User Manual

Calbiochem® cAMP Direct Immunoassay Kit, Colorimetric

100 Tests, 96-well format

116811

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for human or animal consumption.

Product Overview

The Calbiochem® cAMP Direct Immunoassay Kit, Colorimetric is a competitive immunoassay for the quantitative assay of cAMP in cell lysates, tissue extracts, and serum/plasma.

Principles of the Assay

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP) is one of the most important second messengers involved in regulating neuronal, glandular, cardiovascular, immune, and other physiological functions. A number of hormones are known to stimulate the production of cAMP through the action of adenylate cyclase that converts ATP to cAMP.

The Calbiochem® cAMP Direct Immunoassay Kit, Colorimetric uses a polyclonal antibody to cAMP that binds to cAMP in samples in a competitive manner. After a simultaneous incubation at room temperature, the excess reagents are washed away, and substrate is added. After a short incubation time, the reaction is stopped, and the yellow color generated is read on a microplate reader at 405 nm. The intensity of the color is inversely proportional to the concentration of cAMP in standards and samples.

Materials Provided

- Goat anti-Rabbit IgG 96-Well Plate (Kit Component No. KP16701)
  1 plate, 96-wells coated with goat anti-rabbit IgG, supplied as strip wells
- cAMP Direct Conjugate (Kit Component No. KP16702)
  Blue solution containing alkaline phosphatase conjugated to cAMP
- cAMP EIA Antibody (Kit Component No. KP16703)
  Yellow solution containing Anti-cAMP
- 0.1 M HCl (Kit Component No. KP16704)
  0.1 M hydrochloric acid. **Caution:** Wear suitable protective clothing.
- Neutralizing Reagent (Kit Component No. KP16705)
- Wash Buffer Concentrate (Kit Component No. KP16706)
  Tris Buffered Saline (TBS) containing detergents
- Cyclic AMP Standard (Kit Component No. KP16707)
  2,000 pmol/mL cAMP
- pNpp Substrate (Kit Component No. KP16708)
  p-nitrophenyl phosphate
- Stop Solution (Kit Component No. KP16709)
  Trisodium phosphate in water, keep tightly capped. **Caution:** Caustic.
- Triethylamine (Kit Component No. KP16710)
  Caution: Lachrymator, Harmful Vapor, Flammable.
- Acetic Anhydride (Kit Component No. KP16711)
  Caution: Lachrymator, Corrosive, Flammable.
- Plate Sealer (Kit Component No. KP16713)

Materials Required (Not provided)

- Deionized or distilled water
- Precision pipets for volumes between 5 µL and 1000 µL
- Repeater pipets for dispensing 50 µL and 200 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Adsorbent paper for blotting
- Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm

Warnings and Precautions

- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent any azide build-up.
- Some solutions supplied in this kit are caustic. Care should be taken with their use.
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (> 10 mM) such as EDTA and EGTA.
- The cyclic AMP Standard provided (Kit Component KP16707) is supplied in ethanolic buffer at a pH optimized to maintain cAMP integrity. Care should be taken in handling this material because of the known and unknown effects of cAMP.

Recomendations

- Do not mix components from different kit lots.
- Allow all reagents to warm up to room temperature for at least 30 min before opening.
- Standards can be made up in either glass or plastic tubes.
- Keep unused plate strips sealed in bag with desiccant.
- Pre-rinse the pipet tip with the reagent and use fresh pipet tips for each sample, standard and reagent.
- Pipet standards and samples to the bottom of the wells.
- Add the reagents to the side of the well to avoid contamination.
- This kit uses break-apart strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed foil bag. The wells should be used in the frame provided.
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any wash buffer remaining in the wells will cause variations in results.
- All standards and samples should be run in duplicate.
- Bring all reagents to room temperature for at least 30 min prior to opening.

Storage and Stability

Upon arrival store the cAMP Direct Conjugate and the Cyclic AMP Standard at -20 °C and the remaining components of the kit at 4 °C.

Protocol

Preparation Protocol

This kit is compatible with cAMP samples that have been treated with hydrochloric acid to stop endogenous phosphodiesterase activity. Samples in this matrix can be measured directly without evaporation or further treatment. If samples with very low levels of cAMP are to be measured, we have provided reagents to acetylate samples and standards. Please refer to application references for further methods of extraction of cAMP from samples.

Serum and Plasma

For serum samples, add ~ 10 µL concentrated hydrochloric acid per 1 mL of serum. The serum should be allowed to stand for 15 min at room temperature, and then centrifuged at 600 x *g* at room temperature. The supernatants can then be diluted in the 0.1 N HCl provided with the kit. In experiments with serum samples diluted greater than 1:2 in 0.1 N HCl, recoveries of cAMP of greater than 96% were observed. EDTA plasma is not a suitable matrix for the acetylated procedure since it tends to precipitate.

Tissue Extracts

Tissue samples frozen in liquid nitrogen should be ground to a fine powder under liquid nitrogen in a stainless-steel mortar. After the liquid nitrogen has evaporated, weigh the frozen tissue, and homogenize in 10 volumes of 0.1 N HCl. Centrifuge at 600 x *g* at room temperature. The samples can then be diluted in the 0.1 N HCl provided for the assay.

Cell Lysates

Cells grown in tissue culture medium can be treated with 0.1 N HCl after first removing the medium. Incubate for 10 min and visually inspect the cells to verify cell lysis. If adequate lysis has not occurred, incubate for an additional 10 min and re-inspect. Centrifuge at ≥ 600 x *g* at room temperature. Use the supernatant directly in the assay. Cyclic AMP in the medium can be measured after treating the supernatant with concentrated HCl as described above for plasma. Centrifuge at 600 x *g* at room temperature. The supernatants can then be used directly in the assay. In experiments with tissue culture media samples diluted greater than 1:2 in0.1 M HCl, recoveries of cAMP of 98% were seen.

Reagent Protocol

cAMP Standard-Non-Acetylated Version

1. Allow the 2,000 pmol/mL cAMP standard solution to warm to room temperature.
2. Label five 12 x 75 mm glass tubes #1 through #5.
3. Pipet 900 µL of 0.1 N HCl into tube #1 and 750 µL of 0.1 N HCl into tubes #2-5.
4. Add 100 µL of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly.
5. Add 250 µL of tube #1 to tube #2 and vortex thoroughly.
6. Continue this for tubes #3 through #5. The concentration of cAMP in tubes #1 through #5 will be 200, 50, 12.5, 3.12 and 0.78 pmol/mL respectively.

   Diluted standards should be used within 60 min of preparation.

Standard Dilutions (Non-Acetylated)

Std	0.1 N HCl Volume (µL)	Volume Added (µL)	cAMP Conc. (pmol/mL)
1	900	100, Stock	200
2	750	250, Std 1	50
3	750	250, Std 2	12.5
4	750	250, Std 3	3.12
5	750	250, Std 4	0.78

*Acetylation Reagent: Prepare the Acetylating Reagent by adding 0.5 mL of Acetic Anhydride to 1 mL of Triethylamine. Use the reagent within 60 min of preparation.

cAMP Standard-Acetylated Version

1. Allow the 2,000 pmol/mL cAMP standard solution to warm to room temperature.
2. Label five 12 x 75 mm glass tubes #1 through #5.
3. Pipet 990 µL of 0.1 M HCl into tube #1 and 750 µL of 0.1 M HCl into tubes #2-5.
4. Add 10 µL of the 2,000 pmol/mL standard to tube #1. Vortex thoroughly.
5. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 though #5.
   The concentration of cAMP in tubes #1 through #5 will be 20, 5, 1.25, 0.312, and 0.078 pmol/mL respectively.

Standard Dilutions (Acetylated)

Std	0.1 N HCl Volume (µL)	Volume Added (µL)	cAMP Conc. (pmol/mL)
1	990	10, Stock	20
2	750	250, Std 1	5
3	750	250, Std 2	1.25
4	750	250, Std 3	0.312
5	750	250, Std 4	0.078

1. Acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL of standard or sample.
2. Add the reagent directly to the samples and vortex for about 2 s.
3. Label one 12 x 17 mm glass tube as the Zero Standard/NSB tube.
4. Pipet 1 mL of 0.1 N HCl into this tube.
5. Add 50 µL of the Acetylating Reagent to the Zero Standard/NSB tube and use in Step 3 under Application Protocol below. Failure to acetylate the NSB and the Zero Standard will result in inaccurate B/B_0 values. Use the acetylated standards or samples within 30 min of preparation.

Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the concentrate supplied with 95 mL of deionized water. This can be stored at room temperature for 3 months.

Application Protocol

If the Acetylated version of the kit is to be run, acetylate all standards and samples by adding 10 µL of the Acetylating Reagent for each 200 µL of standard or sample. Add 50 µL of the Acetylating Reagent to the Zero Standard/NSB tube and use in Steps 3 and 6 below. (Failure to acetylate the NSB and Zero standard will result in inaccurate B/B_0 values.) Add the reagent directly to the samples and vortex for 2 s. Use the acetylated standards or samples within 30 min.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the Ziploc®. Store unused wells at 4 °C.

Direct cAMP Plate Layout

	A2 Std 1	A3 Std 5	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std 1	B3 Std 5	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 TA	C2 Std 2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 TA	D2 Std 2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 NSB	E2 Std 3	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 NSB	F2 Std 3	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2 Std 4	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2 Std 4	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Kit Lot No.		Exp. Date	Tech.
1st Incubation	Start Time	Temp.	Notes:
	End Time	Temp.	
2nd Incubation	Start Time	Temp.	
	End Time	Temp.	

2. Pipet 50 µL of the Neutralizing Reagent into each well, except the Total Activity (TA) and Blank wells.
3. Pipet 100 µL of 0.1 M HCl into the NSB and the B_0 (0 pmol/mL Standard) wells. When assaying acetylated samples add 100 µL 0.1 M acetylated HCl in place of nonacetylated 0.1 M HCl.
4. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.
5. Pipet 100 µL of the Samples into the appropriate wells.
6. Pipet 50 µL of 0.1 M HCl into the NSB wells. Use non-acetylated 0.1 M HCl for both acetylated or non-acetylated samples.
7. Pipet 50 µL of blue Conjugate into each well except the TA and Blank wells.
8. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

   Note: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.
9. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
10. Empty the contents of the wells and wash by adding 200 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 washes.
11. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
12. Add 5 µL of the blue Conjugate to the TA wells.
13. Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.
14. Add 50 µL of Stop Solution to each well. Read the plate immediately.
15. Blank the plate reader against the Blank wells, read the absorbance at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader cannot be set against the Blank wells, manually subtract the mean absorbance of the blank wells from all readings.

Protocol Summary

Assay Protocol Flow Chart

	Blank	**TA**	**NSB**	**Zero Std (B_0)**	**Stds.**	**Samples**
Well I.D.	**A1, B1**	**C1, D1**	**E1, F1**	**G1, H1**	**A2–B3**	**C3–H12**
Neutralizing Reagent	-	-	50 µL	50 µL	50 µL	50 µL
0.1 N HCl	-	-	150 µL	100 µL	-	-
Std and/or Sample	-	-	-	-	100 µL	100 µL
Conjugate	-	-	50 µL	50 µL	50 µL	50 µL
Antibody	-	-	-	50 µL	50 µL	50 µL
Incubate 2 hours at RT, shaking	→	→	→	→	→	→
Asp. And wash 3 x 200 µL	→	→	→	→	→	→
Conjugate	-	5 µL	-	-	-	-
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incubate 1 hour at RT	→	→	→	→	→	→
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

Data Analysis

Calculations

Several options are available for the calculation of the concentration of cAMP in the samples. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program such as "AssayZap™", sold by Biosoft® for Mac® or PC computers running Windows®. If this type of data reduction software is not readily available, the concentration of cAMP can be calculated as follows:

Calculate the average net absorbance (Abs) bound for each standard and sample by subtracting the average NSB Abs from the average Abs bound:

$$\text{Avg. Net Abs} = \text{Avg. Bound Abs} - \text{Avg. NSB Abs}$$

Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (B_0), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net Abs}}{\text{Net } B_0 \text{ Abs}} \text{ X } 100$$

Using logit-log paper, plot Percent Bound (B/B_0) versus concentration of cAMP for the standards. Approximate a straight line through the points. The concentration of cAMP in the unknowns can be determined by interpolation.

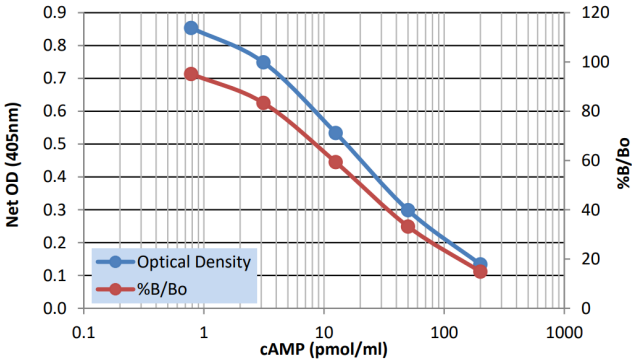
Assay Characteristics and Examples

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

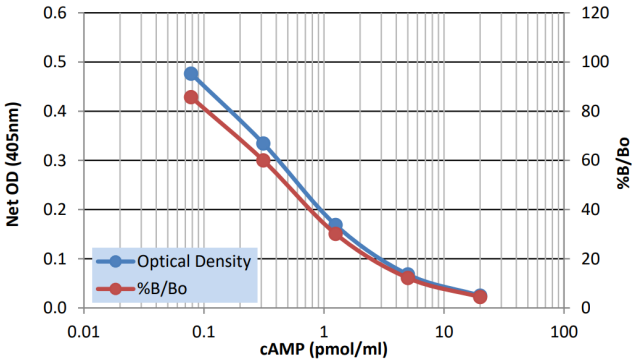
Standard curve

Typical standard curves are shown below. These curves must not be used to calculate cAMP concentrations; each user must run a standard curve for each plate and version used.

Non-acetylated assay format



Acetylated assay format



Product Performance

Example Data

The results shown below are for illustration only and should not be used to calculate results from another assay.

Typical Results

Non-Acetylated Version

Sample	Net Ab	Percent Bound	cAMP (pmol/mL)
Blank Ab	(0.072)	-	-
TA	1.563	-	-
NSB	0.001	0.06%	-
B_0	0.335	100%	0
S1	0.023	6.88%	200
S2	0.050	14.95%	50
S3	0.114	34.08%	12.5
S4	0.212	63.38%	3.125
S5	0.294	87.74%	0.781
Unknown 1	0.093	27.76%	17.89
Unknown 2	0.224	66.87%	2.64

Acetylated Version

Net Ab	Percent Bound	cAMP (pmol/mL)
(0.073)	-	-
1.691	-	-
0.000	0	-
0.257	100	0
0.029	11.31	20
0.064	24.95	5
0.129	50.29	1.25
0.206	80.12	0.312
0.252	98.05	0.078
0.076	29.57	3.73
0.210	81.71	0.27

Sensitivity

390 fmol/mL (non-acetylated), 37 fmol/mL (acetylated)

Sensitivity was calculated by determining the average absorbance bound for sixteen wells run as B_0 and comparing to the average absorbance for sixteen wells run with Standard #5. The detection limit was determined as the concentration of cAMP measured at two standard deviations from the zero along the standard curve.

Sensitivity Calculations

Non-Acetylated Version

Mean Abs for B_0 = 0.340 +/- 0.012 (3.4%)

Mean Abs for Standard #5 = 0.293 +/- 0.007 (2.5%)

Delta absorbance
(0-0.78 pmol/mL) = 0.340-0.293 = 0.047 2 SD's of B_0 = 0.024

$$\text{Sensitivity} = \frac{0.024}{0.048} \times 0.78 \text{ pmol/mL} = 0.39 \text{ pmol/mL}$$

Acetylated Version

Mean Abs for B_0 = 0.313 +/- 0.004 (1.24%).

Mean Abs for Standard #5 = 0.295 +/- 0.009 (2.9%)

Delta absorbance
(0-0.078 pmol/mL) = 0.313-0.295 = 0.017 2 SD's of B_0 = 0.008

$$\text{Sensitivity} = \frac{0.008}{0.017} \times 0.078 \text{ pmol/mL} = 0.39 \text{ pmol/mL}$$

Assay Range

0.78-200 pmol/mL (non-acetylated)

0.078-20 pmol/mL (acetylated)

Recovery

cAMP concentrations were measured in a variety of different samples including tissue culture media and serum. For all of the samples, cAMP was spiked into the undiluted samples that were diluted with the kit 0.1 N HCl and then assayed in the kit. Recovery values were not obtained with urine samples because the endogenous levels of cAMP are high. The following results were obtained:

Sample Recoveries

	Non-Acetylated Version		Acetylated Version	
Sample	**% Recovery**	**Recommended Dilution***	**% Recovery**	**Recommended Dilution***
Tissue Culture Media	85	1:5–1:20		**
Porcine Serum	96	1:10–1:20	102	> 1:50

* See Sample Preparation for details

**RPMI-1640 contains ~ 350 pmol/mL cAMP

Linearity

Non-Acetylated Version A sample containing 15.44 pmol/mL cAMP was serially diluted 4 times 1:2 in the 0.1 N HCl supplied in the kit and measured in the Correlate-EIA assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 0.98 with a correlation coefficient of 0.988. Acetylated Version A sample containing 3.41 pmol/mL cAMP was serially diluted 4 times 1:2 in the 0.1 N HCl supplied in the kit and measured in the Acetylated version of the Correlate-EIA assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration. The line obtained had a slope of 1.226 with a correlation coefficient of 0.999.

Specificity

Cross Reactivities

The cross-reactivities for a number of related compounds were determined. Potential cross-reactants were at concentrations from 2,000 to 2 pmol/mL. These samples were then measured in the cAMP assay, and the measured cAMP concentration at 50% B/B_0 calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Cross Reactivities

Compound	Cross-Reactivity
cAMP	100%
AMP	0.33%
ATP	0.12
cGMP	< 0.001%
GMP	< 0.001%
GTP	< 0.001%
cUMP	< 0.001%
CTP	< 0.001%

Protocol Summary

Assay Protocol Flow Chart

	Blank	TA	NSB	Zero Std (B_0)	Stds.	Samples
Well I.D.	**A1, B1**	**C1, D1**	**E1, F1**	**G1, H1**	**A2–B3**	**C3–H12**
Neutralizing Reagent	-	-	50 µL	50 µL	50 µL	50 µL
0.1 N HCl	-	-	150 µL	100 µL	-	-
Std and/or Sample	-	-	-	-	100 µL	100 µL
Conjugate	-	-	50 µL	50 µL	50 µL	50 µL
Antibody	-	-	-	50 µL	50 µL	50 µL
Incubate 2 hours at RT, shaking	→	→	→	→	→	→
Asp. And wash 3 x 200 µL	→	→	→	→	→	→
Conjugate	-	5 µL	-	-	-	-
Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL
Incubate 1 hour at RT	→	→	→	→	→	→
Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

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