Parameter™

Mouse/Rat cAMP Assay

Catalog Number KGE012

For the quantitative determination of cyclic AMP (cAMP) concentrations in mouse or rat cell culture supernates, cell lysates, tissue homogenates, plasma, and urine.

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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USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
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China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Adenosine 3'5'-cyclic monophosphate (cAMP) is a ubiquitous second messenger that mediates a diverse range of cellular processes in all organisms from bacteria to higher eukaryotes (1, 2). It is converted from adenosine triphosphate (ATP) by adenylyl cyclases (ACs) (3, 4), and is inactivated by phosphodiesterases (PDEs) which catalyze its hydrolysis to 5'-AMP (5). In mammals, upon interaction with extracellular ligands, G protein coupled receptors (GPCRs) linked to $G\alpha_s$ activate the family of nine transmembrane ACs to increase intracellular cAMP. In contrast, GPCRs associated with $G\alpha_{i/o}$ inhibit the synthesis of cAMP by transmembrane ACs. With the exception of AC9, transmembrane ACs can be activated by forskolin, a plant diterpene commonly used to raise cAMP levels in cells (6). A divalent cation-dependent soluble AC (sAC) isoform also exists. It is activated by bicarbonate ions and can be found in the cytosol and in subcellular organelles (7).

The physiological roles of cAMP are mediated via multiple effector molecules (8). Binding of cAMP to protein kinase A (PKA) holoenzyme induces conformational changes and releases the catalytic subunit to phosphorylate target substrates on serine/threonine residues. cAMP binding to the guanine nucleotide exchange factors Epac1/2 (exchange protein activated by cAMP, also known as cAMP-GEF-I and cAMP-GEF-II) mediates the exchange of GDP for GTP on the small molecular weight G proteins Rap-1 and -2. Activated Rap proteins are important in multiple cellular processes including adhesion and exocytosis (8-10). cAMP can also activate cyclic nucleotide-gated ion channels (CNG) by binding directly to the nonselective cation channel proteins that are expressed in various tissues (8, 11). cAMP signaling is spatially and temporally regulated, allowing for the selective activation of a subset of targets. A-kinase anchoring proteins (AKAPs) provide the platform for the assembly of signalsomes consisting of cAMP effectors (PKA and/or Epac) and their substrates, together with signal terminators including phosphatases and PDEs (12-15).

In response to increases in intracellular cAMP, a wide variety of cell types possess mechanisms for exporting cAMP to the extracellular space (16-19). In mammals, plasma and urine cAMP concentrations can become highly elevated under certain physiological conditions. Extracellular cAMP is known to exert physiological actions on diverse cell types, in part through the cAMP-adenosine pathway where cAMP is converted to adenosine via ecto-PDEs and ectonucleotidases.

The R&D Systems cAMP Immunoassay is a 3.5 hour competitive enzyme immunoassay designed to measure cAMP in mouse or rat cell culture supernates, cell lysates, tissue homogenates, plasma, and urine.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique. A streptavidin-coated plate is incubated with a biotinylated monoclonal antibody specific for cAMP. Following a wash to remove excess monoclonal antibody, cAMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cAMP for sites on the monoclonal antibody. This is followed by another 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 cAMP 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 this assay, the possibility of interference cannot be excluded.

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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Streptavidin Coated Microplate	890649	96 well polystyrene microplate (12 strips of 8 wells) coated with streptavidin.	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.*
Mouse/Rat cAMP Conjugate	894125	6 mL of cAMP conjugated to horseradish peroxidase with red dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
cAMP Standard	893353	2400 pmol of cAMP in buffer with preservatives; lyophilized.	
Biotinylated Primary Antibody	894126	6 mL of a biotinylated mouse monoclonal antibody to cAMP in buffer with blue dye and preservatives.	
Calibrator Diluent RD5-55	895398	2 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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.
- 1000 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- cAMP Controls (optional; available from R&D Systems).

If using cell culture supernate, cell lysate, or tissue homogenate samples, the following is also required:

- 1N HCl
- 1N NaOH

PRECAUTIONS

cAMP is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain ProClin® 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. Please 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.

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

Note: *Samples containing ≥ 2 µg/mL of biotin are not recommended for use in this assay. Phosphodiesterase (PDE) inhibitors used in cell culture media may interfere in the assay. If a PDE inhibitor is used, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the effect of the inhibitor on the assay results.*

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

Tissue Homogenates - See Sample Preparation section.

Plasma - Collect plasma using EDTA (≥ 10 mM) as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Serum and heparin plasma are not recommended for use in this assay due to the presence of phosphodiesterases (PDEs).*
Do not use icteric or lipemic samples.
Citrate plasma has not been validated for use in this assay.

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Plasma - Samples require a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-55.

Mouse Urine - Samples require a 300-fold dilution. A suggested 300-fold dilution is 20 µL of sample + 280 µL of Calibrator Diluent RD5-55. Complete the 300-fold dilution by adding 15 µL of this mixture to 285 µL of Calibrator Diluent RD5-55.

Rat Urine - Samples require a 50-fold dilution. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5-55.

Cell Lysates:

1. Wash cells three times in cold PBS.
2. Resuspend cells in cold PBS to a concentration of 1 x 10^7 cells/mL.
3. Immediately add one part of 1N HCl to 9 parts of sample.
4. Incubate at room temperature for 10 minutes.
5. Centrifuge at 600 x g for 10 minutes at 2-8 °C to remove cellular debris.
6. Neutralize the sample with a volume of 1N NaOH equal to the volume of 1N HCl used in step 3.
7. Dilute sample 2-fold with Calibrator Diluent RD5-55.
8. Assay the sample immediately.

A minimum of 250 µL of the diluted cell lysate is required to perform the assay in duplicate. The concentration of cell lysate sample read off the standard curve must be multiplied by the appropriate dilution factor.

Cell Culture Supernates - Samples require an acid treatment to inactivate PDEs. Add 40 µL of 1N HCl to 200 µL of sample. Mix well and incubate for 10 minutes at room temperature. Neutralize the sample by adding 40 µL of 1N NaOH. Add 280 µL of Calibrator Diluent RD5-55 to the treated sample. The concentration of cell culture supernate sample read off the standard curve must be multiplied by the dilution factor, 2.8.

Tissue Homogenates - Rinse organs with PBS and homogenize with a tissue homogenizer in cold 0.1N HCl at a 1:5 ratio (w/v). Centrifuge at 10,000 x g to remove particulates. Neutralize the supernate with 1 N NaOH. Dilute the supernate 2-fold with Calibrator Diluent RD5-55. The concentration of tissue homogenate sample read off the standard curve must be multiplied by the appropriate dilution factor.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

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

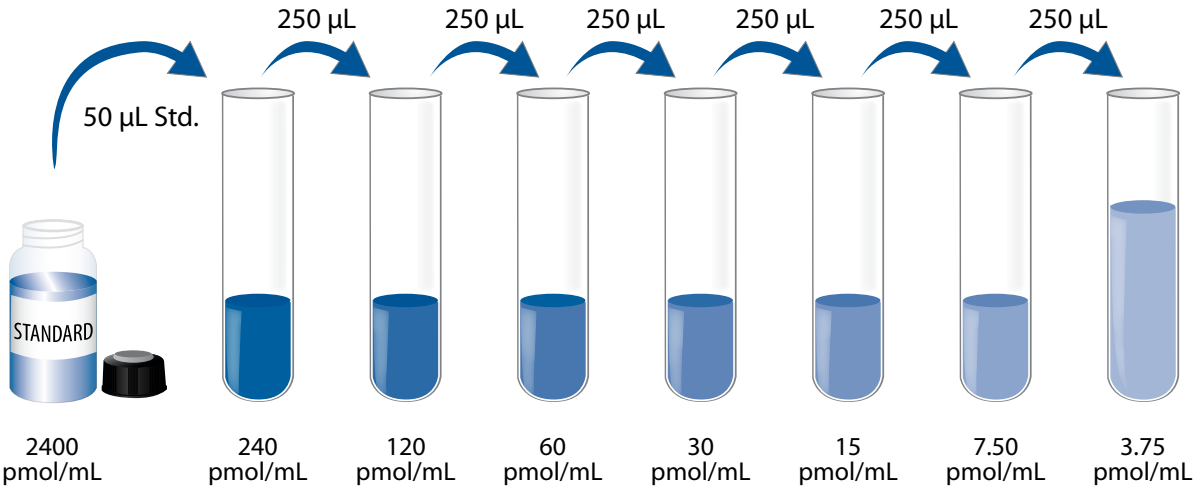
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

cAMP Standard - Reconstitute the cAMP Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 2400 pmol/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-55 into the 240 pmol/mL tube. Pipette 250 µL of Calibrator Diluent RD5-55 into the remaining tubes.

Use the 2400 pmol/mL stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 240 pmol/mL standard serves as the high standard and Calibrator Diluent RD5-55 serves as the zero standard (B_0) (0 pmol/mL).



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. A plate layout is provided to record standards and samples assayed.

Note: *cAMP 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. Wash the Streptavidin Coated Microplate three times. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for 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.
4. Add 50 µL of Biotinylated Primary Antibody to all wells **except** the non-specific binding (NSB) wells. Cover with the adhesive strip provided, and incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm ± 50 rpm.
5. Aspirate each well and wash as in step 3 for a total of 4 washes.

   Note: *Do not allow wells to dry before addition of Mouse/Rat cAMP Conjugate.*
6. **Immediately** add 50 µL of Mouse/Rat cAMP Conjugate to all wells.
7. Add 100 µL of Standard, control, or sample* to the appropriate wells. A plate layout is provided to record standards and samples assayed.
8. Add 100 µL of Calibrator Diluent RD5-55 to the NSB and zero standard (B_0) wells. Cover with a new adhesive strip, and incubate for 2 hours at room temperature on the shaker.
9. Repeat the aspiration and wash as in step 3 for a total of 4 washes.
10. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
11. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
12. 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 require dilution, lysis, or acid treatment. See the 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 paramater 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.

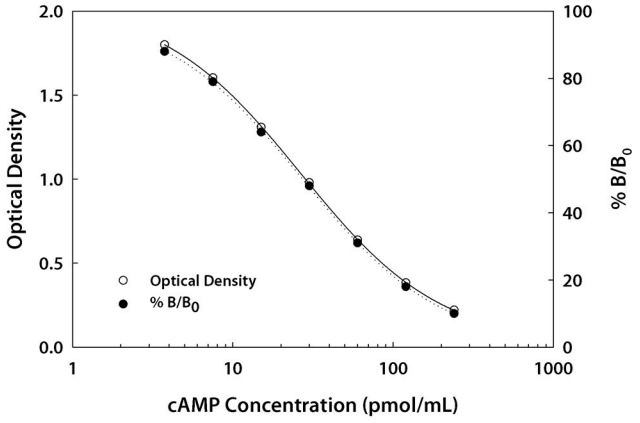
If desired, % B/B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

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

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pmol/mL)	O.D.	Average	Corrected	% B/B_0
NSB	0.012 0.015	0.014	—	—
0 (B_0)	2.023 2.045	2.034	2.020	100
3.75	1.771 1.829	1.800	1.786	88
7.50	1.599 1.606	1.603	1.589	79
15	1.305 1.312	1.309	1.295	64
30	0.973 0.985	0.979	0.965	48
60	0.633 0.642	0.638	0.624	31
120	0.378 0.387	0.383	0.369	18
240	0.217 0.224	0.221	0.207	10

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 twenty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pmol/mL)	27.7	58.7	96.9	28.2	61.7	102
Standard deviation	0.5	1.0	3.9	2.2	4.8	6.5
CV (%)	1.8	1.7	4.0	7.8	7.8	6.4

RECOVERY

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

Sample Type	Average % Recovery	Range %
Cell culture samples (n=4)	100	98-103
Cell lysate (n=2)	106	106-107
Tissue homogenate (n=4)	110	105-115
Mouse EDTA plasma (n=4)	97	92-101
Rat EDTA plasma (n=4)	104	101-108

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of cAMP ranged from 0.01-1.54 pmol/mL. The mean MDD was 0.79 pmol/mL.

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of cAMP were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Cell lysates* (n=4)	Tissue homogenates* (n=4)	Mouse EDTA plasma* (n=4)	Mouse urine* (n=4)
1:2	Average % of Expected	100	99	99	102	103
	Range (%)	88-106	97-100	95-103	95-106	100-105
1:4	Average % of Expected	101	100	100	99	103
	Range (%)	82-110	99-102	96-104	93-106	97-107
1:8	Average % of Expected	100	101	98	95	93
	Range (%)	82-109	99-104	91-103	90-104	89-97
1:16	Average % of Expected	103	92	99	93	93
	Range (%)	87-115	86-98	92-115	———	81-102

		Rat EDTA plasma* (n=4)	Rat urine* (n=4)
1:2	Average % of Expected	96	104
	Range (%)	93-99	100-109
1:4	Average % of Expected	97	104
	Range (%)	93-104	100-111
1:8	Average % of Expected	102	103
	Range (%)	97-107	98-106
1:16	Average % of Expected	104	102
	Range (%)	96-112	99-105

*Samples were diluted or acid treated and diluted prior to assay. See the Sample Preparation section.

SAMPLE VALUES

Plasma/Urine - Samples were evaluated for the presence of cAMP in this assay.

Sample Type	Mean (pmol/mL)	Range (pmol/mL)	Standard Deviation (pmol/mL)
Mouse EDTA plasma (n=20)	115	67.9-157	21.8
Rat EDTA plasma (n=20)	51.0	14.8-102	23.7
Mouse urine (n=20)	32,417	6408-61,782	14,559
Rat urine (n=20)	3801	61.5-26,940	6146

Cell Culture Supernates - Livers from mice were removed, rinsed in 1X PBS, and kept on ice. The tissue was homogenized using a tissue homogenizer and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured for 4 days. An aliquot of the cell culture supernate was removed, prepared as described in the Sample Preparation section, assayed for levels of natural cAMP, and measured 6.87 pmol/mL.

Cell Lysates - Cells were prepared as described in the Sample Preparation section.

Cell Line	(pmol/mL)
NIH-3T3 mouse embryonic fibroblasts	29.8
RAW 264.7 mouse monocyte/macrophage cells	6.30

Tissue Homogenates - Homogenates were prepared as described in the Sample Preparation section. An aliquot of each homogenate was assayed for levels of cAMP.

Tissue	(pmol/mL)
Mouse brain	384
Mouse heart	181
Mouse kidney	108
Mouse liver	143
Mouse lung	487
Mouse spleen	1234

SPECIFICITY

The factors listed below were prepared at 24,000 pmol/mL in Calibrator Diluent RD5-55 and assayed for cross-reactivity. Preparations of the following factors at 24,000 pmol/mL in a mid-range cAMP standard were assayed for interference. No significant cross-reactivity or interference was observed.

AMP	CTP	GMP	UMP
ATP	GTP	cGMP	

Phosphodiesterase inhibitors used in cell culture media were tested in this assay by diluting them in RPMI with 10% fetal bovine serum. Denbufylline, Ro-20-1724, rolipram, pentoxifylline, IBMX, and etazolate hydrochloride did not cross-react or interfere in this assay.

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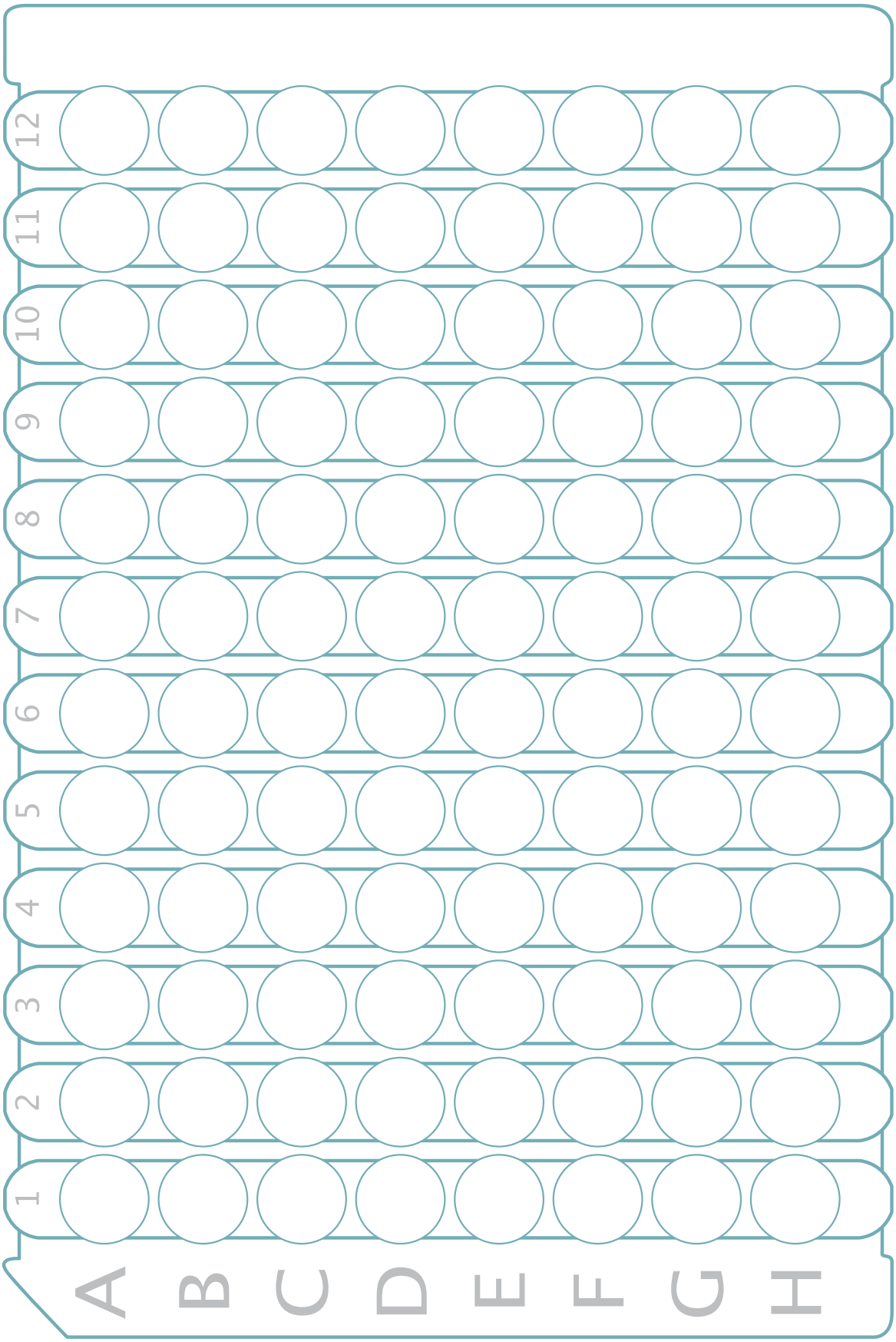
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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