ab65356

cGMP Direct Immunoassay Kit

Instructions for Use

For the rapid, sensitive and accurate measurement of cGMP levels in various samples.

This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

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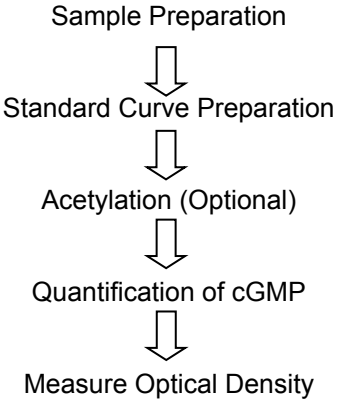
1. Overview

Adenosine and guanosine 3',5'-cyclic monophosphate (cAMP and cGMP) are important "second messengers" involved in many physiological processes. Abcam's cGMP Direct Immunoassay Kit provides a direct competitive immunoassay for sensitive and quantitative determination of cGMP level in biological samples.

The kit utilizes the recombinant Protein G coated plate to anchor cGMP polyclonal antibody. cGMP-HRP conjugates directly compete with cGMP in samples for binding to the cGMP specific antibody on the plate. After incubation and washing, the amount of cGMP-HRP bound to the plate can easily be determined by reading OD_{450nm}. The intensity of OD_{450nm} is inversely proportional to the concentration of cGMP in samples.

The kit provides a new acetylation procedure that improves detection signal significantly. The kit can detect 0.04-10 pmol/well (0.008-2 µM) cGMP samples.

2. Protocol Summary



3. Components and Storage

A. Kit Components

Item	Quantity	Storage Temp
Assay Buffer XXXVI/10X cGMP Assay Buffer	25 mL	+4°C
cGMP Standard/Standard cGMP (10 nmol)	1 vial	-20°C
Neutralization Buffer III/Neutralizing Buffer	7.5 mL	+4°C
Acetylating Reagent A	0.75 mL	+4°C
Acetylating Reagent B**	1.5 mL	+4°C
Anti-cGMP Ab/Anti-cGMP pAb/BSA	1 vial	-20°C
cGMP-HRP/cGMP-HRP/BSA	1 vial	-20°C
TMB Substrate I/HRP Developer	10 mL	+4°C
Protein G Coated Plate	1 each	-20°C

* Store kit at -20°C.

- Dilute the 10X Assay Buffer XXXVI/cGMP Assay Buffer to 1X Assay Buffer with ultra-pure water. Store at 4°C.

- Reconstitute the cGMP Standard/Standard cGMP (pellet may not be visible) in 1 ml of 0.1M HCl (not provided), vertex for 10 seconds to generate 10 pmol/µl cGMP stock standard solution.

- Reconstitute anti-cGMP Ab/anti-cGMP pAb/BSA with 1.1 ml of the 1X Assay Buffer XXXVI/cGMP Assay Buffer as stock solutions.

- Reconstitute cGMP-HRP/cGMP-HRP/BSA in 22 µl of water to prepare 50X cGMP-HRP stock solution. Divide in two aliquots and store at -20 °C. Use one of the aliquots within a month. Use the second aliquot within 2 months.

- Unused well strips can be kept at –20°C with the desiccants, stable for up to 1 month.

- The kit should be stored at –20°C. After reconstitution, some components may be stored at 4°C as instructed above, stable for up to 1- 2 months.

***NOTE:** Acetylating Reagent B is very volatile and hence the vial has to be tightly capped and stored only at +4°C.

B. Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader
- 96 well plate
- Orbital shaker
- HCl

4. Assay Protocol

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1. Sample Preparation:

a. Urine, Plasma and Culture Medium Samples: Urine, plasma, and culture media may be tested directly after adding 1/10 volume of 1M HCl and remove precipitates if occur.

b. Cell Samples:

For **suspension cells** collect by centrifugation. Add 1 ml of 0.1M HCl for every 35 cm^2 of surface area (e.g., 10 cm plate at 70% confluency is ~110 cm^2, so use ~3.1 ml). Incubate for 20 minutes on ice.

For **adherent cells** add the HCl directly, scrape cells off the surface. Dissociate the sample by pipetting up and down until the suspension is homogeneous. Transfer to a centrifuge tube

and centrifuge at top speed for 10 min. The supernatant can be assayed directly. Protein concentration >1 mg/ml is recommended for reproducible results.

c. **Tissue Samples:** Cyclic nucleotides may be metabolized quickly in tissue, so it is important to rapidly freeze tissues after collection (e.g., using liquid nitrogen). Weigh the frozen tissue and add 5-10 volume of 0.1M HCl.
Homogenize the sample on ice using a Polytron-type homogenizer. Spin at top speed for 5 min and collect the supernatant. The supernatant may be assayed directly.

Notes:

a) cGMP samples in 0.1 M HCl (final concentration) are stable and can be used directly in the assay. Make dilutions of your sample with 0.1 M HCl to the range of 0.04-10 pmol/well (0.008-2 µM). Urine and tissue culture supernatant can be diluted in 10% 1M HCl and assayed directly.

b) Plasma, serum, whole blood, and tissue homogenates often contain phosphodiesterases and large amount of immunoglobulins (Igs) which may interfere with the assay. However, preparing samples in 0.1 M HCl can generally inactivate phosphodiesterases and lower the concentration of Igs, making the samples suitable for the assay. Both phosphodiesterases and Igs can also be removed by 5% TCA

precipitation or by 10 kDa molecular weight cut off microcentrifuge filters (**ab93349**).

c) To determine whether interference is present in your sample, you can make two different dilutions. If the two different dilutions of sample show good correlation of the final calculated cGMP concentration, purification is not required. If you do not see good correlation of the different dilutions, deproteinize the sample by using TCA or 10 kDa molecular cut off microcentrifuge filters. Organic solvents in samples may interfere with the assay, which may need to be removed prior to the assay.

2. Standard Curve and Sample Preparation:

a) Add 200 µl of the 10 pmol/µl cGMP Standard/standard cGMP stock into 800 µl of 0.1M HCl to generate 2 pmol/µl cGMP working solution. The diluted cGMP should be used within 1 hour.

b) Label 11 microcentrifuge tubes, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0, 0_B pmol/50 µl (these concentrations represent what will finally be in the wells after the dilutions mentioned below).

c) Add 200 µl of the 2 pmol/µl cGMP into the tube labeled 10 pmol (enough for 20 tests), add100 µl 0.1M HCl into the rest of tubes.

d) Transfer 100 µl from the 10 pmol tube into the labeled 5 pmol tube, mix. Continue the serial dilution by transferring 100 µl from the 5 pmol tube to 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 pmol tubes. Discard 100 µl from the 0.039 pmol tube. The diluted cGMP should be used within 1 hour.

e) Label new tubes for test samples, add 100 µl each test sample per tube. We suggest using different dilutions for each sample (dilute with 0.1M HCl).

f) Add 50 µl of Neutralization Buffer/Neutralizing Buffer to each tube to neutralize the HCl in the samples and standards.

g) Prepare Acetylating Reagent Mix (5 µl is needed for each assay): Mix 1 volume of Acetylating Reagent A (Violet cap) with two volumes of Acetylating Reagent B (Black cap) in a microtube. Prepare just enough for the experiment. Use within 1 hour.

h) Add 5 µl of the Acetylating Reagent Mix directly into each test solution (both standard and sample), IMMEDIATELY vortex 2-3 seconds following each addition without delay, one tube at a time and incubate at room temperature for 10 min.

i) Add 845 µl 1X Assay Buffer into each tube, mix well. Use for below quantification.

Notes:

The acetylation step improves the assay sensitivity significantly and avoids the interferences of many components in unpurified samples. If cGMP concentrations in your samples are very low, the acetylation reagents can be dried after step h, without dilution step i to minimize the volume. Then reconstitute in a 50-100 µl volume of Assay Buffer.

3. Quantification of cGMP:

a) Add 50 µl of the acetylated cGMP Standard/Standard cGMP and test samples from step 2.i to each well of the Protein G coated 96-well plate. We suggest duplicate assays for each sample and standard.

b) Add 10 µl of the reconstituted cGMP antibody per well to the standard cGMP and sample wells except the well with 0_B pmol cGMP.

Note 1:

Do not add cGMP antibody into the well with 0_B pmol cGMP, instead add 10 µl of 1X Assay Buffer for background reading.

Note 2:

Using a repeating pipette is recommended for minimizing pipetting errors.

c) Incubate for 1 hour at room temperature with gentle agitation.

d) Dilute 50X cGMP-HRP stock solution in assay buffer to generate 1X cGMP-HRP working solution. Just make enough working solution for the number of samples.

e) Add 10 µl of cGMP-HRP to each well and incubate for 1 hr at room temperature with gentle agitation.

f) Wash 5 times with 200 µl 1X Assay Buffer each time. Completely empty the wells by tapping the plate on a fresh paper towel after each wash step.

g) Add 100 µl of TMB Substrate I/HRP developer and Read the OD 650 nm at kinetic mode for 1 hour at room temperature with agitation.
Note: You should stop the reaction when the OD 650 nm of 0 pmol cGMP reaches 0.8-1.0.

h) Stop the reaction by adding 100 µl of 1M HCl (not provided) to each well (sample color should change from blue to yellow).

i) Read the plate at OD 450 nm.
Note: The OD450 nm reading may vary significantly among experiments depend on lot numbers, kit storage and experiment conditions. Therefore, samples and standard curve must be performed at the same time and using the same kit reagents.

5. Data Analysis

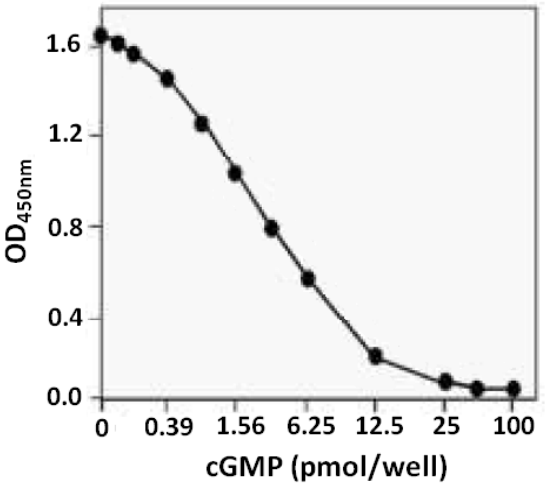
Subtract OD_{450nm} background reading (the well with 0_B pmol cGMP) from all samples and standards.

Plot standard curve to observe the linear portion, then replot only the linear portion and in Excel add a trendline, then use the trend line linear formula (y=mx+b).

Calculate amount of cGMP in samples after correcting the for dilution factors:

cGMP Concentration = Sa / Sv (pmol/µl or nmol/ml or µM)

Where:
Sa is cGMP amount (pmol) from standard curve.
Sv is sample volume (µl) added into the assay wells after dilution factor correction.



cGMP Standard Curve: The assay was performed following the kit protocol.

6. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Problem	Reason	Solution
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)***
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)