KAMIYA BIOMEDICAL COMPANY

Cyclic Adenosine Monophosphate (cAMP) ELISA

For the quantitative determination of cAMP in serum, plasma, tissue homogenates and other biological fluids

Cat. No. KT-12516

For Research Use Only. Not for use in diagnostic procedures.

Product Information

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INTENDED USE

This ELISA kit is a competitive enzyme immunoassay for the *in vitro* quantitative measurement of cAMP in serum, plasma, tissue homogenates and other biological fluids. For research use only. Not for use in diagnostic procedures.

COMPONENTS

Reagents	Quantity
Pre-coated, ready to use 96-well plate	1
Calibrators (liquid)	2
Calibrator Diluent	1 × 20 mL
Detection Reagent A	1 × 120 µL
Assay Diluent A (2 X concentrate)	1 x 6 mL
TMB Substrate	1 × 9 mL
Stop Solution	1 × 6 mL
Wash Buffer (20 X concentrate)	1 × 20 mL
Plate sealer for 96 wells	4

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microplate reader with 450 ± 10 nm filter.
2. Precision single and multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

STORAGE

All reagents should be stored according to their label. The **Calibrators**, **Detection Reagent A** and the **96-well plate** should be stored at -20°C upon receipt. The unused strips should be kept in a sealed bag with the desiccant provided to minimize exposure to damp air. Opened test kits will remain stable until the expiration date, provided they are stored as above.

PRINCIPLE

This assay employs the competitive inhibition immonassay technique. A polyclonal antibody specific for cAMP has been pre-coated onto the microtiter plate. Calibrators, samples and HRP labeled cAMP are then added to the appropriate microtiter plate wells. The HRP labeled cAMP competes with the unlabeled cAMP from the calibrators and samples to bind the pre-coated anti-cAMP antibody. Then the TMB substrate solution is added to each well and a change in color will be exhibited. The higher the concentration of cAMP present in the sample the less HRP labeled cAMP will have bound to the antibody and therefore there will be less color development. The absorbance will have an inverse relationship to the concentration of cAMP present in the sample; the higher absorbance samples will have the lowest concentration of cAMP present in the original sample. The color development is stopped and the intensity of the color is measured.

SAMPLE COLLECTION AND STORAGE

Serum
Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4℃ before centrifugation for 20 minutes at approximately 1000 x g. Assay freshly prepared serum immediately or store samples in aliquots at -20℃ or -80℃ for later use. Avoid repeated freeze/thaw cycles.

Plasma
Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 x g at 2℃ - 8℃ within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquots at -20℃ or -80℃ for later use. Avoid repeated freeze / thaw cycles.

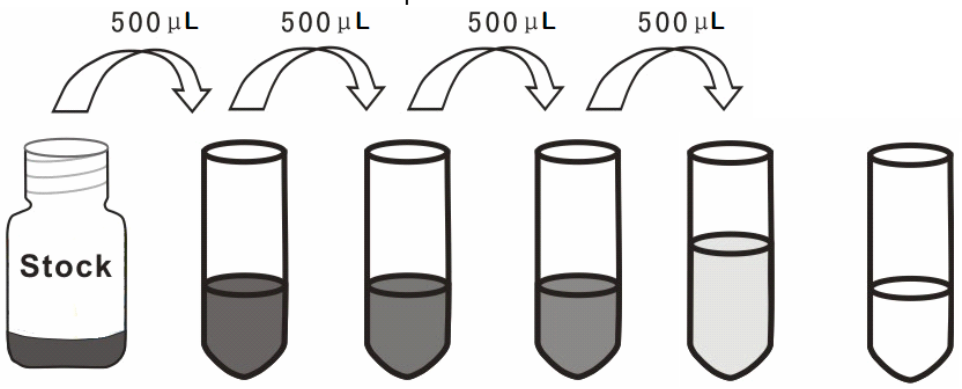
Other Biological Fluids
Centrifuge samples for 20 minutes at 1000 x g. Remove particulates and assay immediately or aliquot samples and store at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue Homogenate
The preparation of tissue homogenates will vary depending upon tissue type. As an example, rinse tissue with 1X PBS to remove excess blood, homogenize in 5~10 mL of 1X PBS and store overnight at ≤ -20℃. Perform two freeze-thaw cycles to break the cell membranes and centrifuge the homogenates for 5 minutes at 5000 x g. Remove the supernatant and assay immediately or aliquot and store at -20℃.

Note:
1. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Before performing the assay, slowly bring samples to room temperature.
3. Avoid hemolysis as excessive hemolysis will impact the result.

REAGENT PREPARATION
Bring all kit components and samples to room temperature (18-25°C) before use.

Calibrators
Reconstitute the **Calibrator** with 0.8 mL of **Calibrator Diluent** and allow it to sit for 10 minutes at room temperature. Then mix uniformly but gently, avoid foaming. The concentration of the stock calibrator solution is now at 250 pmol/mL. Use the stock calibrator solution and the **Calibrator Diluent** to produce a triple dilution series. Pipette 1 mL of **Calibrator Diluent** into each tube. Then perform a serial dilution, beginning with the stock calibrator solution, to create the other 4 calibrators (as shown below). Make sure to use a new pipette tip for each transfer and mix each tube thoroughly before the next transfer. The five calibrators will have concentrations of 250, 83.3, 27.8, 9.3, 3.1 pmol/mL, and the last tube filled with only **Calibrator Diluent** will serve as the blank at 0 pmol/mL.



Tube	1	2	3	4	5	6
pmol/mL	250	83.3	27.8	9.3	3.1	0

Assay Diluent A
Dilute 6 mL of Assay Diluent A (2X) with 6 mL of deionized or distilled water to prepare 12 mL of Assay Diluent A. **The prepared working dilution can not be frozen.**

Detection Reagent A
Briefly spin or centrifuge the stock Detection Reagent A before use. Dilute to the working concentration with working **Assay Diluent A** (1:100).

Wash Solution
Dilute 20 mL of Wash Solution Concentrate (30X) with 580 mL of deionized or distilled water to prepare 600 mL of Wash Solution (1X).

TMB Substrate
Aspirate the needed dosage of the solution with sterilized tips and do not return the residual solution to the vial.

⚠ Note:
1. Prepare the calibrators within 15 minutes of beginning the assay. Do not dissolve the reagents at 37°C.
2. Do not perform your serial dilutions directly in the wells.
3. Carefully reconstitute Calibrators or working Detection Reagent A according to the instruction. Avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to draw more than 10µl for pipetting.
4. The reconstituted Calibrators and Detection Reagent A can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30X), warm to room temperature and mix gently until the crystals have completely dissolved.

ASSAY PROCEDURE

Estimate the sample cAMP concentration before assaying. If the estimated values are not within the range of the calibration curve, users must determine the optimal sample dilutions for their particular experiments.

1. Determine wells to be used for diluted calibrators, blank and samples. Prepare 5 wells for calibrators, 1 well for blank and other wells for samples. Add 50 µL of each calibrator, blank and sample into the appropriate wells.

2. Immediately add 50 µL of Detection Reagent A to all wells. Shake the plate gently, cover with a plate sealer and incubate for 1 hour at 37℃. (Detection Reagent A may appear cloudy. Warm to room temperature and mix gently until solution appears uniform.

3. Aspirate the solution and wash each well with 400 µL of 1X Wash Solution using a squirt bottle, multi-channel pipette, manifold dispenser or auto-washer, and let sit for 1~2 min. Remove the remaining liquid from all wells completely by sharply striking the plate on absorbent paper. Repeat wash 3 - 5 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

4. Add 90 µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 – 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light.

5. Add 50 µL of **Stop Solution** to each well to stop the reaction. Mix the liquid by gently tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

6. Remove any drops of solution or fingerprints on the bottom of the plate and confirm there are no bubbles on the surface of the liquid. Then, run the microplate reader and conduct measurements at 450 nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Unused strips should be resealed and stored at 4℃ until the expiration date.
2. **Sample or reagent additions:** Carefully add samples to wells and mix gently to avoid foaming. Do not touch the well walls if possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all calibrators and samples, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each calibrator level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. After reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drops of solution or fingerprints on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 5 minutes)**,** if the color is too dark, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Protect from light.

CALCULATION OF RESULTS

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between cAMP concentration in the sample and the assay signal intensity. Low levels of cAMP result in a high luminescence intensity, while a high concentration of cAMP results in a low signal.

Average the duplicate readings for each calibrator and sample. Create a calibration curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a calibration curve by plotting the mean absorbance for each calibrator on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the cAMP concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. It is recommended to use some related software to do this calculation. If samples have been diluted, the concentration read from the calibration curve must be multiplied by the dilution factor.

PERFORMANCE

Detection Range

The detection range is: 3.1 – 250 pmol/mL.

The calibration curve concentrations used for the ELISA's were 250, 83.3, 27.8, 3.9, 3.1 pmol/mL.

Sensitivity

The minimum detectable dose of cAMP is typically less than 1.2 pmol/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by the mean O.D. value of 20 replicates of the zero calibrator plus three standard deviations.

Specificity

This assay has high sensitivity and excellent specificity for detection of cAMP.

IMPORTANT NOTES

1. The final experimental results will be closely related to the operative skills of the end users and the experimental environments. Please make sure that sufficient samples are available.
2. This assay is designed to eliminate interference by soluble receptors, ligands, binding proteins, and other factors present in biological samples. However, until all factors have been tested the possibility of interference cannot be eliminated.
3. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
4. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be tightly closed to prevent the evaporation or contamination by microorganisms.
5. There may be some foggy substance in the plate wells when first opened. This will have no effect on the final assay results. Do not remove microtiter plate from the storage bag until needed.
6. A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. or greater at 450nm wavelength is acceptable for use in absorbance measurement.
7. The Stop Solution used with this kit is an acid. Wear eye, hand, face, and clothing protection when using this material.

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