APExBIO
An Apoptosis and Epigenetics Company

Product Information

cAMP Direct Immunoassay Kit (Colorimetric)

I. Kit Contents:

Components	K2044-100 100 assays	Cap Color	Storage Temperature	Part Number
10X cAMP Assay Buffer	25 ml	WM	+4℃	K2044-C-1
Standard cAMP (10 nmol)	1 vial	Yellow	-20℃	K2044-C-2
Neutralizing Buffer	7.5 ml	NM	+4℃	K2044-C-3
Acetylating Reagent A	0.75 ml	Violet	+4℃	K2044-C-4
Acetylating Reagent B	1.5 ml	Black	+4℃	K2044-C-5
Rabbit Anti-cAMP pAb	1 vial	Red	-20℃	K2044-C-6
cAMP-HRP	1 vial	Green	-20℃	K2044-C-7
HRP Developer	10 ml	Amber	+4℃	K2044-C-8
Protein G Coated Plate	1 each	-	-20℃	K2044-C-9

II. Introduction:

Cyclic adenosine monophosphate (cAMP) is a second messenger that plays an important role in intracellular signal transduction and is synthesized from ATP by adenylate cyclase.

The cAMP Direct Immunoassay Kit (Colorimetric) provides a highly sensitive and convenient way for detection of cAMP level in various biological samples based on direct competitive immunoassay and colorimetric method. The recombinant Protein G coated 96-well plate is utilized to efficiently anchor cAMP polyclonal antibody on the plate. After incubation and washing, the amount of cAMP-HRP bound to the plate can be easily determined by reading HRP activity at $OD_{450\ nm}$. The intensity of $OD_{450\ nm}$ is inversely proportional to the concentration of cAMP in samples. In addition, the assay provides a new acetylation method that significantly improves detection sensitivity. The assay can detect ~0.1-10 pmol/5 µl (or ~ 0.02-2 µM) cAMP levels.

III. Reagent Preparation:

Dilute the 10X cAMP Assay Buffer to 1X Assay Buffer with MilliQ water. Store at 4℃.

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

Dilute the rabbit anti-cAMP pAb and cAMP-HRP each with 1.1 ml of the 1X Assay Buffer as stock solutions, keep frozen.

Unused Protein G coated strips can be kept at –20℃ with descants, stable for up to 1 month after opening.

The kit should be stored at –20℃. After opening and reconstitution, components can be stored as instructed in the kit contents above, stable for 1 to 2 months.

IV. General Consideration:

Esterases in samples may degrade cAMP. Therefore, prepare samples in 0.1N HCl to inactivate esterase, and store at -80℃. Dilute your samples to ~ 0.1 - 10 pmol/well (0.02 - 2 µM) cAMP range.

Urine and tissue culture supernatant can be diluted in 10% 1M HCl and assayed directly.

Plasma, serum, whole blood, and tissue homogenates often contain phosphodiesterases and large amount of immunoglobulins (IgGs) which may interfere with the assay. However, diluting these samples with 0.1M HCl can generally inactivate phosphodiesterases and lower the concentration of IgGs, making the samples suitable for the assay. Phosphodiesterases and IgGs can also be removed by 5% TCA precipitation or 10 kD molecular weight cut off micro centrifuge filters. To determine whether interference is presence in your sample, you may make two different dilutions. If the two different dilutions of sample show good correlation in the final calculated cAMP concentrations, purification is not required; otherwise use TCA precipitation or 10 Kd molecular weight cut off microcentrifuge filters to remove any enzymes from samples. Organic solvents in samples may interfere with the assay, which may need to be removed prior to the assay.

V. Sample Preparation:

Urine, Plasma and Culture Medium Samples: Urine and plasma may be tested directly with 1:20 to 1:100 dilutions in 0.1M HCl. Culture medium can also be tested with 1:10 to 1:200 dilutions in 0.1M HCl. (Note: RPMI medium may contain >350 fmol/µl cAMP).

Cell Samples: Aspirate medium. Add 1 ml of 0.1M HCl for every 35 cm^2 of surface area. Incubate at room temperature for 20 minutes. Scrape cells off the surface with a cell scraper. Dissociate sample by pipetting up and down until suspension is homogeneous. Transfer to a centrifuge tube and centrifuge at top speed for 10 min. The supernatant can be assayed directly. Protein concentration >1 mg/ml is recommended for reproducible results.

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

VI. cAMP Assay Protocol:

The procedure described here includes an acetylation step which makes the cAMP assay much more sensitive and avoid the interferences of many components in samples. However, for routine assay of the well known samples, non-acetylation procedure may also be used, just skip the acetylation steps (Step 7 and 8).

Prepare cAMP Standard Curve and Samples:

1. Dilute 25 µl of the 10 pmol/µl standard cAMP stock with 975 µl of 0.1M HCl to generate 0.25 pmol/µl cAMP working solution. The diluted cAMP should be fresh, and used within 1 hour.
2. Label 8 microcentrifuge tubes, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0 , 0_B pmol/50 µl. (Note: these concentrations represent what will finally be in the wells after the dilutions mentioned below).
3. Add 200 µl of the 0.25 pmol/µl cAMP standard into the tube labeled 1.25 pmol tube (enough for 20 assays). Add100 µl 0.1M HCl into the rest of tubes.
4. Transfer 100 µl from the tube labeled 1.25 pmol tube to the labeled 0.625 pmol tube, mix, then transfer 100 µl into the labeled 0.3125 pmol tube. Continue the serial dilution by transferring 100 µl to 0.156, 0.078, 0.039 pmol tubes. Discard 100 µl from the 0.039 pmol tube. The diluted cAMP should be used within 1 hour.
5. Label new tubes for test samples, add 100 µl each test sample per tube. We suggest using different dilutions for each sample (dilute with 0.1M HCl).
6. Add 50 µl of Neutralizing Buffer to each tube (all standards cAMP and testing samples).
7. Prepare Acetylating Reagent Mix: Mix 1 volume of Acetylating Reagent A (Violet cap) with two volumes of Acetylating Reagent B (Black cap) in a microtube. Prepare enough for the experiment (need 5 µl each sample and standard tubes). Use within 1 hour.
8. Add 5 µl of the Acetylating Reagent Mix directly into each test solution (all standards and samples), IMMEDIATELY vortex 2-3 seconds following each addition without delay, one tube at a time, and incubate at room temperature for 10 min to acetylate cAMP.
9. Add 845 µl 1X Assay Buffer into each tube to dilute the acetylation reagents, mix well. The acetylated standard and samples are ready for quantification. (If cAMP in your samples are very low, the acetylation reagents can be dried after step 8, without dilution step 9 to minimize the volume increase. Then reconstitute in a 50 -100 µl of Assay Buffer).

Quantification cAMP:

1. Add 50 µl of the acetylated Standard cAMP and test samples from Step 9 to the Protein G coated 96-well plate.
2. Add 10 µl of the reconstituted cAMP antibody per well to the standard cAMP and sample wells except the well with 0_B pmol cAMP. (Note: Do not add cAMP antibody into the well with 0_B pmol cAMP, instead add 10 µl of 1X Assay Buffer for background reading). Incubate for 1 hour at room temperature with gentle agitation.

   Note: It is recommended to use a repeating pipette to minimize pipette errors.
3. Add 10 µl of cAMP-HRP to each well, incubate for 1 hr at room temperature with agitation.
4. Wash 5 times with 200 µl 1X Assay Buffer each wash. Completely empty the wells by tapping the plate on a new paper towel after each wash step
5. Add 100 µl of HRP developer and develop for 1 hour at room temperature with agitation.
6. Stop the reaction by adding 100 µl of 1M HCl (not provided) to each well (sample color should change from blue to yellow).
7. Read the plate at $OD_{450\ nm}$.

   Note: The $OD_{450\ nm}$ reading may vary significantly among experiments depend on lot numbers, kit storage and experiment conditions. Therefore, samples and standard curve must be performed at the same time and using the same kit reagents.
8. Subtract $OD_{450\ nm}$ background reading (the well with 0_B pmol cAMP) from all samples and standards. Plot standard curve to observe the linear portion, then replot only the linear portion and in Excel add a trendline, then use the trend line linear formula (y=mx+b) to calculate your sample concentrations. Calculate amount of cAMP in samples after correcting the for dilution factors.
9. Calculations:

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

   Where: Sa is cAMP amount (pmol) from standard curve.

   Sv is sample volume (µl) added into the assay wells after dilution factor correction.

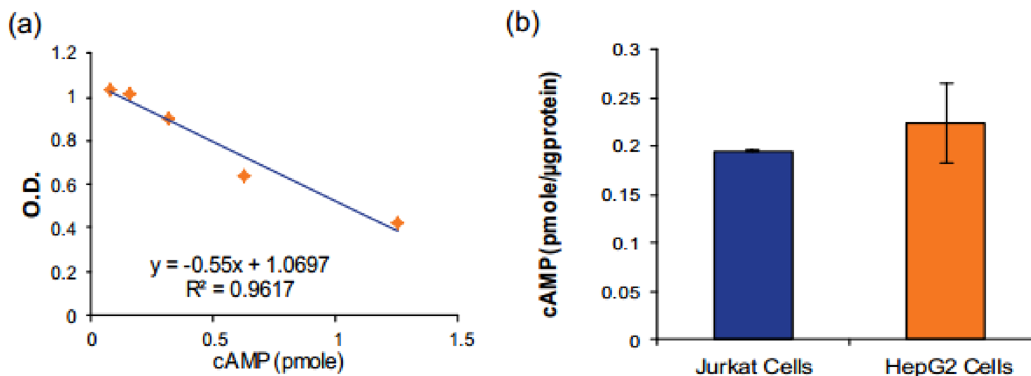

Figure: (a) cAMP Standard Curve. (b) Measurement of cAMP in Jurkat Cells and HepG2 Cells. For suspension cultures, use between 2-4 x 10^6 cells. Resuspend in ~ 500 µl 0.1 N HCl and prepare cell lysate as mentioned in the protocol. Use 100 µl of this cell lysate for acetylation reaction. Add 50 µl of the final acetylated reaction to the protein G coated wells and make up the volume to 50 µl using 1X Assay Buffer. Assay was performed following the kit protocol.

General Troubleshooting Guide:

Problems	Cause	Solution
Assay not working	• Use of a different buffer • Omission of a step in the protocol	• Assay buffer must be at room temperature • Refer and follow the data sheet precisely

	• Plate read at incorrect wavelength • Use of a different 96-well plate	• Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type • Samples prepared in a different buffer • Samples were not deproteinated • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	• Refer data sheet for details about incompatible samples • Use the Nucleotide releasing buffer provided in the kit or refer data sheet for instructions • Use the 10 kDa spin cut-off filter or PCA precipitation as indicated or use 0.1 M HCl to inactivate phosphodiesterases • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used	• Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer data sheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots	• Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Samples/standards aceylated and treated one at a time through this treatment. • Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		

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Our promise

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