Parameter™

Cyclic AMP Assay

Catalog Number KGE002
SKGE002
PKGE002

For the quantitative determination of cyclic AMP (cAMP) concentrations in cell culture supernates, serum, plasma, saliva, urine, and cell lysates.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

TELEPHONE: +49 (0)6122 90980
FAX: +49 (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

R&D Systems Europe
77 boulevard Vauban
59041 LILLE CEDEX
France

FREEPHONE: +0800 90 72 49
FAX: +0800 77 16 68
E-MAIL: info@RnDSystems.co.uk

INTRODUCTION

Adenosine 3',5'-cyclic monophosphate (cAMP) is a ubiquitous second messenger involved in various cellular activities in many cell and tissue types. It is converted from adenosine triphosphate (ATP) via adenylyl cyclases (AC). There are at least 10 AC isoforms found in mammals, 9 of which are multi-transmembrane domain proteins (AC_1 - AC_9), and one soluble form has been described (sAC) (1 - 5). Membrane subtypes may be activated by the stimulatory G protein a subunit (G_sa) (5). Some examples of G protein-coupled receptors known to activate this pathway include those for the ligands epinephrine, dopamine, PGE_2, adenosine, and glucagon (5). In contrast, the G_ia subclass suppresses the activity of ACs (6 - 9). The intrinsic GTPase activity of the G_s subtype is classically suppressed by cholera toxin, while the $G_{i/o}$ subtypes are inhibited by pertussis toxin (10 - 12). Ca^{2+} and its downstream effectors are also regulators of AC activity. Depending on the AC subtype, this regulation might be activating or inhibitory (13 - 15). Similarly, specific AC isoforms appear to be positively or negative regulated by Gbg subunits (16, 17). Most isoforms are also potently activated exogenously by forskolin (5). sAC is structurally and biochemically distinct from membrane ACs. It is insensitive to forskolin and may be activated by bicarbonate (18 - 20). A large family of cyclic nucleotide phosphodiesterases regulates the levels of cAMP and guanosine 3',5'-cyclic monophosphate (cGMP) (21).

cAMP may affect cellular function through several different mechanisms including the activation of cAMP-dependent Protein Kinase (PKA), Guanine Nucleotide Exchange Factors (GEFs), and Cyclic Nucleotide-gated (CNG) channels. It may also be extruded by certain cell types and have extracellular roles as well.

PKA is a heterotetramer consisting of 2 regulatory (R) subunits and 2 catalytic (C) subunits. Two cAMP molecules bind cooperatively to 2 sites on each R subunit, releasing the active C subunit monomers to phosphorylate a range of downstream substrates (9, 22 - 25). GEFs facilitate the exchange of GDP for GTP and, therefore, promote the activity of G proteins. Exchange Protein Activated by cAMP (Epac) 1 and 2 are GEFs activated upon binding to cAMP. Epac 1 and 2 have been implicated in regulating the activity of the small GTPase Rap-1 (26, 27). CNG channels are cation channels activated by cGMP and/or cAMP. These channels regulate membrane potential, and due to their Ca^{2+} permeability, can alter the levels of intracellular Ca^{2+}. Their regulation of olfactory sensory neuron and photoreceptor activity has been well studied (28). In response to stimulating factors that include glucagon, epinephrine, and PGE_2, several cell types have been shown to extrude cAMP including hepatocytes, adipocytes, smooth muscle cells, glomerular cells, and glial cells (29, 30). Consequently, cAMP may be found in circulation, urine, and cell culture supernates (29, 30).

R&D Systems' cAMP Immunoassay is a 3.5 hour competitive enzyme immunoassay designed to measure cAMP in cell culture supernates, plasma, serum, saliva, urine, and cell lysates.

PRINCIPLE OF THE ASSAY

This assay is based on the competitive binding technique in which cAMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cAMP for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse 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 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.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and 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. 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

Description	Part #	Cat. # KGE002	Cat. # SKGE002
Goat Anti-mouse Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.	892575	1 plate	6 plates
cAMP Conjugate - 6 mL/vial of cAMP conjugated to horseradish peroxidase with red dye and preservatives.	892970	1 vial	6 vials
cAMP Standard - 1 mL/vial (2000 pmol/mL) of cAMP in buffer with preservatives.	892972	1 vial	6 vials
Primary Antibody Solution - 6 mL/vial of mouse monoclonal antibody to cAMP in buffer with blue dye and preservatives.	892971	1 vial	6 vials
Calibrator Diluent RD5V - 21 mL/vial of a buffered protein base with preservatives.	895425	2 vials	12 vials
Cell Lysis Buffer 5 Concentrate - 21 mL/vial of a 5-fold concentrated buffered solution with preservatives.	895890	1 vial	6 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
Plate Covers - Adhesive strips.	——	4 strips	24 strips

KGE002 contains sufficient materials to run an ELISA on one 96 well plate.
SKGE002 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PKGE002). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at ≤ -20° C in a manual defrost freezer. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Calibrator Diluent RD5V</td></tr>
<tr><td>Cell Lysis Buffer 5</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Primary Antibody Solution</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Conjugate</td><td>Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Microplate Wells</td><td>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.*</td></tr>
</table>

*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.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples (Salivette™ or equivalent).
- cAMP Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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

Salivette™ is a trademark of Sarstedt, Inc.

SAMPLE COLLECTION AND STORAGE

Samples containing mouse IgG may interfere with this assay.

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

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or 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: *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. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

Cell Lysates - Cells must be lysed before assaying according to the following directions.

1. Wash cells three times in cold PBS.
2. Resuspend cells in Cell Lysis Buffer 5 (1X) 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.

SAMPLE PREPARATION

Serum and plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 100 µL sample + 400 µL Calibrator Diluent RD5V.

Cell culture supernate and saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 200 µL sample + 200 µL Calibrator Diluent RD5V.

Urine samples require a 100-fold dilution. A suggested 100-fold dilution is 10 µL sample + 990 µL Calibrator Diluent RD5V.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *cAMP is found in saliva. We recommend using a face mask and gloves 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. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

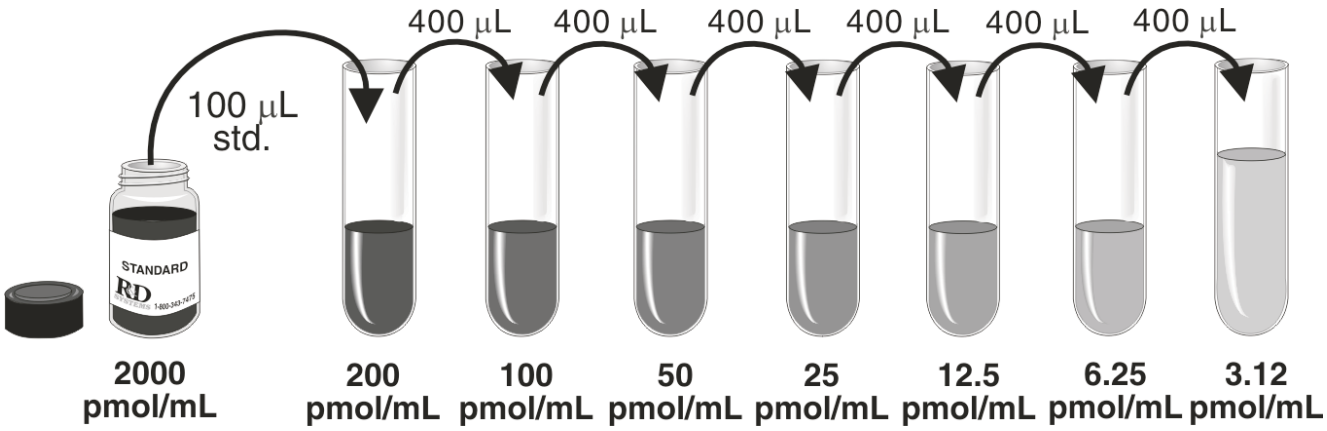
Cell Lysis Buffer 5 (1X) - Dilute 20 mL of Cell Lysis Buffer 5 Concentrate into 80 mL of deionized or distilled water to prepare 100 mL of Cell Lysis Buffer 5 (1X).

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.

cAMP Standard - Thaw the 2000 pmol/mL Standard and allow to sit for a minimum of 15 minutes after thawing with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5V into the 200 pmol/mL tube. Pipette 400 µL of Calibrator Diluent RD5V into the remaining tubes.

Use the 2000 pmol/mL Standard to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 200 pmol/mL standard serves as the high standard and Calibrator Diluent RD5V 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.

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

3. Add 150 µL of Calibrator Diluent RD5V into the NSB wells.

4. Add 100 µL of Calibrator Diluent RD5V to the zero standard ($B_{0)}$ wells.

5. Add 100 µL of Standard, control, or sample* to the appropriate wells.

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 blue in color.

7. Add 50 µL of cAMP Conjugate to all wells. All wells except the NSB wells will now be violet in color. Cover with the adhesive strip provided.

8. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

9. 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 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.

10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**

11. 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.

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. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 150 µL Calibrator Diluent RD5V to the NSB wells.

3. Add 100 µL Calibrator Diluent RD5V to the zero standard (B_0) wells.

4. Add 100 µL Standard, control or sample* to the remaining wells.

5. Add 50 µL Primary Antibody Solution to all wells. (excluding NSB wells).

6. Add 50 µL cAMP Conjugate to all wells. Incubate for 3 hours on the shaker at RT.

7. Aspirate and wash 4 times.

8. Add 200 µL Substrate Solution to each well. Incubate for 30 minutes at RT. **Protect from light.**

9. Add 50 µL Stop Solution to each well.
Read at 450 nm within 30 min.
λ correction 540 or 570 nm

*Samples 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.

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.

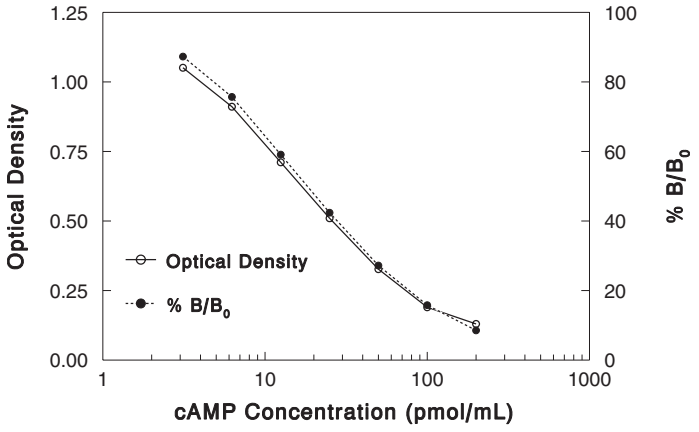
If desired, % B/B_0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.

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	OD	Average	Corrected	% B/B_0
NSB	0.008 0.012	0.010	—	—
0 (B_0)	1.128 1.299	1.214	1.204	—
3.12	1.045 1.076	1.061	1.051	87.3
6.25	0.916 0.925	0.921	0.911	75.7
12.5	0.721 0.721	0.721	0.711	59.1
25	0.522 0.517	0.520	0.510	42.4
50	0.338 0.335	0.337	0.327	27.2
100	0.200 0.200	0.200	0.190	15.8
200	0.113 0.112	0.113	0.103	8.6

PRECISION

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

Inter-assay Precision (Precision between assays)
Four samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision				Inter-assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	40	40	40	40
Mean (pmol/mL)	5.40	16.7	29.7	79.9	5.91	18.2	32.3	81.9
Standard deviation	0.85	1.27	1.58	3.35	0.95	1.58	2.20	6.25
CV (%)	15.7	7.6	5.3	4.2	16.1	8.8	6.9	7.6

RECOVERY

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

Sample	Average % Recovery	Range
Cell culture media (n=4)	110	92 - 120%
Serum (n=4)	103	92 - 107%
EDTA plasma (n=4)	108	88 - 127%
Heparin plasma (n=4)	100	89 - 107%
Urine (n=4)	96	86 - 102%
Saliva (n=4)	113	96 - 132%
Cell Lysis Buffer 5 (1X) (n=1)	113	108 - 121%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of cAMP were serially diluted with Calibrator Diluent RD5V to produce samples with values within the dynamic range of the assay.

		Cell culture media* (n=4)	Serum* (n=4)	Heparin plasma* (n=4)	EDTA plasma* (n=4)	Urine** (n=4)	Saliva* (n=4)	Cell Lysis Buffer 5* (n=1)
1:2	Average % of Expected	89	92	97	100	97	95	94
	Range (%)	85 - 92	89 - 99	94 - 104	100 - 101	92 - 104	86 - 111	—
1:4	Average % of Expected	88	88	93	93	98	101	86
	Range (%)	87 - 90	85 - 94	90 - 97	90 - 96	92 - 104	85 - 111	—
1:8	Average % of Expected	87	90	87	86	92	—	90
	Range (%)	81 - 98	82 - 97	83 - 93	80 - 98	85 - 97		—
1:16	Average % of Expected	93	88	90	87	91	—	97
	Range (%)	77 - 107	82 - 102	84 - 94	81 - 91	88 - 93		—

*Samples were diluted prior to assay. See Sample Preparation section.
**Urine samples were diluted 50-fold prior to assay.

SENSITIVITY

Twenty-six assays were evaluated and the minimum detectable dose (MDD) of cAMP ranged from 0.58 - 3.00 pmol/mL. The mean MDD was 1.43 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.

SAMPLE VALUES

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

Sample Type	Mean (pmol/mL)	Standard Deviation (pmol/mL)	Range (pmol/mL)
Serum* (n=36)	60	26	27 - 174
EDTA plasma* (n=36)	65	27	32 - 194
Heparin plasma* (n=36)	58	20	28 - 125
Urine* (n=26)	4098	2956	598 - 11955
Saliva* (n=10)	36	20	14 - 75

*Samples were diluted prior to assay. See Sample Preparation section.

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf 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 supernate were removed and assayed for levels of cAMP.

Condition	Day 1 (pmol/mL)	Day 5 (pmol/mL)
Unstimulated	ND	3.9
Stimulated	3.1	4.6

ND = Non-detectable

Cell Lysates - Four samples were assayed for levels of cAMP and measured from 3.72 - 14.6 pmol/mL.

SPECIFICITY

The factors listed below were prepared at 2000 pmol/mL in Calibrator Diluent RD5V and assayed for cross-reactivity. Preparations of the following factors at 2000 pmol/mL in a mid-range cAMP control were assayed for interference. No significant cross-reactivity or interference was observed.

AMP	CTP	GMP	cUMP
ATP	GTP	cGMP	

REFERENCES

1. Hanoune, J. *et al.* (1997) Mol. Cell. Endocrinol. **128**:179.
2. Patel, T.B. *et al.* (2001) Gene **269**:13.
3. Smit, M.J. and R. Iyengar (1998) Adv. Second Messenger Phosphoprotein Res. **32**:1.
4. Sunahara, R.K. *et al.* (1996) Annu. Rev. Pharmacol. Toxicol. **36**:461.
5. Sunahara, R.K. and R. Taussig (2002) Mol Interv **2**:168.
6. Kozasa, T. and A.G. Gilman (1995) J. Biol. Chem. **270**:1734.
7. Taussig, R. *et al.* (1993) Science **261**:218.
8. Taussig, R. *et al.* (1994) J. Biol. Chem. **269**:6093.
9. Tasken, K. and E.M. Aandahl (2004) Physiol. Rev. **84**:137.
10. Bokoch, G.M. *et al.* (1983) J. Biol. Chem. **258**:2072.
11. Codina, J. *et al.* (1983) Proc. Natl. Acad. Sci. USA **80**:4276.
12. Van Heyningen, S. and C.A. King (1975) Biochem. J. **146**:269.
13. Cali, J.J. *et al.* (1994) J. Biol. Chem. **269**:12190.
14. Fagan, K.A. *et al.* (2000) J. Biol. Chem. **275**:40187.
15. Ferguson, G.D. and D.R. Storm. (2004) Physiology **19**:271.
16. Gao, B.N. and A.G. Gilman (1991) Proc. Natl. Acad. Sci. USA **88**:10178.
17. Tang, W.J. and A.G. Gilman (1991) Science **254**:1500.
18. Buck, J. *et al.* (1999) Proc. Natl. Acad. Sci. USA **96**:79.
19. Geng, W. *et al.* (2005) Am. J. Physiol. Cell Physiol. **288**:C1305.
20. Wuttke, M.S. *et al.* (2001) JOP **2**:154.
21. Maurice, D.H. *et al.* (2003) Mol. Pharmacol. **64**:533.
22. Builder, S.E. *et al.* (1980) J. Biol. Chem. **255**:3514.
23. Kopperud, R. *et al.* (2002) J. Biol. Chem. **277**:13443.
24. Taylor, S.S. (1989) J. Biol. Chem. **264**:8443.
25. Seino, S. and T. Shibasaki (2005) Physiol. Rev. **85**:1303.
26. de Rooij, J. *et al.* (2000) J. Biol. Chem. **275**:20829.
27. de Rooij, J. *et al.* (1998) Nature **396**:474.
28. Kaupp, U.B. and R. Seifert (2002) Physiol. Rev. **82**:769.
29. Bankir, L. *et al.* (2002) Am. J. Physiol. Renal Physiol. **282**:F376.
30. Jackson, E.K. and R.K. Dubey (2001) Am. J. Physiol. Renal Physiol. **281**:F597.

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.