Direct Cyclic AMP

Enzyme Immunoassay Kit

User Manual

Catalog # K3019-C **1 Plate Kit**
K3019-C5 **5 Plate Kit**

TABLE OF CONTENTS

Notice to Purchaser

This product is to be used for Research Purposes Only. It is not to be used for Drug or Diagnostic Purposes, nor is it intended for Human Use. B-Bridge products may not be resold, modified for resale, or used to manufacture commercial products without the express written consent of B-Bridge International, Inc.

EXCEPT AS OTHERWISE EXPRESSLY SET FORTH IN THIS USER MANUAL, B-BRIDGE DOES NOT MAKE ANY REPRESENTATION OR WARRANTIES OR CONDITIONS OF ANY KIND, EITHER EXPRESSED OR IMPLIED, WITH RESPECT TO THE PRODUCTS, OR INFORMATION DISCLOSED HEREUNDER, INCLUDING, BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY, FIT FOR A PARTICULAR PURPOSE, OR NONINFRINGEMENT OF THE INTELLECTUAL PROPERTY RIGHTS OF THIRD PARTIES.

INTENDED USE

The B-Bridge Direct Cyclic AMP Enzyme Immunoassay Kit is species independent and designed to quantitatively measure cAMP present in lysed cells, EDTA and heparin plasma, urine, saliva and tissue culture media samples. Please read the complete kit insert before performing this assay.

BACKGROUND

Adenosine-3',5'-cyclic monophosphate, or cyclic AMP (cAMP), $C_{10}H_{12}N_5O_6P$, is one of the most important second messengers and a key intracellular regulator. Discovered by Sutherland and Rall in 19571, it functions as a mediator of activity for a number of hormones, including epinephrine, glucagon, and ACTH. Adenylate cyclase is activated by the hormones glucagon and adrenaline and by G protein. Liver adenylate cyclase responds more strongly to glucagon, and muscle adenylate cyclase responds more strongly to adrenaline. cAMP decomposition into AMP is catalyzed by the enzyme phosphodiesterase. In the Human Metabolome Database there are 166 metabolic enzymes listed that convert cAMP.

Other biological actions of cAMP include regulation of innate immune functioning, axon regeneration, cancer, and inflammation.

ASSAY PRINCIPLE

The B-Bridge Direct Cyclic AMP (cAMP) Enzyme Immunoassay kit is designed to quantitatively measure cAMP present in lysed cells, EDTA and heparin plasma, urine, saliva and tissue culture media samples.

For tissue samples, saliva and urine, where the levels of cAMP are expected to be relatively high, the regular format for the assay can be used. For plasma samples and some dilute cell lysates an optional acetylation protocol can be used.

The kit is unique in that all samples and standards are diluted into an acidic Sample Diluent, which contains special additives and stabilizers. This allows plasma, urine, and saliva samples to be read in an identical manner to lysed cells. Acidified samples of cAMP are stable and endogenous phosphodiesterases are inactivated in the Sample Diluent.

1. Plate primer added to microtiter plate wells followed by sample or standard (either with or without acetylation).
2. The reaction is initiated with the addition of the cAMP-peroxidase conjugate and the binding reaction is initiated by the additional of a sheep antibody to cAMP in each well.
3. After a 2 hour incubation the plate is washed and the substrate is added.
4. The reaction is stopped and a plate reader measuring 450 nm wavelength is used to detect the generated color.
5. The concentration of cAMP is calculated.

KIT COMPONENTS

Conponent:	Cat #	K3019-C	K3019-C5
Coated white 96-well plate		1 plate	5 plates
Cyclic AMP Standard (1,500 pmol/mL)		125 µl	625 µl
Cyclic AMP Antibody		3 ml	13 ml
Cyclic AMP Conjugate		3 ml	13 ml
Sample Diluent Concentrate		12 ml	60 ml
Plate Primer		25 ml	25 ml
Acetic Anhydride **WARNING: Corrosive Lachrymator**		2 ml	2 ml
Triethylamine **WARNING: Corrosive Lachrymator**		4 ml	4 ml
20X Wash Buffer Concentrate		30 ml	125 ml
TMB Substrate		11 ml	55 ml
Stop Solution		5 ml	25 ml
Plate Sealer		1 each	5 each

Store all components at 4°C

MATERIALS REQUIRED BUT NOT SUPPLIED

- Deionized or distilled water
- Repeater pipet with disposable tips capable of dispensing 25 uL, 50 uL and 100 uL.
- Microplate Shaker
- Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.
- Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturer's Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer.

The supplied Sample Diluent and Sample Diluent Concentrate are acidic. The Stop Solution is 1M HCl. These solutions should not come in contact with skin or eyes. Take appropriate precautions when handling these reagents.

The kit uses acetic anhydride and triethylamine as acetylation reagents. Triethylamine and acetic anhydride are lachrymators. **Caution - corrosive, flammable, and harmful vapor. Use in hood with proper ventilation and wear appropriate protective safety wear.**

INTERFERENTS

A variety of detergents were tested as possible interfering substances in the assay. CHAPS, and Tween 20 at 0.1% increased measured cAMP by 8.9 and decreased measured cAMP by 0.9% respectively. Triton X-100 at 2% increased measured cAMP by 1.8% and CTAC at 0.05% increased measured cAMP by 6.3%. Samples containing SDS above 0.01% should not be used in the assay.

SAMPLE PREPARATION

This assay has been validated for saliva, urine, serum, EDTA and heparin plasma samples and for tissue culture media samples. Samples should be stored at -70°C for long term storage. 24-Hour urine samples may need to have 1 mL concentrated hydrochloric acid added for every 100 mL volume to act as a preservative. Samples containing visible particulate should be centrifuged prior to using.

Cyclic AMP is identical across all species and we expect this kit may measure cAMP from sources other than human. The end user should evaluate recoveries of cAMP in other samples being tested.

After dilution in the Sample Diluent there may be some precipitation of proteins and the supernatant from the centrifuged samples used. After being diluted in Sample Diluent the samples can be assayed directly within 2 hours, or frozen at ≤ -70°C for later analysis. Severely hemolyzed samples should not be used in this kit.

For samples containing low levels of cAMP and for all plasma samples, the acetylated assay protocol must be used due to its enhanced sensitivity.

All standards and samples should be diluted in glass test tubes.

Cells
Cell lysis buffers containing high concentrations of SDS or other detergents may not be compatible with this assay or may require extra dilution. Please read the Interferents section above for more information.

This kit is compatible with either adherent or non-adherent cells. The cells can be grown in any suitable sterile containers such as Petri dishes, 12-, 48- or 96-well culture plates or flasks. The cells must be isolated from the media prior to being lysed with the provided Sample Diluent. The acidic Sample Diluent contains detergents to lyse the cells, inactivate endogenous phosphodiesterases and stabilize the cAMP. Some cell types are extremely hardy and the end user should optimize the lysis conditions utilizing freeze-thaw cycles and ultrasonic treatments to fully lyse their cells.

For adherent cells, the media should be aspirated from the cells and the cells washed with PBS. The adherent cells should be treated directly with the Sample Diluent for 10 minutes at room temperature. Cells can be scraped to dislodge them from the plate surface and cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥ 600 x g at 4°C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cAMP as outlined below.

For non-adherent cells, pellet and wash the cells with PBS by centrifuging the samples at ≥ 600 x g at 4°C for 15 minutes as described above. Treat the aspirated, washed pellet directly with the Sample Diluent for 10 minutes at room temperature. Cells should be inspected to ensure lysis. Detergent has been added to the Sample Diluent to help lysis occur. Centrifuge the samples at ≥ 600 x g at 4 °C for 15 minutes and assay the supernatant directly. If required, the TCM can be assayed for cAMP as outlined below.

Tissue Samples
Tissues samples should be frozen in liquid nitrogen and stored at -80°C if analysis is not to be carried out immediately.

Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of Sample Diluent for every 100 mg of tissue. Incubate in the Sample Diluent for 10 minutes on ice, and then centrifuge at ≥600 x g at 4°C for 15 minutes. Collect the supernatant and run in the assay immediately or store frozen at ≤ -70°C.

For samples that require concentration and delipidation, a trichloroacetic acid (TCA)/ether protocol can be used. Grind the frozen tissue in a stainless steel mortar under liquid nitrogen until it is a fine powder. Allow the liquid nitrogen to evaporate and weigh the powdered tissue. Add 1 mL of ice cold 5% TCA (weight/volume) for every 100 mg of tissue and grind in a glass-Teflon mortar. Incubate in the TCA for 10 minutes on ice, and then centrifuge at ≥600 x g at 4°C for 15 minutes. Collect the supernatant.

For every 1 mL of TCA supernatant add 3 mL of water saturated diethyl ether* and shake in a glass vial. Allow the ether to separate as the top layer, remove it and discard the ether. Dry the aqueous layer by lyophilization or using a vacuum centrifuge. Reconstitute by adding 1 mL of Sample Diluent for every mL of 5% TCA used to extract and run in the assay immediately or store at ≤ -70°C.
Diethyl ether is extremely flammable and should be used in a hood.

Tissue Culture Media
For measuring cAMP in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM.

Plasma Samples
Plasma samples should be diluted ≥ 1:10 with the supplied Sample Diluent and acetylated prior to running in the Acetylated Format assay

Urine Samples
Urine samples should be diluted ≥ 1:20 with the supplied Sample Diluent prior running in the assay. Due to the high concentration of cAMP in urine, samples may need to be diluted further.

Saliva Samples
Saliva samples should be diluted ≥ 1:4 with the supplied Sample Diluent prior running in the assay..

Use all samples within 2 hours of dilution in Sample Diluent.

REGULAR FORMAT ASSAY

Use this format for urine, saliva and some cell lysates. Do NOT use for plasma samples.

REGULAR FORMAT REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30-60 minutes.

We recommend that all standards and samples be run in for accurate determination of cAMP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Standard Preparation
All standards and samples should be diluted in glass test tubes

1. Label six test tubes as #1 through #6.
2. Pipet 270 uL of Sample Diluent into tube #1 and 200 ul into tubes #2 through #6.
3. Carefully add 30 ul of the cAMP stock solution to tube #1 and vortex completely.
4. Take 100 uL of the cAMP solution in tube #1 and add it to tube #2 and vortex completely.
5. Repeat the serial dilutions for tubes #3 through #5. The concentration of Cyclic AMP in tubes #1 through #6 will be 150, 50, 16.67, 5.56, 1.85, and 0.617 pmol/mL.

Use all Standards within 1 hour of preparation.

Reagent	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
Sample Diluent (ul)	270	200	200	200	200	200
cAMP Standard (ul)	30	-	-	-	-	-
Standard 1 (ul)	-	100	-	-	-	-
Standard 2(ul)	-	-	100	-	-	-
Standard 3(ul)	-	-	-	100	-	-
Standard 4(ul)	-	-	-	-	100	-
Standard 5(ul)	-	-	-	-	-	100
Final Concentration (pM/mL)	150	50	16.67	5.56	1.85	0.617

REGULAR FORMAT ASSAY PROTOCOL

1. Add 25 uL of Plate Primer into all wells used.
   Failure to Add Plate Primer to <u>ALL</u> Wells First Will Cause Assay To Fail.

2. Pipet 75 uL Sample Diluent into the non-specific binding (NSB) wells.

3. Pipet 50 uL of Sample Diluent into wells to act as maximum binding wells (B0 or 0 pg/mL).

4. Pipet 50 uL of samples or standards into wells in the plate.

NOTE: Sample Diluent will turn from orange to bright pink upon sample or standard addition to the Plate Primer in the wells.

5. Add 25 uL of the cAMP Conjugate to each well using a repeater pipet.

6. Add 25 uL of the cAMP Antibody to each well, **except the NSB wells**, using a repeater pipet.

7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 2 hours. If the plate is not shaken, signals bound will be approximately 25% lower.

8. Aspirate the plate and wash 4 times with 300 ul wash buffer. Tap the plate dry on absorbent towels.

9. Add 100 uL of the TMB Substrate to each well, using a repeater pipet.

10. Incubate the plate at room temperature for 30 minutes without shaking.

11. Add 50 ul of the Stop Solution to each well using a repeater pipet.

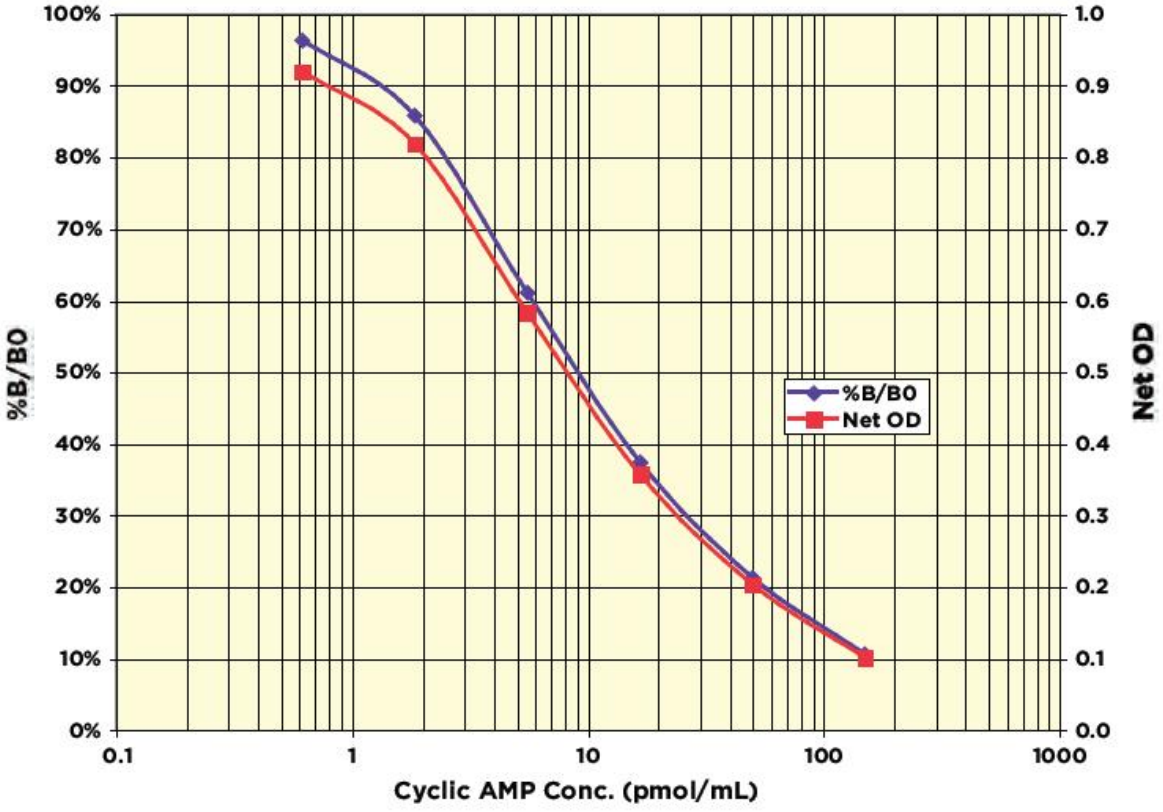
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

13. Use the plate reader's built-in 4PLC software capabilities to calculate cAMP concentration for each sample.

CALCULATIONS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

REGULAR FORMAT TYPICAL STANDARD CURVE: EXAMPLE



Always run your own standard curve for calculation of results.
Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection
Sensitivity was calculated by comparing the OD's for nineteen wells run for each of the B0 and standard #6. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.
Sensitivity was determined as 0.64 pmol/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration human urine sample.
Limit of Detection was determined as 0.20 pmol/mL

ACETYLATED ASSAY

This format must be used for plasma, some cell lysates and any sample with low cAMP concentrations.

Prior to running the acetylated assay, all standards, samples and the Sample Diluent used for the B0 and NSB wells must be acetylated. Acetylation is carried out by adding 10 uL of the Acetylation Reagent (as prepared below) for each 200 uL of the standard, sample and Sample Diluent. After addition of the Acetylation Reagent immediately vortex each treated standard, sample or Sample Diluent and **use within 30 minutes of preparation**.

Note: Upon Acetylation, all of the standards and samples diluted in the orange Sample Diluent will change to a pale yellow color.

ACETYLATED ASSAY REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30-60 minutes.

We recommend that all standards and samples be run in for accurate determination of cAMP concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Wash Buffer
Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at room temperature for 3 months.

Acetylation Reagent
Working in a fume hood mix one part of Acetic Anhydride with 2 parts of Triethylamine in a glass test tube. Use the following table to help determine the amount of Acetylation Reagent to make.
Use the Acetylation Reagent within 1 hour of preparation.

	Number of Samples to be Tested		
Reagent	**20**	**40**	**100**
Acetic Anhydride Volume (uL)	200	400	1,000
Triethylamine Volume (uL)	400	800	2,000
Acetylation Reagent Volume (mL)	0.6	1.2	3

Standard Preparation
All standards and samples should be diluted in glass test tubes.

1. Label eight glass test tubes as #1 through #7. Label one tube as Working Stock.
2. Pipet 270 ul of Sample Diluent into the Working Stock tube.
3. Pipet 560 uL of Sample Diluent into tube #1 and 300 into tubes #2 through #7.
4. **The Cyclic AMP stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 30 uL of the cAMP stock solution to the Working Stock tube and vortex completely.
5. Carefully add 40 uL of the Working Stock tube to tube #1 and vortex completely.
6. Take 300 uL of the cAMP solution in tube #1 and add it to tube #2 and vortex completely.
7. Repeat the serial dilutions for tubes #3 through #8. The concentration of Cyclic AMP in tubes #1 through #7 will be 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 pmol/mL.

Reagent	Working Stock	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
Sample Diluent (ul)	**270**	560	300	300	300	300	300	300
cAMP Standard (ul)	**30**	-	-	-	-	-	-	-
Working Stock (ul)	**-**	40	-	-	-	-	-	-
Standard 1 (ul)	**-**	-	300	-	-	-	-	-
Standard 2 (ul)	**-**	-	-	300	-	-	-	-
Standard 3 (ul)	**-**	-	-	-	300	-	-	-
Standard 4 (ul)	**-**	-	-	-	-	300	-	-
Standard 5 (ul)	**-**	-	-	-	-	-	300	-
Standard 6 (ul)	**-**	-	-	-	-	-	-	300
Final Concentration (pM/mL)	**150**	10	5	2.5	1.25	0.625	0.313	0.156

STANDARD AND SAMPLE ACETYLATION

1. Pipet 300 uL of Sample Diluent into a glass tube to act as the Zero standard/NSB tube.
2. Add 15 uL of Acetylation Reagent to this tube and vortex immediately. Proceed to assay within 30 minutes.
3. Pipet 200 uL of each standard and sample to be tested into glass tubes.
4. Add 10 uL of the Acetylation Reagent into each tube and vortex immediately. Proceed to assay within 30 minutes.

NOTE: Samples and Sample Diluent will turn from orange to pale yellow upon acetylation.
Use Acetylated Standards and Samples within 30 minutes of preparation

ACETYLATED ASSSAY PROTOCOL

1. Add 50 uL of Plate Primer into all wells used. **Failure To Add Plate Primer To ALL Wells First Will Cause Assay To Fail.**

2. Pipet 75 uL acetylated Sample Diluent into the non-specific binding (NSB) wells.

3. Pipet 50 uL of acetylated Sample Diluent into wells to act as maximum binding wells (B0 or 0 pg/mL).

4. Pipet 50 uL of acetylated samples or standards into wells in the plate.

5. Add 25 uL of the cAMP Conjugate to each well using a repeater pipet.

6. Add 25 uL of the cAMP Antibody to each well, **except the NSB wells**, using a repeater pipet.

7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 2 hours. If the plate is not shaken, signals bound will be approximately 25% lower.
Note: Wells will turn from very pale yellow to pale pink during incubation.

8. Aspirate the plate and wash 4 times with 300 ul wash buffer. Tap the plate dry on absorbent towels.

9. Add 100 uL of the TMB Substrate to each well, using a repeater pipet.

10. Incubate the plate at room temperature for 30 minutes without shaking.

11. Add 50 ul of the Stop Solution to each well using a repeater pipet.

12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

13. Use the plate reader's built-in 4PLC software capabilities to calculate cAMP concentration for each sample.

CALCULATIONS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

ACETYLATED FORMAT TYPICAL STANDARD CURVE: EXAMPLE

Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection - Acetylated
Sensitivity was calculated by comparing the OD's for nineteen wells run for each of the acetylated B0 and standard #6. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve.
Sensitivity was determined as 0.083 pmol/mL.

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of acetylated zero standard and a low concentration acetylated human sample.
Limit of Detection was determined as 0.078 pmol/mL. This is equivalent to 3.9 fmol cAMP per sample.