Thermo SCIENTIFIC

The world leader in serving science

CERTIFICATE OF ANALYSIS

Cyclic GMP Competitive ELISA
Lysate and Homogenate Samples

Ordering Code: EMSCGMPL

Lot Number: RA229061

Kit Storage: Store the cAMP-AP Conjugate and the cAMP Standard at -20ºC. Store the remaining components at 2-8ºC.

Expiration Date: 30/Nov/2016

Product: 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.

cGMP COMPETITIVE ELISA KIT COMPONENTS - LYSATE AND HOMOGENATE SAMPLES

***Allow all Kit Components to come to room temperature for at least 30 minutes prior to opening.*

Description	Size	Form	Component Usage	Lot
Anti-Rabbit IgG Plate	1 plate	96-well strip plate coated with antibody specific to rabbit IgG.	▪ Unused wells must be kept desiccated at 2 - 8°C in the sealed foil bag. ▪ Use wells in the frame provided.	RA229061A
cGMP Antibody	5 ml	A yellow solution of a rabbit polyclonal antibody to cGMP.	Ready to Use.	RA229061D
cGMP-AP Conjugate	5 ml	A blue solution of cGMP conjugated to alkaline phosphatase.	Ready to Use.	RA229061C
0.1 M HCl	30 ml	0.1 M hydrochloric acid in water.	Ready to Use. CAUTION: Acid, wear suitable protective clothing.	RA229061I
Neutralizing Reagent	6 ml	A clear, colorless solution.	Ready to Use.	RA229061B
Triethylamine	2 ml	A solution of triethylamine.	Ready to Use. Caution: Lachrymator, Harmful Vapor, Flammable	RA229061K
Acetic Anhydride	1 ml	A solution of acetic anhydride.	Ready to Use. Caution: Lachrymator, Corrosive, Flammable.	RA229061J
cGMP Standard	0.5 ml	A solution of 5000 pmol/ml cGMP	**Allow the standard to come to room temperature.** Follow procedures for either Acetylated or Non-Acetylated detailed below.	RA229061E
10X Wash Buffer	30 ml	Tris buffered saline containing detergents and a preservative.	▪ Dilute 10 ml of the 10X Wash Buffer with 90 ml of deionized water. ▪ 1X Wash Buffer can be stored for up to 3 months at room temperature.	RA229061F
p-NPP Substrate Solution	20 ml	A solution of p-nitrophenyl phosphate in buffer.	Ready to Use.	RA229061G
Stop Solution	6 ml	A solution of trisodium phosphate in water.	Ready to Use. Keep tightly capped. Caution: Caustic	RA229061H
Plate Sealer	1 each		Ready to Use.	

Pierce Biotechnology | PO Box 117 | (800) 874-3723 | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 | www.thermo.com/pierce

Thermo SCIENTIFIC

The world leader in serving science

cGMP COMPETITIVE ELISA KIT - LYSATE AND HOMOGENATE SAMPLES PROCEDURAL NOTES

- Materials needed but not supplied with the kit include: deionized or distilled water; precision pipets for volumes between 5 µl and 1,000 µl; repeat pipets for dispensing 50 µl and 200 µl; glass or polypropylene 12 x 75 mm tubes; disposable beaker for diluting buffer concentrates; graduated cylinders; a microplate shaker; absorbent paper for blotting; and a microplate reader capable of reading at 405 nm, preferable with correction between 570 and 590 nm.
- 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) which may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells to prevent variation in assay results.

STANDARDS AND ACETYLATING REAGENT

Non-Acetylated Standards

- Label five 12 x 75 mm glass or polypropylene tubes 1 - 5.
- Pipet 900 µl of 0.1 M HCl into tube 1.
- Pipet 800 µl of 0.1 M HCl into tubes 2 - 5.
- Add 100 µl of stock cGMP standard to tube 1 and vortex.
- Add 200 µl of tube #1 to tube 2 and vortex. Continue this for tubes 3 - 5.
- The concentration of cGMP in tubes 1 - 5 will be 500, 100, 20, 4, and 0.8 pmol/ml, respectively.
- Use diluted standards within 60 minutes.

Acetylating Reagent Preparation

- Add 0.5 ml of acetic anhydride to 1 ml triethylamine and mix well.
- Use within 60 minutes of preparation.

Acetylated Standards

- Label five 12 x 75 mm glass or polypropylene tubes 1 - 5.
- Pipet 990 µl of 0.1 M HCl into tube 1.
- Pipet 800 µl of 0.1 M HCl into tubes 2 - 5.
- Add 10 µl of stock cGMP standard to tube 1 and vortex.
- Add 200 µl from tube 1 to tube 2 and vortex. Continue this for tubes 3 - 5.
- The concentration of cGMP in tubes 1 - 5 will be 50, 10, 2, 0.4, and 0.08 pmol/ml, respectively.
- Acetylate all of the Acetylated Version standards and samples by adding 10 µl of Acetylating Reagent to each 200 µl of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds.
- Label one 12 x 17 mm glass tube as the Zero Standard/NSB tube. Pipet 1 ml 0.1 M HCl into this tube. Add 50 µl of the Acetylating Reagent to the Zero Standard/NSB tube. NOTE: Failure to acetylate the NSB and Zero standard will result in inaccurate B/B_0 values.
- Use the Acetylated Standards or samples within 30 minutes.

SAMPLE HANDLING

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. Samples in this matrix can be measured directly without evaporation or further treatment. Reagents to acetylate samples and standards for samples with very low levels of cGMP are provided.

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 0.1 M HCl provided for the assay.

Cells grown in tissue culture media can be treated with 0.1 M 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 another 10 minutes and inspect. Centrifuge at ≥ 600 x g at room temperature for 10 minutes, 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 0.1 M HCl prior to use. When used in this concentration range, the detergent will not interfere with acetylation or 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 media can be measured by treating 1 ml of supernatant media with 10 µl concentrated HCl. Centrifuge at 600 x g at room temperature for 10 minutes. The supernatants can then be used directly in the assay.

Pierce Biotechnology | PO Box 117 | (800) 874-3723 | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 | www.thermo.com/pierce

Thermo SCIENTIFIC

The world leader in serving science

cGMP COMPETITIVE ELISA - LYSATE AND HOMOGENATE SAMPLES ASSAY PROCEDURE

All standards and samples should be run in duplicate. If acetylated version of the kit is to be run, acetylate both the standards and samples by adding 10 µl of the Acetylating Reagent to each 200 µl of standard or sample.

1. Pipet 50 µl of the pink Neutralizing Reagent into each well, **except** the TA and Blank wells.
2. Pipet 100 µl of 0.1 M HCl into the NSB and B_0 (0 pmol/ml Standard) wells.
3. Pipet 100 µl of Standards into the appropriate wells.
4. Pipet 100 µl of the Samples into the appropriate wells.
5. Pipet 50 µl of 0.1 M HCl into the NSB wells.
6. Pipet 50 µl of the blue cGMP-AP conjugate into each well, **except** the Total Activity (TA) wells and the blank.
7. Pipet 50 µl of the yellow cGMP antibody into each well, **except** the Blank, TA and NSB wells.

   NOTE: Every well used should be BROWN in color except the NSB wells which should be PURPLE. The Blank and TA wells are empty at this point and have no color.
8. Incubate the plate at room temperature (22 - 25°C) on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired. If using the **Acetylated Overnight Format**, incubate for 18 - 24 hours at 2 - 8°C.
9. Empty the contents of the wells and wash by adding 200 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 the p-NPP Substrate Solution to every well. Incubate at room temperature (22 - 25°C) for 1 hour without shaking.
13. Add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm 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.

cGMP Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Std 1	Std 1									
B	Blk	Std 2	Std 2									
C	TA	Std 3	Std 3									
D	TA	Std 4	Std 4									
E	NSB	Std 5	Std 5									
F	NSB											
G	B_0											
H	B_0											

Definition of Key Terms

Total Activity (TA): total enzymatic activity of the cGMP-AP.

NSB (Non-Specific Binding): non-immunological binding of the cGMP-AP in the well.

B_0 (Maximum Binding): maximum amount of the cGMP-AP that the antibody can bind in the absence of free cGMP.

%B/B_0 (% Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Average Net OD = Average Bound OD – Average NSB OD

$$\textit{Percent Bound} = \frac{\text{Net OD}}{\text{Net } B_0 \text{ OD}} \times 100$$

Pierce Biotechnology | PO Box 117 | (800) 874-3723 | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 | www.thermo.com/pierce

Thermo SCIENTIFIC

The world leader in serving science

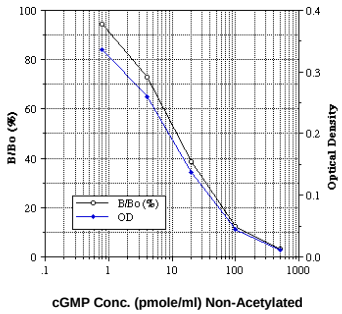
CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of cGMP in the sample. It is recommended that the data be analyzed by a weighted 4 parameter logistic curve fitting program. If data reduction software is not available, the concentration of the cGMP can be calculated. Calculate the average Net OD bound for each standard and sample. Calculate the Percent Bound of each pair of standard wells as a percentage of the maximum binding wells (B_0). Using Logit-Log paper plot Percent Bound (B/B_0) versus Concentration of cGMP for the standards. Approximate a straight line through the points. The concentration of cGMP in the samples can be determined by interpolation.

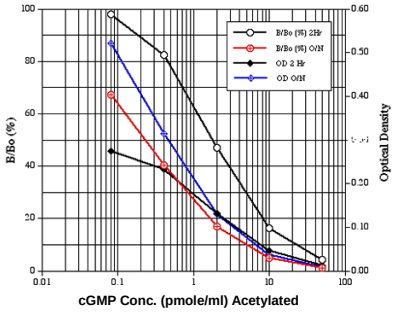
Typical Standard Curve

Typical standard curves are shown. This curve must not be used to calculate cGMP concentrations; a standard curve must be run with every assay.

Non-Acetylated Version



Acetylated Version



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.

Linearity

Non-Acetylated Version: A sample containing 96 pmol/ml cGMP was diluted 7 times 1:2 in 0.1 M HCl and measured in the assay. The data was plotted graphically as actual cGMP concentration versus measured cGMP concentration.

The line obtained had a slope of 1.000 and a correlation coefficient of 0.999.

Acetylated Version: A sample containing 16.0 pmol/ml cGMP was serially diluted 7 times 1:2 in 0.1 M HCl and measured in the Acetylated 2 Hour Version of the assay. The data was plotted graphically as actual cGMP concentration versus measured cGMP concentration.

The line obtained had a slope of 1.001 and a correlation coefficient of 0.998.

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 3 samples of low, medium and high concentrations in multiple assays (n=8). 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 Version		Acetylated Version-2 Hour	
Intra-assay	**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	**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

Sensitivity

Sensitivity was calculated by determining the average OD for 16 wells run as B_0, and comparing to the average OD for 16 wells run with Standard #5 in the Non-Acetylated or with Standard #5 with the Acetylated version. The detection limit was determined as the concentration of cGMP measured at 2 standard deviations from the 0 along the standard curve.

Non-Acetylated Version Sensitivity	604 fmol/ml
Acetylated Version Sensitivity-2 hour	0.59 fmol/ml
Acetylated Overnight Format	25 fmol/ml

Pierce Biotechnology | PO Box 117 | (800) 874-3723 | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 | www.thermo.com/pierce

Thermo SCIENTIFIC

The world leader in serving science

CALCULATION OF RESULTS

Cross-Reactivities

The cross-reactivities for a number of related compounds were determined by dissolving the cross reactant in Reagent Diluent 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 Recoveries

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	% Recovery	Suggested Dilution	% Recovery	Suggested Dilution
	Non-Acetylated Version		Acetylated Version	
Tissue Culture Media	95.9	None	86.8	None

Quality Control Signature

Pierce Biotechnology | PO Box 117 | (800) 874-3723 | (815) 968-7316 fax
3747 N. Meridian Road | Rockford, IL 61105 USA | (815) 968-0747 | www.thermo.com/pierce