Prepare cultured cells

1. Remove tissue culture media from cells, and treat with 0.1 M HCl.

   Note: Cell lysis can be enhanced by adding 0.1% to 1% Triton™ X-100 to the 0.1 M HCl prior to use. The detergent does not interfere with acetylation or the binding portion of the assay in this concentration range, but a modest increase in the optical density will be observed.
2. Incubate for 10 minutes and verify cell lysis by visual inspection. Incubate for an additional 10 minutes if adequate lysis has not occurred.
3. Centrifuge at ≥600 × *g* at room temperature for 10 minutes.
4. Use the supernatant directly in the assay.

Prepare cell culture supernatant

1. Treat 1 mL cell or tissue culture supernatant with 10 µL of **concentrated** HCl.
2. Centrifuge at 600 × *g* at room temperature for 10 minutes.
3. Use the supernatant directly in the assay.

Sample preparation guidelines

- Samples in matrix treated with hydrochloric acid can be measured directly without evaporation or further treatment.
- Acetylate samples and standards for samples with very low levels of cGMP.
- To perform the **acetylated version** of the assay, acetylate samples by adding 10 µL of the Acetylating Reagent for every 200 µL of sample.
- Use **acetylated samples** within 30 minutes.

Prepare Acetylating Reagent

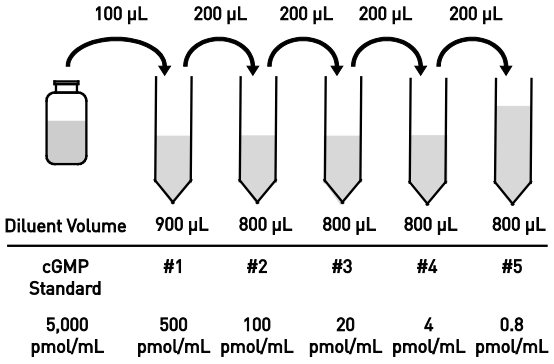
1. Add 0.5 mL of acetic anhydride to 1 mL triethylamine and mix well.
2. Use reagent within 60 minutes of preparation.

Standard preparation guidelines

If using samples that are acetylated, or treated with Triton™ X-100, ensure the standard curve is prepared in the same type of matrix.

Dilute non-acetylated standards

1. Allow the 5,000 pmol/mL cGMP standard to warm to room temperature.
2. Label five 12 × 75 mm glass tubes #1 through #5.
3. Add 900 µL 0.1 M HCl to Tube #1.
4. Add 800 µL 0.1 M HCl to Tubes #2 to #5.
5. Add 100 µL cGMP Standard to Tube #1 and vortex thoroughly.
6. Make serial dilutions of the standard as shown in the dilution diagram below.
   Use the non-acetylated standards within 60 minutes.



Dilute acetylated standards

1. Allow the 5,000 pmol/mL cGMP standard to warm to room temperature.
2. Label five 12 × 75 mm glass tubes #1 through #5.
3. Add 990 µL 0.1 M HCl to Tube #1.
4. Add 800 µL 0.1 M HCl to Tubes #2 to #5.
5. Add 10 µL cGMP Standard to Tube #1.
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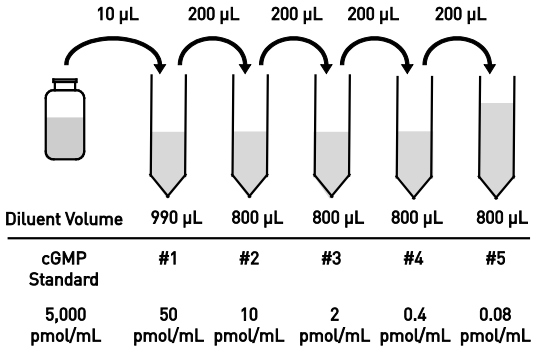


7. For each tube, add 10 µL of the Acetylating Reagent for every 200 µL of standard and immediately vortex for 2 seconds.
8. Add 1 mL of 0.1 M HCl and 50 µL of Acetylating Reagent to a Zero Standard/NSB tube.
9. Use the acetylated standards within 30 minutes.

ELISA procedure

Bring all reagents to room temperature for at least 30 minutes prior to use. Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2 to 8°C for future use.

Run all standards and samples in duplicate.

1. Add 50 µL of Neutralizing Reagent into each well, **except** the Total Activity (TA) and Blank wells.
2. Add 100 µL of 0.1 M HCl to NSB and B_0 (0 pmol/mL Standard) wells.
3. Add 100 µL of Standards #1 through #5 into the appropriate wells.
4. Add 100 µL of the Samples into the appropriate wells.
5. Add 50 µL of 0.1 M HCl to NSB wells.
6. Add 50 µL of the blue cGMP-AP Conjugate into each well, **except** the TA and Blank wells.
7. Add 50 µL of the yellow cGMP Antibody into each well, **except** the TA, Blank, 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 and incubate at room temperature on a plate shaker (~500 rpm) for 2 hours.

   NOTE: If performing the acetylated version of the assay, incubation can be carried out at 2°C to 8°C for 18–24 hours).
9. Empty the contents of the wells and wash by adding 400 µL of the 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes.
10. 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.
11. Add 5 µL of the blue cGMP-AP Conjugate to the TA wells.
12. Add 200 µL of p-NPP Substrate Solution to each well, and incubate at room temperature for 1 hour (no shaking).
13. Add 50 µL of Stop Solution to every well and read the plate immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculations

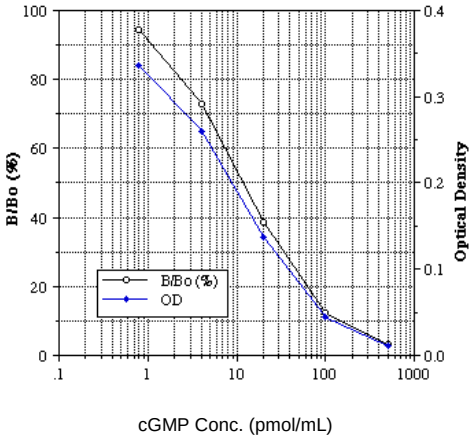
Several options are available for the calculation of the concentration of cGMP in the samples. It is recommended that the data be analyzed by a 4 parameter logistic curve-fitting program. If data reduction software is not available, the concentration of the cGMP can be calculated by plotting % Bound [(Net OD/Net B_0 OD) × 100] versus Concentration of cGMP for the standards. Approximate a straight line through the points. The concentration of cGMP in the unknowns can be determined by interpolation.

Performance characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

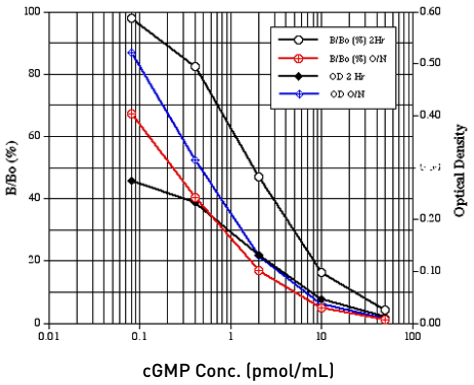
Typical standard curve

A typical standard curve for the **non-acetylated version** of the assay is shown below. This curve must not be used to calculate cGMP concentrations; a standard curve must be run with every assay.



Typical standard curve

A typical standard curve for the **acetylated version** of the assay is shown below. This curve must not be used to calculate cGMP concentrations; a standard curve must be run with every assay.



Performance characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

Sensitivity

The minimum detectable dose of cGMP:

Assay	Sensitivity
Non-acetylated version	0.604 pmol/mL
Acetylated version (2-hour incubation)	0.059 pmol/mL
Acetylated version (overnight incubation)	0.025 pmol/mL

This was determined by adding two standard deviations to the mean O.D. obtained from the average OD bound for 16 wells run as B_0, compared to the average OD for 16 wells run with Non-Acetylated Standard #5, or Acetylated Standard #5, respectively.

Linearity

For the **non-acetylated version** of the assay, a sample containing 96 pmol/mL cGMP was serially diluted 7 times 1:2 in 0.1 M HCl. Linear regression analysis of samples versus the expected concentration yielded a line with a slope of 1.000 and correlation coefficient of 0.999.

For the **acetylated version** of the assay, a sample containing 16 pmol/mL cGMP was serially diluted 7 times 1:2 in 0.1 M HCl. Linear regression analysis of samples versus the expected concentration yielded a line with a slope of 1.001 and correlation coefficient of 0.998.

Precision

The precision numbers listed below represent the percent coefficient of variation for the concentrations of cGMP determined in these assays (with 2-hour incubation) as calculated by a 4 parameter logistic curve fitting program.

Intra-assay (n=24)	Non-acetylated		Acetylated	
	cGMP (pmol/mL)	%CV	cGMP (pmol/mL)	%CV
Low	1.85	4.43	0.58	9.57
Medium	9.88	7.90	1.38	3.55
High	115.3	6.57	5.38	3.49

Inter-assay (n=8)	Non-acetylated		Acetylated	
	cGMP (pmol/mL)	%CV	cGMP (pmol/mL)	%CV
Low	2.14	5.96	0.349	10.89
Medium	8.53	9.85	3.51	8.35
High	97.0	6.88	10.3	4.57

Cross-reactivity

The cross-reactivities for a number of related compounds were determined by dissolving the cross reactant in 0.1 M HCl 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/B_0 calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

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

Sample recovery

cGMP concentrations were measured in tissue culture media. cGMP was spiked into the undiluted sample which was diluted with the kit 0.1 M HCl and then assayed in the kit.

Sample	Non-acetylated		Acetylated	
	% Recovery	Dilution	% Recovery	Dilution
Tissue Culture Media	95.9	None	86.8	None

Limited product warranty

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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

Manufacturer's address: Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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