Version 09-12/24

User's Manual

Mouse/Rat cAMP ELISA Kit

REF **DEIA2964**

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RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

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

General Description

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. It is converted from adenosine triphosphate (ATP) by adenylyl cyclases (ACs), and is inactivated by phosphodiesterases (PDEs) which catalyze its hydrolysis to 5'-AMP. In mammals, upon interaction with extracellular ligands, G protein coupled receptors (GPCRs) linked to Gαs activate the family of nine transmembrane ACs to increase intracellular cAMP. In contrast, GPCRs associated with Gαi/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. 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.

The physiological roles of cAMP are mediated via multiple effector molecules. 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. 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. 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.

In response to increases in intracellular cAMP, a wide variety of cell types possess mechanisms for exporting cAMP to the extracellular space. 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.

Principles of Testing

This assay is based on the competitive binding technique. A streptavidin-coated plate is incubated with a biotinylated monoclonal antibody specific for mouse and rat 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.

Reagents And Materials Provided

1. Streptavidin Coated Microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated with streptavidin.

   Note: Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal.
2. Mouse/Rat cAMP Conjugate: 6 mL of cAMP conjugated to horseradish peroxidase with preservatives.
3. cAMP Standard: cAMP in a buffer with preservatives; lyophilized. Refer to the vial label for reconstitution volume.
4. Biotinylated Primary Antibody: 6 mL of a biotinylated mouse monoclonal antibody to cAMP in buffer with preservatives.
5. Calibrator Diluent: 2 vials (21 mL/vial) of a buffered protein base with preservatives.
6. Cell Lysis Buffer 5: 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. May turn yellow over time.
7. Wash Buffer Concentrate: 2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.
8. Color Reagent A: 12 mL of stabilized hydrogen peroxide.
9. Color Reagent B: 12 mL of stabilized chromogen (tetramethylbenzidine).
10. Stop Solution: 23 mL of diluted hydrochloric acid.
11. Plate Sealers: 4 adhesive strips.

Materials Required But Not Supplied

1. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
2. Pipettes and pipette tips
3. Deionized or distilled water
4. Squirt bottle, manifold dispenser, or automated microplate washer
5. 50 mL and 1000 mL graduated cylinders
6. Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
7. Test tubes for dilution of standards and samples
8. Mouse/Rat cAMP Controls

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

9. 1N HCl
10. 1N NaOH

Storage

Store unopened kit at ≤ -20 °C in a manual defrost freezer and at 2-8 °C for up to 1 month. Do not use past kit expiration date.

Specimen Collection And Preparation

Sample Collection

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 ≥ 2.0 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 × 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.

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

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

Cell Lysates:

1. Wash cells three times in cold PBS.
2. Resuspend cells in cold 0.1N HCl/Cell Lysis Buffer (diluted 1:5, see Reagent Preparation section.) to a concentration of 1×10^7 cells/mL.
3. Incubate at room temperature for 10 minutes with gentle mixing.
4. Neutralize the sample with 1N NaOH at a 1:10 (v/v) of total sample volume.

   Note: Confirm cell lysis using Trypan Blue and a microscope. Lysed cells will be blue. If cells are not lysed, freeze/thaw cells as needed.

5. Centrifuge at 600 × g for 10 minutes at 2-8 °C to remove insoluble cellular debris.
6. Dilute the sample 2-fold with Calibrator Diluent.
7. Assay the sample immediately.

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 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 × g to remove particulates. Neutralize the supernate with 1 N NaOH. Dilute the supernate 2-fold with Calibrator Diluent.

Reagent Preparation

Bring all reagents to room temperature before use.

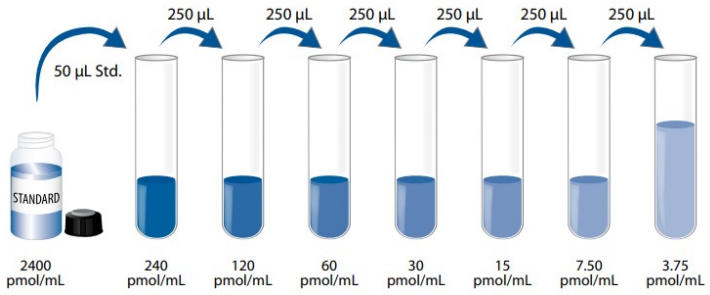
Note: cAMP is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.

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 960 mL of 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.

0.1N HCl/Cell Lysis Buffer 5 (diluted 1:5) - Add 10 mL of Cell Lysis Buffer 5 to 35 mL deionized or distilled water. Add 5.0 mL 1 N HCl to prepare 50 mL of 0.1N HCl/Cell Lysis Buffer 5 (diluted 1:5).

Mouse/Rat cAMP Standard - Refer to the vial label for reconstitution volume. Reconstitute the Mouse/Rat cAMP Standard with 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 into the 240 pmol/mL tube. Pipette 250 µL of Calibrator Diluent 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 serves as the zero standard (B0) (0 pmol/mL).



Assay Procedure

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples 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 to the NSB and zero standard (B0) 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

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 B0 in the standard curve.

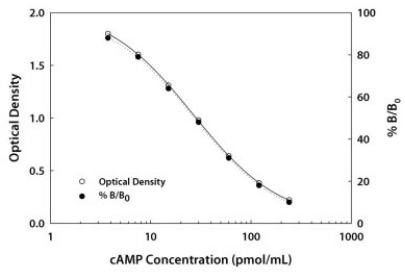
If desired, % B/B0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B0 O.D. and multiplying by 100.

Calculate the concentration of mouse/rat 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 appropriate dilution factor.

Typical Standard Curve

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

Reference Values

Sample Values

Plasma/Urine - Samples were evaluated for the presence of mouse/rat 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 1× 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 mg/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 mouse/rat 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 mouse/rat cAMP.

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

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. Assays were performed by at least three technicians using two lots of components.

	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

Sensitivity

Eighty assays were evaluated and the minimum detectable dose (MDD) of mouse/rat 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 O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

Specificity

The factors listed below were prepared at 24,000 pmol/mL in calibrator diluent 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 ATP CTP GTP GMP cGMP UMP

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 does not cross-react or interfere in this assay.

Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse/rat cAMP were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted or acid treated and diluted prior to assay. See the Sample Preparation section.

		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

Recovery

The recovery of mouse/rat 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%

Precautions

1. FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
2. The kit should not be used beyond the expiration date on the kit label.
3. Do not mix or substitute reagents with those from other lots or sources.
4. If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
5. Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
6. Variations in sample collection, processing, and storage may cause sample value differences.
7. 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.
8. When mixing or reconstituting protein solutions, always avoid foaming.
9. 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.

10. Pre-rinse the pipette tip when pipetting.
11. Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
12. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
13. 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.
14. 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.
15. 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.
16. cAMP is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.
17. The Stop Solution provided with this kit is an acid solution.
18. Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.
19. Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.
20. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.