Parameter™

cGMP Assay

Catalog Number KGE003
SKGE003
PKGE003

For the quantitative determination of cyclic GMP (cGMP) concentrations in cell culture supernates, cell lysates, EDTA plasma, saliva, and urine.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Guanosine 3',5'-cyclic monophosphate (cGMP) is a multi-functional second messenger molecule. It is generated via two pathways distinguished by the types of Guanylyl Cyclase (GC) that mediate its conversion from guanosine triphosphate (GTP) (1). In the soluble pathway, cGMP is generated via cytoplasmic nitric oxide (NO)-activated GC (2). GCs in the particulate pathway share some homology with those activated by NO but are transmembrane proteins with extracellular ligand-binding domains (3). The ligands for a subset of membrane GCs are members of the Natriuretic Peptide (NP) hormone family including Atrial NP hormone, B-type NP hormone, and C-type NP hormone (4-7).

cGMP primarily affects cellular activities through four different pathways. These include cGMP-dependent Protein Kinases (PKG/GK), cyclic nucleotide-gated (CNG) channels, cAMP-dependent Protein Kinase (PKA), and Phosphodiesterases.

PKGs (PKG I and PKG II) are serine/threonine kinases activated by cGMP. PKG I has several putative targets, many of which are involved in the regulation of smooth muscle cell (SMC) contractility. cGMP/PKG I-dependent relaxation of SMCs results from mechanisms that include lowering the levels of intracellular Ca^{2+} and the phosphorylation of proteins involved in actin/myosin contraction. PKG I substrates involved in lowering Ca^{2+} levels include the IP3 Receptor and associated protein IRAG (8), and Ca^{2+}-activated K^{+} Channels (BK) that hyperpolarize the membrane and lead to a decrease in voltage-gated Ca^{2+} channel activity (9). Other cGMP/PKG I substrates that affect contractility include the myosin-binding subunit (MBS) of MLC Phosphatase and the small GTPase Rho (10, 11). In addition to its role in smooth muscle relaxation, cGMP/PKG I may also regulate cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, and angiogenesis (12-19). In contrast to PKG I, the activities of PKG II are less well known. The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a substrate for PKG II. Phosphorylation activates the channel, and/or leads to insertion in the membrane, resulting in chloride secretion and fluid loss (20, 21). Other putative roles for PKG II include regulating circadian rhythms and endochondral bone development (22-24).

The CNG channels are non-selective cation channels activated by cAMP and cGMP. Their regulation of photoreceptor and olfactory sensory neuron activity has been studied extensively (25, 26). cGMP-gated channels may also be expressed in other neuronal cell types, as well as non-neuronal cells and tissues including spermatozoa and kidney (27-29).

There appears to be crosstalk between the cAMP and cGMP systems. Although more efficiently activated by cAMP, PKA may also be activated by cGMP (30). The levels of cGMP and cAMP are regulated by members of the Phosphodiesterase gene family (30, 31). In turn, the activities of Phosphodiesterases are regulated by cGMP, cAMP, and associated signaling pathways. cGMP may regulate Phosphodiesterase activity via PKG-mediated phosphorylation, and/or direct binding of cGMP to the enzyme (31-35).

The Parameter™ cGMP Immunoassay is a 3.5 hour competitive enzyme immunoassay designed to measure cGMP in cell culture supernates, EDTA plasma, saliva, urine, and cell lysates.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which cGMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cGMP for sites on a rabbit polyclonal antibody. During the incubation, the polyclonal antibody becomes bound to the goat anti-rabbit antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of cGMP in the sample.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Parameter™ Immunoassay, the possibility of interference cannot be excluded.
- **Samples containing rabbit IgG may interfere with this assay.**

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pre-rinse the pipette tip when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution. Gently tap the side of the plate to ensure thorough mixing.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	CATALOG # KGE003	CATALOG # SKGE003	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Goat Anti-rabbit Microplate	892943	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-rabbit polyclonal antibody.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
cGMP Conjugate	892947	1 vial	6 vials	6 mL/vial of cGMP conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
cGMP Standard	892949	1 vial	6 vials	cGMP in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Primary Antibody Solution	892948	1 vial	6 vials	6 mL/vial of a rabbit polyclonal antibody for cGMP in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-5	895485	2 vials	12 vials	21 mL/vial of a buffered protein base with preservatives.	
Cell Lysis Buffer 5	895890	1 vial	6 vials	21 mL/vial of a concentrated buffered solution with preservatives. *Use diluted 1:5 in this assay. May contain crystals. Warm to room temperature and mix well to dissolve.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12.5 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

KGE003 contains sufficient materials to run an ELISA on one 96 well plate.
SKGE003 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PKGE003). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples (Salivette® or equivalent).
- Test tubes for dilution of standards and samples.
- cGMP Controls (optional; R&D Systems®, Catalog # QC54).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Samples containing rabbit IgG may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Prior to assay, cells must be lysed according to the directions in the Cell Lysis Procedure section.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Heparin plasma and serum are not recommended for use in this assay.*
Citrate plasma has not been validated for use in this assay.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

EDTA plasma samples require a 20-fold dilution. A suggested 20-fold dilution is 25 µL of sample + 475 µL of Calibrator Diluent RD5-5.

Urine and saliva samples require a 10-fold dilution. A suggested 10-fold dilution is 50 µL of sample + 450 µL of Calibrator Diluent RD5-5.

CELL LYSIS PROCEDURE

1. Wash cells three times in cold PBS.
2. Resuspend cells in Cell Lysis Buffer 5 (diluted 1:5)* to a concentration of 1 x 10^7 cells/mL.
3. Freeze cells at ≤ -20 °C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle once. Trypan Blue and a microscope can be used to confirm cell lysis. Lysed cells will be blue. If cells are not lysed, repeat the freeze/thaw cycle as needed.
4. Centrifuge at 600 x g for 10 minutes at 2-8 °C to remove cellular debris.
5. Assay the supernate immediately or aliquot and store at ≤ -20 °C.

The above method can be modified for Tissue Culture plates (6, 12, 24, 48, or 96 well). Incubate cells in appropriate media overnight in a 37 °C, 5% CO_2 incubator, prior to step 1.

A minimum of 200 µL of cell lysate is required to perform the assay in duplicate.

Note: *When running cell lysates, use Cell Lysis Buffer 5 (diluted 1:5) instead of calibrator diluent to produce the standard dilution series and as the zero standard. There will be a small change in binding associated with running the standards and samples in Cell Lysis Buffer.*

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *cGMP is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

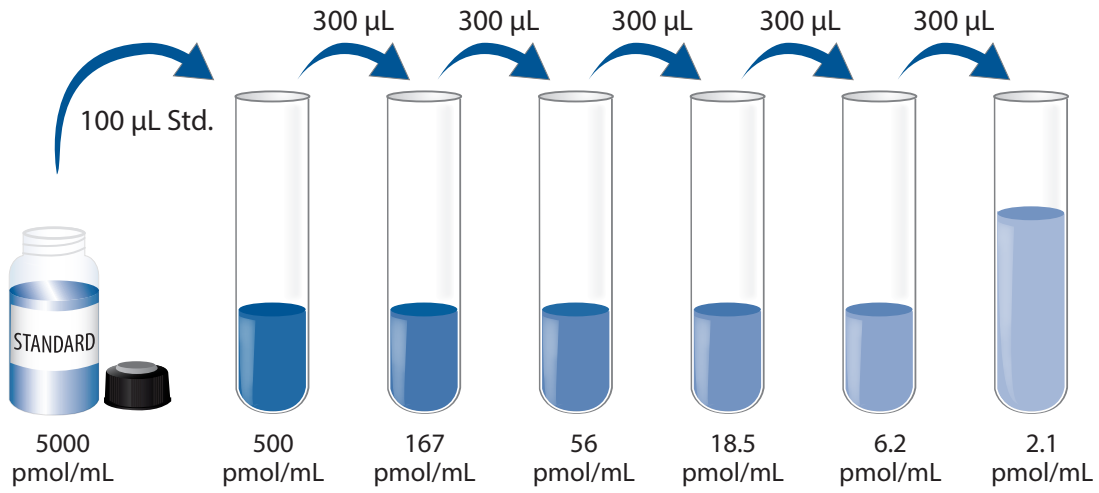
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Cell Lysis Buffer 5 (diluted 1:5) - Add 20 mL of Cell Lysis Buffer 5 to 80 mL of deionized or distilled water to prepare 100 mL of Cell Lysis Buffer 5 (diluted 1:5). *May contain crystals. Warm to room temperature and mix well to dissolve before diluting.*

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

cGMP Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the cGMP Standard with deionized or distilled water. This reconstitution produces a stock solution of 5000 pmol/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-5 (*for cell culture supernates/plasma/saliva/urine samples*) or Cell Lysis Buffer 5 (diluted 1:5) (*for cell lysate samples*) into the 500 pmol/mL tube. Pipette 600 µL of the appropriate diluent into the remaining tubes. Use the standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 500 pmol/mL standard serves as the high standard. Calibrator Diluent RD5-5 or Cell Lysis Buffer 5 (diluted 1:5) serves as the zero standard (B_0) (0 pmol/mL).
Use diluted standards within 60 minutes of preparation.



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *cGMP is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 150 µL of the appropriate diluent to the non-specific binding (NSB) wells and 100 µL to the zero standard (B_0) wells. Use Calibrator Diluent RD5-5 for all cell culture supernates, plasma, saliva, and urine samples. **Use Cell Lysis Buffer 5 (diluted 1:5) for cell lysates.**
4. Add 100 µL of standard, control, or sample* to the remaining wells. A plate layout is provided to record standards and samples assayed.
5. Add 50 µL of cGMP Conjugate to each well. Wells will now be red in color.
6. Add 50 µL of the Primary Antibody Solution to each well **(excluding the NSB wells)**. All wells except the NSB wells will now be violet in color. Cover with the adhesive strip provided.
7. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
8. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 mL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
9. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
10. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample then subtract the average NSB optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

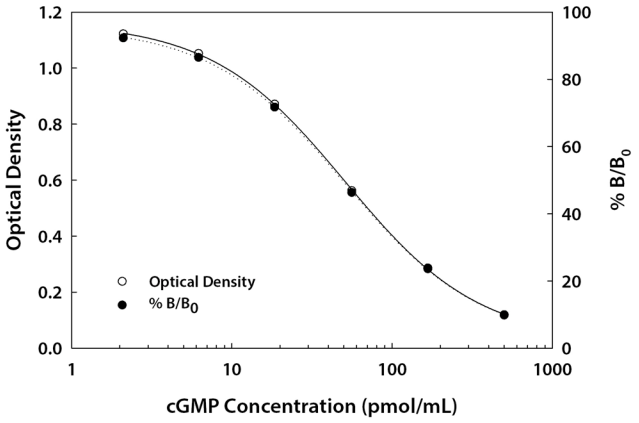
Calculate the concentration of cGMP corresponding to the mean absorbance from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

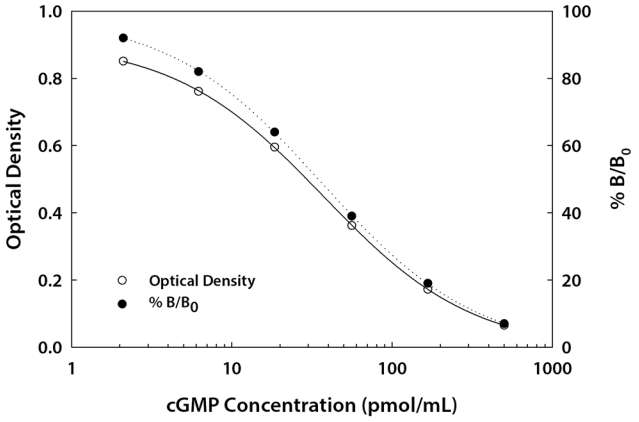
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/PLASMA/SALIVA/URINE ASSAY



(pmol/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.028 0.028	0.028	—	—
0 (B_0)	1.160 1.270	1.215	1.187	—
2.1	1.130 1.170	1.150	1.122	92.3
6.2	1.060 1.100	1.080	1.052	86.5
18.5	0.880 0.917	0.899	0.871	71.7
56	0.578 0.601	0.590	0.562	46.3
167	0.303 0.325	0.314	0.286	23.6
500	0.143 0.151	0.147	0.119	9.81

CELL LYSATE ASSAY



(pmol/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.040 0.040	0.040	—	—
0 (B_0)	0.963 0.973	0.968	0.928	—
2.1	0.890 0.891	0.891	0.851	92.0
6.2	0.784 0.818	0.801	0.761	82.0
18.5	0.631 0.638	0.635	0.595	64.0
56	0.398 0.406	0.402	0.362	39.0
167	0.210 0.214	0.212	0.172	19.0
500	0.104 0.106	0.105	0.065	7.00

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE/PLASMA/SALIVA/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pmol/mL)	85.2	184	265	80.7	188	273
Standard deviation	6.9	12.5	14.0	8.0	16.5	22.4
CV (%)	8.1	6.8	5.3	9.9	8.8	8.2

CELL LYSATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pmol/mL)	57.0	72.0	178	52.0	67.0	195
Standard deviation	2.2	4.0	9.5	4.0	4.4	12.7
CV (%)	3.9	5.6	5.3	7.7	6.6	6.5

RECOVERY

The recovery of cGMP spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	100	91-108%
EDTA plasma (n=4)	91	74-105%
Urine (n=4)	90	77-98%
Saliva (n=4)	102	80-123%
Cell lysates (n=1)	92	80-98%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of cGMP were serially diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell Lysate* (n=1)	EDTA plasma* (n=4)	Saliva* (n=4)	Urine* (n=4)
1:2	Average % of Expected	95	105	102	103	99
	Range (%)	88-99	———	98-104	97-105	98-100
1:4	Average % of Expected	101	119	104	107	101
	Range (%)	98-106	———	99-108	103-109	98-104
1:8	Average % of Expected	101	111	110	107	105
	Range (%)	95-104	———	104-115	99-116	97-113

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-three assays were evaluated and the minimum detectable dose (MDD) of cGMP ranged from 0.56-3.06 pmol/mL. The mean MDD was 1.14 pmol/mL.

The MDD was determined by subtracting two standard deviations from the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of cGMP in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pmol/mL)	Range (pmol/mL)	Standard Deviation (pmol/mL)
EDTA plasma* (n=45)	152	75-219	35
Urine* (n=24)	675	96-1940	545

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of cGMP. No detectable levels were observed.

Cell Lysates - Four peripheral blood mononuclear cell samples were lysed and assayed for levels of cGMP. No detectable levels were observed.

SPECIFICITY

The factors listed below were prepared at 50 nmol/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 2000 pmol/mL in a mid-range cGMP control were assayed for interference. No significant cross-reactivity or interference was observed.

AMP	CTP
ATP	GTP
cAMP	GMP
cUMP	

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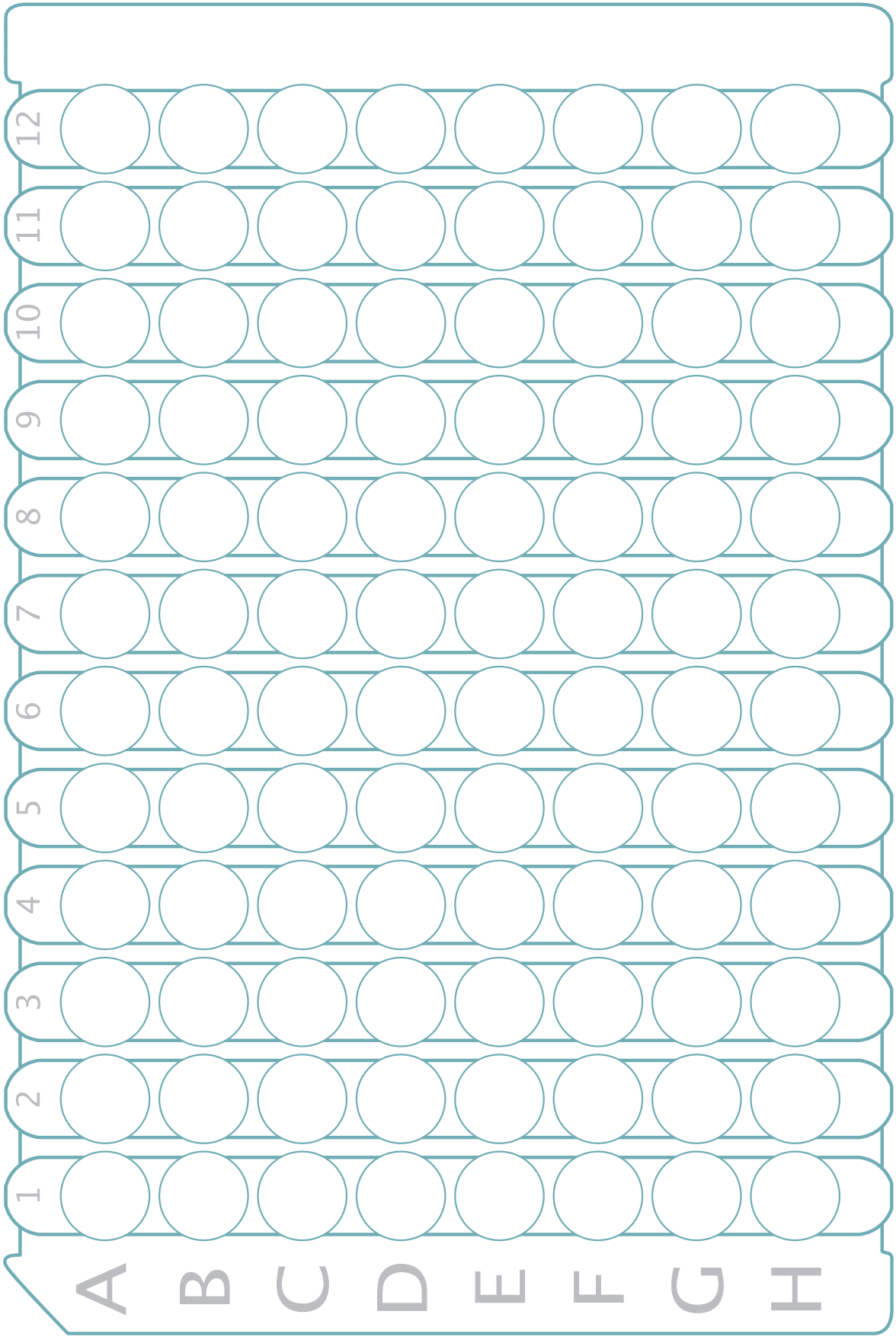
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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