—A helping hand for your research

Product Manual

Monoclonal Anti-cGMP Antibody Based Direct cGMP ELISA Kit (New Non-acetylated Version)

Catalog No. 80103

96 Well Kit

NewEast Biosciences
24 Whitewoods Lane
Malvern , PA 19355

FAX: 610-945-2008
Phone: 610-945-2007
Web: www.neweastbio.com
E-mail: sale@neweastbio.com

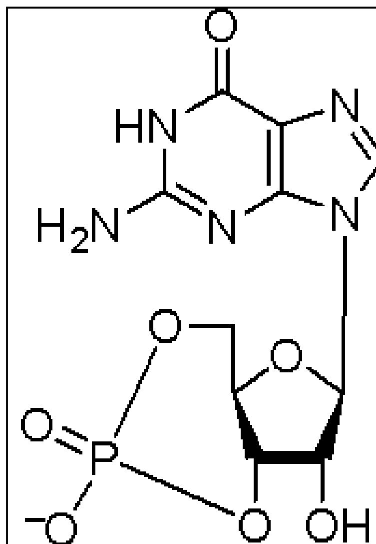
Table of Content

Product Description	3
Principle Outline	4
Background	5
Materials Supplied	5
Storage	6
Materials Needed but Not Supplied	6
Sample Handling	7
Procedural Notes	8
Reagent Preparation	8
Assay Procedure	9
Calculation of Results	11
Typical Standard Curve	12
Sensitivity	13
Linearity	13
Cross Reactivities	13
References	14

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDUR

Product Description

Guanosine 3', 5'-cyclic monophosphate (cyclic GMP; cGMP) plays critical regulatory roles in many physiological processes. cGMP is produced from GTP by guanylyl cyclases and is degraded by phosphodiesterases. Stimulation of guanylyl cyclases or inhibition of phosphodiesterases can increase cellular cGMP concentrations. Inhibitors of the cGMP-specific phosphodiesterases are used for treating human diseases. For example, inhibitors of cGMP specific phosphodiesterase type 5 (such as Viagra and Cialis) are used for treating erectile dysfunction.



To screen for inhibitors of phosphodiesterases or stimulators of guanylyl cyclases, it is essential to have a fast, sensitive, selective and reproducible method to measure the cGMP concentrations. This is especially true for the initial screenings given the possible weaker effects of larger pools of compounds.

Currently available other ELISA kits measuring cGMP levels are based on the non-affinity-purified polyclonal anti-cGMP antibody. Despite the claimed selectivity, these polyclonal anti-cGMP antibodies display certain cross-reactivity with cAMP or GTP. In most cell types, cGMP is present at levels ~100 fold lower than cAMP.

NewEast Biosciences cGMP ELISA kit is based on the **unique mouse monoclonal anti-cGMP antibody**. This monoclonal anti-cGMP antibody displays >10^8 fold of selectivity over cAMP, GTP, and other nucleoside analogues. NewEast Biosciences cGMP ELISA kit provides significantly **improved sensitivity and selectivity** over other kits based on polyclonal anti-cGMP antibodies. Our monoclonal anti-cGMP antibody-based ELISA kit also avoids the batch-to-batch variations associated with polyclonal antibody productions from animals, thus providing the **reproducibility** in the long run.

Furthermore, while polyclonal anti-cGMP antibodies used in other ELISA kits have higher affinity for acetylated cGMP than non-acetylated cGMP, NewEast Biosciences monoclonal anti-cGMP antibody has similar affinities to non-acetylated and acetylated cGMP molecules. Therefore, acetylation treatments of samples and standards are not needed in NewEast Biosciences cGMP ELISA kit. This significantly **reduces the time for the assay**. The avoidance of organic reagents used in the acetylation process provides **a safe and healthy work environment**.

Principle Outline

NewEast Biosciences cGMP ELISA Kit is a competitive immunoassay to measure the cGMP levels, either from cell extracts or from in vitro guanylyl cyclase assays. Briefly, multi-well plates are coated with goat-anti-mouse serum. cGMP in cell extracts or in vitro guanylyl cyclase assays will competitively bind to the monoclonal anti-cGMP antibody in the presence of fixed amounts of cGMP-conjugated horse-radish peroxidase. Known amounts of cGMP are used as standards to generate the calculation curve. After a short incubation, the excess reagents are washed away and substrate is added. The multiwell plates are

then read on a microplate reader at 450 nm. The intensity of the yellow color is inversely proportional to the concentration of cGMP in samples. The measured optical density is used to calculate the concentration of cGMP in samples based on the curve from the cGMP standards.

Background

cGMP is a ubiquitous second messenger mediating cellular responses to various exogenous and endogenous signaling molecules. cGMP controls diverse physiological functions such as relaxation of vascular smooth muscles, phototransduction, epithelial electrolyte transport, bone growth, leukocyte migration, axonal guidance, sperm motility, platelet spreading, and vascular permeability (1-9). cGMP regulates physiological processes by activating protein kinases, gating specific ion channels, and modulating cellular cyclic nucleotide concentrations through phosphodiesterases (8).

The conversion of GTP to cGMP is catalyzed by guanylyl cyclases (GCs). There are two types of GCs in mammals: the soluble and the membrane-bound GCs (8,10,11). The soluble GCs are generally activated when NO (nitric oxide) binds to the attached prosthetic heme group. Seven membrane-bound GCs (also named transmembrane or particulated GCs) have been identified in the human genome (8). GC-A and GC-B are natriuretic peptide receptors. GC-C can be activated by bacterial heat-stable enterotoxins, guanylin and uroguanylin. The activity of transmembrane GCs can also be modulated by other receptor signals through intracellular signaling pathways (12).

Materials Supplied

1. **Goat anti-Mouse IgG Coated Microtiter Plate, One Plate of 96 Wells, Catalog No. 30101**

A plate using break-apart strips coated with goat antibody specific to mouse IgG.

2. **cGMP Direct Conjugate, 6 mL, Catalog No. 30102**
   A solution of horse radish peroxidase conjugated with cGMP (a 1000 X stock solution and dilution solution are provided).
3. **cGMP Direct Antibody, 6 mL, Catalog No. 26001-2**
   A solution of a mouse monoclonal antibody to cGMP (a 1000 X stock solution and dilution solution are provided).
4. **Neutralizing Reagent, 6 mL, Catalog No. 30103**
5. **10X Wash Buffer Concentrate, 15 mL, Catalog No. 30106**
   Phosphate buffered saline containing detergents.
6. **Cyclic GMP Standard, 0.25 mL, Catalog No. 30114**
   A solution of 5,000 pmol/mL cGMP.
7. **Substrate A, 10 mL, Catalog No. 30107**
8. **Substrate B, 10 mL, Catalog No. 30108**
9. **Stop Solution, 6 mL, Catalog No. 30110**
   A solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic.**

Storage

For long-term best results, store stocks of the anti-cGMP antibody and cGMP-HRP conjugate at -80°C. Right before use, dilute these stocks (take 6 µl) with the provided dilution buffers (6 ml).
All other components of this kit are stable at 4°C until the kit's expiration date.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Concentrated HCl.

3. A microplate shaker.
4. Microplate reader capable of reading at 450 nm, preferably with correction between 570 and 590 nm.

Sample Handling

NewEast Biosciences EIA is compatible with cGMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity. Samples in this matrix can be measured directly without evaporation or further treatment.

Tissue samples should be frozen in liquid nitrogen. The tissue should be ground to a fine powder under liquid nitrogen in a stainless steel mortar. After the liquid nitrogen has evaporated, weigh the frozen tissue and homogenize in 10 volumes of 0.1M HCl. Centrifuge at > 600 x g at room temperature. The samples can then be diluted in the 0.1M HCl.

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 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. Cyclic GMP in the media can be measured after treating 1 mL of the supernatant media with 10 mL of concentrated hydrochloric acid. Centrifuge at 600 x g at room temperature. The supernatants can then be used directly in the assay.

Procedural Notes

1. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
2. **Pre-rinse the pipet tip with reagent**, use fresh pipet tips for each sample, standard and reagent.
3. Pipet standards and samples to the bottom of the wells.
4. Add the reagents to the side of the well to avoid contamination.
5. 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.
6. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells.**

Reagent Preparation

1. cGMP Standard – Non-Acetylated Version

Allow the 5,000 pmol/mL cGMP standard solution to warm to room temperature. Label seven (or more) tubes #1 through #7. Pipet 475 µL 0.1M HCl into tube #1 and 400 µL 0.1M HCl into tubes #2-7. Add 25 µL of the 5,000 pmol/mL standard to tube #1. Vortex thoroughly. Add 100 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #7.

The concentration of cGMP in tubes #1 through #7 will be 250, 50, 10, 2, 0.4, 0.08, and 0.016 pmol/mL respectively. See Direct cGMP Assay Layout Sheet for dilution details.

Diluted standards should be used within 30 minutes of preparation.

Label one tube as the Zero Standard/NSB tube. Pipet 600 µl 0.1M HCl into this tube.

2. Wash Buffer

Prepare the Wash Buffer by diluting 15 mL of the supplied concentrate with 135 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.
Add the reagent directly to the samples and vortex for 2 seconds immediately after the addition.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

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

3. Pipet 100 µL of 0.1M HCl into the NSB and the Bo (0 pmol/mL Standard) wells.

4. Pipet 100 µL of Standards into the appropriate wells.
5. Pipet 100 µL of the Samples into the appropriate wells.
6. Pipet 50 µL of 0.1 M HCl into the NSB wells.
7. Pipet 50 µL of Conjugate into each well **except** the TA and Blank wells.
8. Pipet 50 µL of Antibody into each well, **except** the Blank, TA and NSB wells.
9. Incubate the plate at room temperature for 2 hours on a plate shaker at 250~500 rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 washes.**
11. 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.
12. Add 5 µL of the Conjugate to the TA wells.
13. Add 200 µL of the Substrate solution to every well. Incubate at room temperature for 5~30 minutes without shaking. A gradient of blue color should become visible during the incubation period.
    (**Substrate A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light.**)
14. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
15. Blank the plate reader against the Blank wells, read the optical

density at 450 nm (for HRP), preferably with correction between 570 and 590 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.

Calculation of Results

Several options are available for the calculation of the concentration of cGMP in the samples. The X-axis is the concentration of cGMP for the standards. The Y-axis is either the Average Net Optical Density or the Percent Bound.

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:

   Average Net OD = Average Bound OD − Average NSB OD

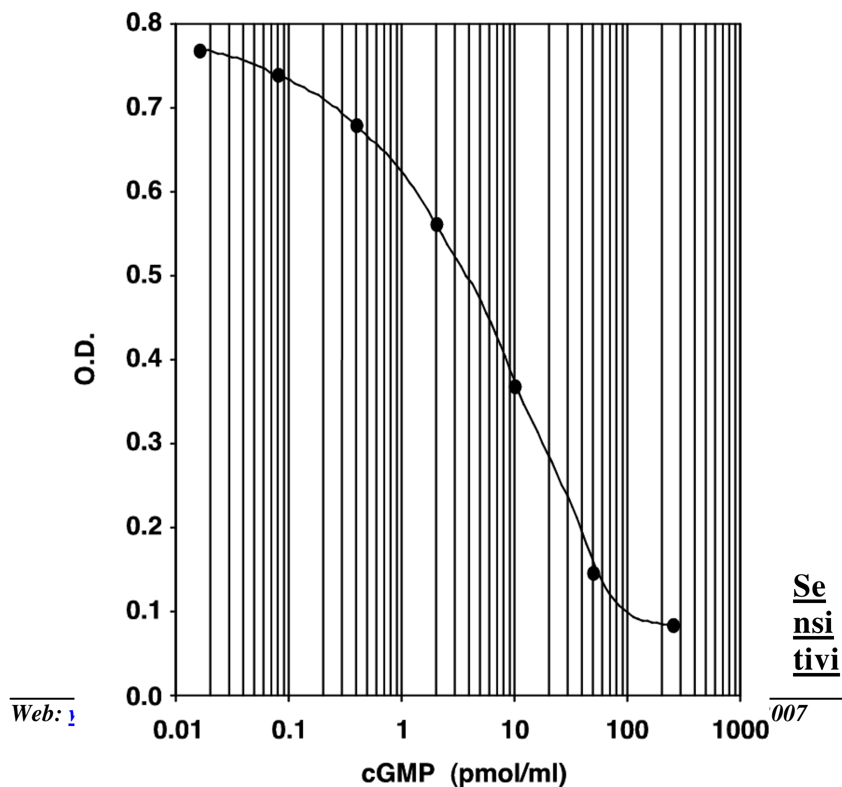
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

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

3. Using Logit-Log paper plot Average Net OD or Percent Bound (B/Bo) versus concentration of cGMP for the standards. The concentration of cGMP in the unknowns can be determined by interpolation.

Typical Standard Curves

These curves **must not** be used to calculate cGMP concentrations; each user must run a standard curve for each assay and version used.



Se nsi tivi

ty

Sensitivity was calculated by determining the average optical density bound for ten wells run with the Bo, and comparing to the average optical density for ten wells run with Standard #6. The detection limit was determined as the concentration of cGMP measured at two standard deviations from the zero along the standard curve.

Non-Acetylated Version

Mean OD for Bo =	0.847± 0.0103
Mean OD for Standard #6 =	0.729 ± 0.0013
Delta Optical Density =	0.118
2 SD's of the Zero Standard =	0.0206
Sensitivity $= \frac{0.0206}{0.118} \times 0.08$ pmol/mL =	**14 fmol/mL**

Linearity

A sample containing 16.0 pmol/mL cGMP was serially diluted 7 times 1:2 in the 0.1M HCl and measured. The data was plotted graphically as actual cGMP concentration versus measured cGMP concentration. The line obtained had a slope of 1.000 with a correlation coefficient of 0.999.

Cross Reactivities

The cross reactivities for a number of related compounds were determined by competition ELISA assays. 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.

Compound	Cross Reactivity
cGMP	100%
GMP	<0.0001%
GTP	<0.0001%
cAMP	<0.0001%
AMP	<0.0001%
ATP	<0.0001%
cUMP	<0.0001%
CTP	<0.0001%

REFERENCES:

1. Estensen, R. D., Hill, H. R., Quie, P. G., Gogan, N., and Goldberg, N. D. (1973) *Nature* **245**, 458-460
2. Elferink, J. G., and VanUffelen, B. E. (1996) *Gen Pharmacol* **27**, 387-393
3. Uemura, Y., and Okamoto, K. (1997) *Biochem Mol Biol Int* **41**, 1085-1092
4. Massberg, S., Sausbier, M., Klatt, P., Bauer, M., Pfeifer, A., Siess, W., Fassler, R., Ruth, P., Krombach, F., and Hofmann, F. (1999) *J Exp Med* **189**, 1255-1264
5. Schmidt, H., Werner, M., Heppenstall, P. A., Henning,

M., More, M. I., Kuhbandner, S., Lewin, G. R., Hofmann, F., Feil, R., and Rathjen, F. G. (2002) *J Cell Biol* **159**, 489-498

6. Li, Z., Xi, X., Gu, M., Feil, R., Ye, R. D., Eigenthaler, M., Hofmann, F., and Du, X. (2003) *Cell* **112**, 77-86
7. Manahan, C. L., Iglesias, P. A., Long, Y., and Devreotes, P. N. (2004) *Annu Rev Cell Dev Biol* **20**, 223-253
8. Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000) *Pharmacol Rev* **52**, 375-414
9. Finn, J. T., Grunwald, M. E., and Yau, K. W. (1996) *Annu Rev Physiol* **58**, 395-426
10. Wedel, B., and Garbers, D. (2001) *Annu Rev Physiol* **63**, 215-233
11. Maack, T. (1992) *Annu Rev Physiol* **54**, 11-27
12. Guo, D., Tan, Y. C., Wang, D., Madhusoodanan, K. S., Zheng, Y., Maack, T., Zhang, J. J., and Huang, X. Y. (2007) *Cell* **128**, 341-355