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CERTIFICATE OF ANALYSIS

Cyclic AMP Competitive ELISA
Lysate and Homogenate Samples

Ordering Code: EMSCAMPL
Lot Number: OB182458
Kit Storage: Store the cAMP-AP Conjugate and the cAMP Standard at -20ºC. Store the remaining components at 2-8ºC.
Expiration Date: 01/31/2014

Product: A competitive immunoassay for the quantitative determination of cyclic AMP (cAMP) in samples treated with 0.1 M HCl. The assay is based on the competition between cAMP in the standard or sample and Alkaline Phosphatase conjugated cAMP (cAMP-AP) for a limited amount of cAMP monoclonal antibody bound to an Anti-Rabbit IgG precoated 96-well plate. As the concentration of cAMP in the sample increases, the amount of cAMP-AP captured by the coating antibody decreases. Thus, there is an inverse relationship between optical density (OD) and the amount of analyte in the sample.

cAMP COMPETITIVE ELISA KIT COMPONENTS - LYSATE AND HOMOGENATE SAMPLES

***Allow all Kit Components to come to room temperature for at least 30 minutes prior to opening.*

Description	Size	Form	Component Usage	Lot
Anti-Rabbit IgG Plate	1 plate	96-well strip plate coated with antibody specific to rabbit IgG.	§ Unused wells must be kept desiccated at 2 - 8°C in the sealed foil bag. § Use wells in the frame provided.	OB182458A
cAMP Antibody	5 ml	A yellow solution of a rabbit polyclonal antibody to cAMP.	Ready to Use.	OB182458K
cAMP-AP Conjugate	5 ml	A blue solution of cAMP conjugated to alkaline phosphatase.	Ready to Use.	OB182458C
0.1 M HCl	30 ml	0.1 M hydrochloric acid in water.	Ready to Use. CAUTION: Acid, wear suitable protective clothing.	OB182458H
Neutralizing Reagent	6 ml	A clear, colorless solution	Ready to Use.	OB182458B
Triethylamine	2 ml	A solution of triethylamine.	Ready to Use. Caution: Lachrymator, Harmful Vapor, Flammable	OB182458I
Acetic Anhydride	1 ml	A solution of acetic anhydride.	Ready to Use. Caution: Lachrymator, Corrosive, Flammable.	OB182458J
cAMP Standard	0.5 ml	A solution of 2000 pmol/ml cAMP.	**Allow the standard to come to room temperature.** Follow procedures for either Acetylated or Non-Acetylated detailed below.	OB182458D
10X Wash Buffer	30 ml	Tris buffered saline containing detergents and a preservative.	§ Dilute 10 ml of the 10X Wash Buffer with 90 ml of deionized water. Mix well. § 1X Wash Buffer can be stored for up to 3 months at room temperature.	OB182458E
p-NPP Substrate Solution	20 ml	A solution of p-nitrophenyl phosphate in buffer.	Ready to Use.	OB182458F
Stop Solution	6 ml	A solution of trisodium phosphate in water.	Ready to Use. Keep tightly capped. Caution: Caustic	OB182458G
Plate Sealer	1 each		Ready to Use.	

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cAMP COMPETITIVE ELISA KIT - LYSATE AND HOMOGENATE SAMPLES PROCEDURAL NOTES

- Materials needed but not supplied with the kit include: deionized or distilled water; precision pipets for volumes between 5 µl and 1,000 µl; repeat pipets for dispensing 50 µl and 200 µl; glass or polypropylene 12 x 75 mm tubes; disposable beaker for diluting buffer concentrates; graduated cylinders; a microplate shaker; absorbent paper for blotting; and a microplate reader capable of reading at 405 nm, preferable with correction between 570 and 590 nm.
- Do not mix components from different kit lots or use reagents beyond the kit expiration date.
- Care must be taken to minimize contamination by endogenous alkaline phosphatase (AP) which 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 to prevent variation in assay results.

STANDARDS AND ACETYLATING REAGENT

Non-Acetylated Standards

§ Label five 12 x 75 mm glass or polypropylene tubes 1 - 5.
§ Pipet 900 µl of 0.1 M HCl into tube 1.
§ Pipet 750 µl of 0.1 M HCl into tubes 2 - 5.
§ Add 100 µl of stock cAMP standard to tube 1 and vortex.
§ Add 250 µl of tube #1 to tube #2 and vortex. Continue this for tubes 3 - 5.
§ The concentration of cAMP in tubes 1 - 5 will be 200, 50, 12.5, 3.12, and 0.78, pmol/ml, respectively.
§ Use diluted standards within 60 minutes.

Acetylating Reagent Preparation

§ Add 0.5 ml of acetic anhydride to 1 ml triethylamine and mix well.
§ Use within 60 minutes of preparation.

Acetylated Standards

§ Label five 12 x 75 mm glass or polypropylene tubes 1 - 5.
§ Pipet 990 µl of 0.1 M HCl into tube 1.
§ Pipet 750 µl of 0.1 M HCl into tubes 2 - 5.
§ Add 10 µl of stock cAMP standard to tube 1 and vortex.
§ Add 250 µl from tube #1 to tube #2 and vortex. Continue this for tubes 3 - 5.
§ The concentration of cAMP in tubes 1 - 5 will be 20, 5, 1.25, 0.312, and 0.078 pmol/ml, respectively.
§ Acetylate all of the Acetylated Version standards and samples by adding 10 µl of Acetylating Reagent to each 200 µl of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds.
§ Label one 12 x 17 mm glass tube as the Zero Standard/NSB tube. Pipet 1 ml 0.1 M HCl into this tube. Add 50 µl of the Acetylating Reagent to the Zero Standard/NSB tube. NOTE: Failure to acetylate the NSB and Zero standard will result in inaccurate B/B_0 values.
§ Use the Acetylated Standards or samples within 30 minutes.

SAMPLE HANDLING

The cAMP Competitive ELISA for lysate and homogenate samples 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. Reagents to acetylate samples and standards for samples with very low levels of cAMP are provided.

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 M HCl. Centrifuge at 600 x g at room temperature for 10 minutes. The samples can then be diluted in the 0.1 M HCl provided for the assay.

Cells grown in tissue culture media can be treated with 0.1 M HCl after first removing the media. Incubate for 10 minutes and visually inspect the cells to verify cell lysis. If adequate lysis has not occurred incubate for another 10 minutes and inspect. Centrifuge at ≥ 600 x g at room temperature for 10 minutes, then use the supernatant directly in the assay. Cell or tissue lysis can be enhanced by adding 0.1% - 1% Triton X-100 to the 0.1 M HCl prior to use. When used in this concentration range, the detergent will not interfere with acetylation or the binding portion of the assay, however, there will be a modest increase in the optical density. Samples containing Triton should be evaluated against a standard curve diluted in the same for the

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most accurate determination. Cyclic AMP in media can be measured by treating 1 ml of supernatant media with 10 µl concentrated HCl. Centrifuge at 600 x g at room temperature for 10 minutes. The supernatants can then be used directly in the assay. Note: RPMI-1640 media contains high concentrations (>350 pmol/ml) of endogenous cAMP and residual media on the cells will effect the measurement of intracellular cAMP levels.

cAMP COMPETITIVE ELISA - LYSATE AND HOMOGENATE SAMPLES ASSAY PROCEDURE

All standards and samples should be run in duplicate. If acetylated version of the kit is to be run, acetylate both the standards and samples by adding 10 µl of the Acetylating Reagent to each 200 µl of standard or sample.

1. Pipet 50 µl of the Neutralizing Reagent into each well, **except** the TA and Blank wells.
2. Pipet 100 µl of 0.1 M HCl into the NSB and B_0 (0 pmol/ml Standard) wells.
3. Pipet 100 µl of Standards into the appropriate wells.
4. Pipet 100 µl of the Samples into the appropriate wells.
5. Pipet 50 µl of 0.1 M HCl into the NSB wells.
6. Pipet 50 µl of the blue cAMP-AP conjugate into each well, **except** the Total Activity (TA) wells and the blank.
7. Pipet 50 µl of the yellow cAMP 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.

8. Incubate the plate at room temperature (22 - 25°C) on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 200 µl of the 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes.
10. 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.
11. Add 5 µl of the blue cAMP-AP conjugate to the TA wells.
12. Add 200 µl of the p-NPP Substrate Solution to every well. Incubate at room temperature (22 - 25°C) for 45 minutes without shaking.
13. Add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

cAMP Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Std 1	Std 1									
B	Blk	Std 2	Std 2									
C	TA	Std 3	Std 3									
D	TA	Std 4	Std 4									
E	NSB	Std 5	Std 5									
F	NSB											
G	B_0											
H	B_0											

Definition of Key Terms

Total Activity (TA): total enzymatic activity of the cAMP-AP.

NSB (Non-Specific Binding): non-immunological binding of the cAMP-AP in the well.

B_0 (Maximum Binding): maximum amount of the cAMP-AP that the antibody can bind in the absence of free cAMP.

%B/B_0 (% Bound/Maximum Bound): ratio of the absorb ance of a particular sample or standard well to that of the maximum binding (B_0) well.

Average Net OD = Average Bound OD – Average NSB OD

Percent Bound = $\frac{\text{Net OD}}{\text{Net } B_0 \text{ OD}} \times 100$

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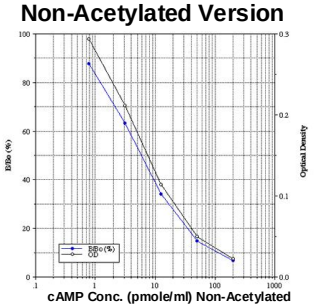
CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of cAMP in the sample. It is recommended that the data be analyzed by a weighted 4 parameter logistic curve fitting program. If data reduction software is not available, the concentration of the cAMP can be calculated. Calculate the average Net OD bound for each standard and sample. Calculate the Percent Bound of each pair of standard wells as a percentage of the maximum binding wells (B_0). 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 samples can be determined by interpolation.

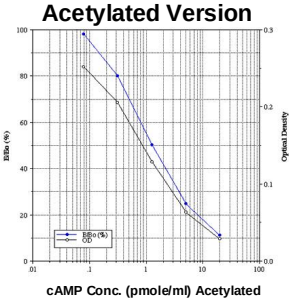
Typical Standard Curve

Typical standard curves are shown. This curve must not be used to calculate cAMP concentrations; a standard curve must be run with every assay.

Non-Acetylated Version



Acetylated Version



PERFORMANCE CHARACTERISTICS

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

Linearity

Non-Acetylated Version: A sample containing 15.44 pmol/ml cAMP was diluted 4 times 1:2 in 0.1 M HCl and measured in the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration.

The line obtained had a slope of 0.98 and a correlation coefficient of 0.988.

Acetylated Version: A sample containing 3.41 pmol/ml cAMP was serially diluted 4 times 1:2 in 0.1 M HCl and measured in the Acetylated Version of the assay. The data was plotted graphically as actual cAMP concentration versus measured cAMP concentration.

The line obtained had a slope of 1.226 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of cAMP and running these samples multiple times (n ≥24) in the same assay. Inter-assay precision was determined by measuring 3 samples of low, medium and high concentrations in multiple assays (n ≥8). The precision numbers listed below represent the percent coefficient of variation for the concentrations of cAMP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	Non-Acetylated Version		Acetylated Version-2 Hour	
Intra-assay	**cAMP pmol/ml**	**%CV**	**cAMP pmol/ml**	**%CV**
Low	1.24	8.9	0.679	4.6
Medium	6.31	4.3	3.58	8.4
High	35.29	8.3	-	-
Inter-assay	**cAMP pmol/ml**	**%CV**	**cAMP pmol/ml**	**%CV**
Low	1.18	13.1	1.29	13.6
Medium	5.52	4.2	5.62	7.8
High	30.36	11.6	-	-

Sensitivity

Sensitivity was calculated by determining the average OD for 16 wells run as B_0, and comparing to the average OD for 16 wells run with Standard #5 in the Non-Acetylated or with Standard #5 with the Acetylated version. The detection limit was determined as the concentration of cAMP measured at 2 standard deviations from the 0 along the standard curve.

Non-Acetylated Version Sensitivity	0.39 fmol/ml
Acetylated Version Sensitivity	0.037 fmol/ml

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CALCULATION OF RESULTS

Cross-Reactivities

The cross-reactivities for a number of related compounds were determined by dissolving the cross reactant in Reagent Diluent at concentrations from 500,000 to 500 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.

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

Sample Recoveries

cAMP concentrations were measured in tissue culture media. cAMP was spiked into the undiluted sample which was diluted with the kit 0.1 M HCl and then assayed in the kit.

Sample	% Recovery	Suggested Dilution	% Recovery	Suggested Dilution
	Non-Acetylated Version		Acetylated Version	
Tissue Culture Media	85	1:5 – 1:20		*

*RPMI-1640 contains ≈350 pmol/ml cAMP.

Quality Control Signature

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