Product Manual

Direct cAMP ELISA Kit

Catalog #: ADI-900-066A

1 x 96 well assay

For the latest product information, including support documentation, visit us online:
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Please read entire booklet before proceeding with the assay.

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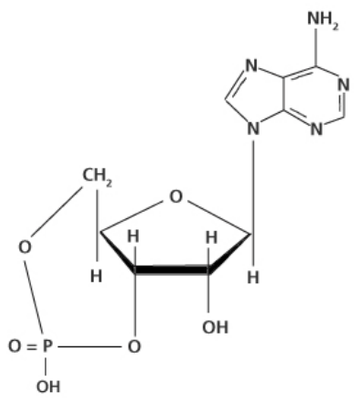
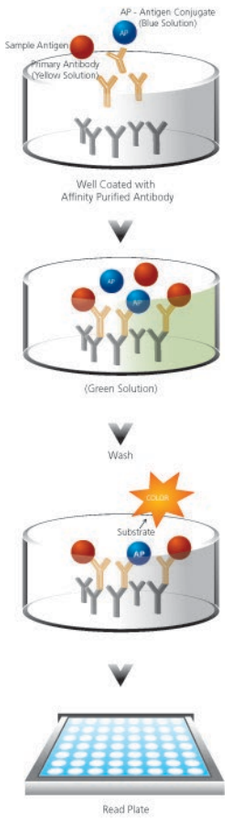
INTRODUCTION

The Direct cyclic AMP Enzyme-Linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of cyclic AMP in cell and tissue lysates treated with HCl. The optional acetylated assay format provides a significant increase in sensitivity and is ideal for samples with extremely low levels of cAMP. If expected levels of cAMP are unknown, the investigator may evaluate a few samples in the non-acetylated format in order to determine if higher sensitivity is required.

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important "second messengers" involved as a modulator of physiological processes [5]. cAMP is also involved in regulating neuronal, glandular, cardiovascular, immune and other functions [6-9]. A number of hormones are known to activate cAMP through the action of the enzyme adenylate cyclase which converts ATP to cAMP. These hormones include a variety of anterior pituitary peptide hormones such as corticotropin (ACTH), glucagon, calcitonin, thyroid stimulating hormone (TSH), and luteinizing hormone (LH). Because cAMP has been shown to be involved in the cardiovascular and nervous systems, immune mechanisms, cell growth and differentiation, and general metabolism[10-12], there remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures. The investigation of cAMP may help to provide a clearer understanding of the physiology and pathology of many disease states.

cyclic AMP





PRINCIPLE

1. Standards and samples are added to wells coated with GxR IgG antibody. A blue solution of cAMP conjugated to alkaline phosphatase is then added, followed by a yellow solution of rabbit polyclonal antibody to cAMP.
2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the cAMP in the sample or conjugate. The plate is washed, leaving only bound cAMP.
3. pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the cAMP conjugate.
4. Stop solution is added. The yellow color is read at 405nm. The amount of signal is indirectly proportional to the amount of cAMP in the sample.

MATERIALS SUPPLIED

HCl is caustic. Use caution and keep tightly capped.

1. **0.1M HCl, 27mL**
   Component Number 80-0080
   0.1M hydrochloric acid in water.

2. **cAMP Standard, 0.5mL**
   Component Number 80-0056
   A solution of 2,000 pmol/mL cyclic AMP.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

3. **Acetylation Kit, 2 vials**
   Component Number 950-001
   a. **Triethylamine**
      2 mL, Component No. 80-0063
   b. **Acetic Anhydride**
      1 mL, Component No. 80-0064

The acetylation reagents are lachrymators. **Caution**: corrosive, flammable and harmful vapor.

4. **Goat anti-Rabbit IgG Clear Microtiter Plate**
   One plate of 96 wells, Component Number 80-0060
   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody.

5. **Neutralizing Reagent, 5mL**
   Component Number 80-1475
   A proprietary solution used to neutralize HCl.

6. **cAMP Antibody, 5mL**
   Component Number 80-3000
   A yellow solution of rabbit polyclonal antibody to cAMP.

7. **cAMP Conjugate, 5mL**
   Component Number 80-3002
   A blue solution of cAMP conjugated to alkaline phosphatase.

8. **Wash Buffer Concentrate, 27mL**
   Component Number 80-1286
   A 20X solution of tris buffered saline containing detergents.

9. **pNpp Substrate, 20 mL**
   Component Number 80-0075
   A solution of p-nitrophenyl phosphate.

10. **Stop Solution, 5 mL**
    Component Number 80-0247
    A solution of trisodium phosphate in water.

11. **cAMP Direct Assay Layout Sheet, 1 each**
    Component Number 30-0359

12. **Plate sealer, 1 each**
    Component Number 30-0012

Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.

Activity of conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Stop solution is caustic. Use caution and keep tightly capped.

This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided.

STORAGE

All components of this kit, **except the Conjugate and Standard**, are stable at 4ºC until the kit's expiration date. The Conjugate and Standard should be stored at -20ºC upon receipt.

OTHER MATERIALS NEEDED

1. Deionized or distilled water.
2. Precision pipets for volumes between 1 µL and 1,000 µL.
3. Repeater pipet for dispensing 50 µL and 200 µL.
4. Disposable beakers for dilution buffer concentration.
5. Graduated cylinders.
6. Microplate shaker.
7. Lint-free paper toweling for blotting.
8. Microplate reader capable of reading at an optical density of 405nm.
9. Triton X-100 (optional for sample preparation).

10. Liquid nitrogen, mortar & pestle and concentrated HCl (optional – for tissue samples).

Samples must be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

SAMPLE HANDLING

Treatment of cells and tissue with HCl will stop endogenous phosphodiesterase activity and allow for the direct measurement of these samples in the assay without evaporation or further processing. Recommended treatment protocols follow. Samples containing rabbit IgG will interfere in the assay.

Please note that some samples may contain high levels of cAMP and additional dilution may be required. Samples with low levels of cAMP may be assayed in the acetylated format or the samples may be concentrated. Tissue and cell lysates should be diluted in HCl, when necessary, and run directly in the assay. When using the non-acetlyated protocol, a minimum 1:4 dilution is required for both tissue and cell lysates. When using the acetylation protocol, a minumum 1:32 dilution is required for both tissue and cell lysates. Please see Sample Recoveries section for detailed information. These are the minimum dilutions required to remove matrix interference of these samples.

Biological fluids (e.g. plasma, saliva, serum) should be used in the cAMP ELISA (Cat # ADI-900-067A, ADI-901-067A) or the cAMP Complete ELISA Kit (Cat # ADI-900-163A, ADI-901-163A).

SAMPLE RECOVERIES

Linearity

Cell and tissue lysates were diluted to their respective minimum recommended dilutions (MRD), spiked with cAMP and serially diluted 1:2 in 0.1M HCl. For the acetylated samples, cell and tissue lysates were diluted to their respective MRDs, spiked with cAMP, acetylated and serially diluted 1:2 in 0.1M HCl. All samples were run in the assay and compared to the standard curve. Results for both the non-acetylated and acetylated samples are shown in the tables below.

Dilutional Linearity, % (non-acetylated)			
Dilution	**Cell lysate**	**Tissue lysate**	**Tissue lysate, treated**
Neat	--	--	--
1:2	--	--	--
1:4	100	100	100
1:8	100	84	110
1:16	139	123	120
1:32	148	125	121
1:64	134	122	110
1:128	151	131	104
1:256	139	138	87
1:512	134	--	82
1:1024	--	--	--
1:2048	--	--	--
1:4096	--	--	--
1:8192	--	--	--

Dilutional Linearity, % (acetylated)			
Dilution	**Cell lysate**	**Tissue lysate**	**Tissue lysate, treated**
Neat	--	--	--
1:2	--	--	--
1:4	--	--	--
1:8	--	--	--
1:16	--	--	--
1:32	100	100	100
1:64	99	121	145
1:128	177	189	196
1:256	145	188	175
1:512	97	146	168
1:1024	139	186	170
1:2048	158	199	183
1:4096	175	179	207
1:8192	--	--	--

Spike and Recovery

cAMP was spiked at three concentrations into cell and tissue lysates at their respective MRDs. For the acetylated samples, cAMP was spiked at the same three concentrations into cell and tissue lysates at their respective MRDs, acetylated and run in the assay. Matrix background was subtracted and the recovery was compared to the recovery of cAMP spiked into 0.1M HCl. The average percent recovery for each matrix at the minimum recommended dilution is indicated below. Results for both non-acetylated and acetylated samples are shown in the tables below.

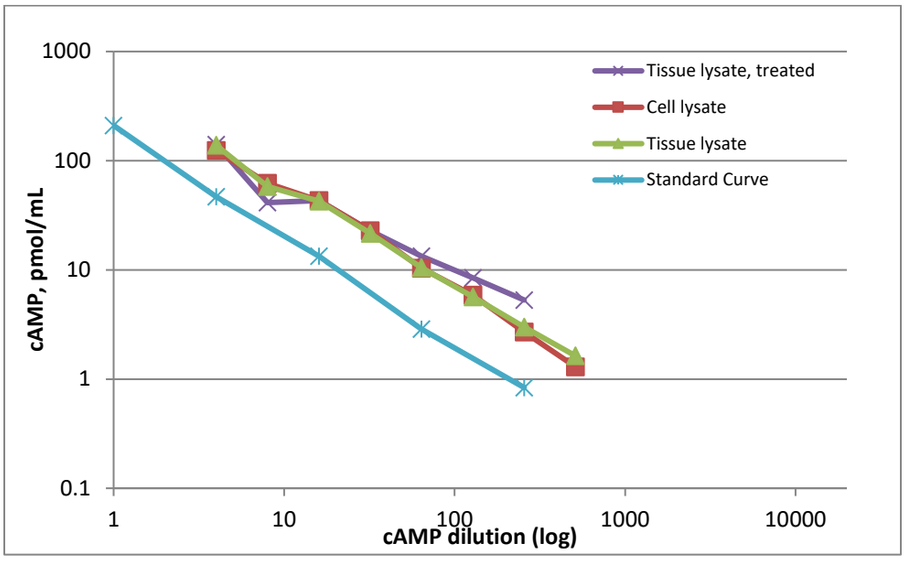
Sample (non-acetylated)	Spike Concentration, pmol/mL	% Recovery	Minimum Recommended Dilution
Cell lysate	200	74	1:4
	50	93	
	20	101	
Tissue lysate	200	86	1:4
	50	104	
	20	111	
Tissue lysate, treated	200	86	1:4
	50	100	
	20	110	

Sample (acetylated)	Spike Concentration, pmol/mL	% Recovery	Minimum Recommended Dilution
Cell lysate	20	97	1:32
	5	94	
	2	150	
Tissue lysate	20	131	1:32
	5	150	
	2	146	
Tissue lysate, treated	20	112	1:32
	5	150	
	2	139	

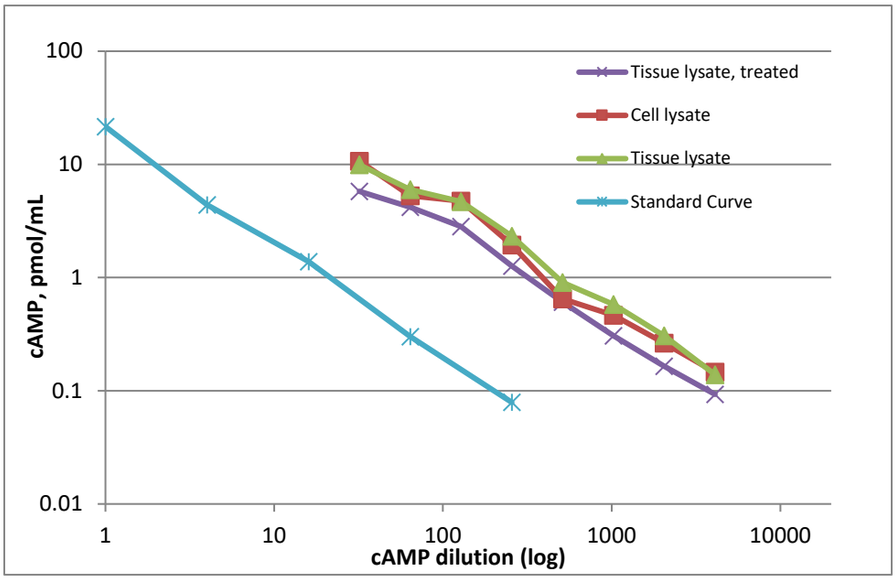
Parallelism

To assess parallelism, cell and tissue lysates were spiked with cAMP at their respective MRDs and serially diluted in 0.1M HCl. All of the samples were then run in the assay. The cAMP concentration in each sample was determined from the standard curve. Concentrations were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted samples matrices. Results for both the regular protocol and the acetylation protocol are shown in the graphs below.

Non-acetylated assay format



Acetylated assay format



PROTOCOL FOR CELL LYSATES

The concentration of cells used must be optimized for the specific cell line and treatment conditions. Cells may be grown in typical containers such as Petri dishes, culture plates (e.g., 48-well, 12-well, or 96-well), culture flasks, etc. Some cells are particularly hardy (e.g., bacteria) and may require the addition of 0.1 to 1% Triton X-100 to the 0.1M HCl for enhanced lysis. If Triton X-100 is added to samples it should also be added to the standard dilution as a modest increase in optical density may occur.

1. Pellet **suspension cells** and aspirate the media. Treat cells with 0.1M HCl. A general starting concentration of 1 x 10^6 cells per mL of 0.1M HCl is recommended. Remove the media from **adherent cells** and add enough 0.1M HCl to cover the bottom of the plate. Avoid over-diluting the sample with an excessive volume of HCl. Please note that the culture media may be saved and assayed separately, if desired.
2. Incubate the cells in 0.1M HCl for 10 minutes at room temperature.
3. Inspect the cells under a microscope to ensure uniform lysis. Continue incubating for an additional 10 minutes, if necessary.
4. Centrifuge ≥600 x g to pellet the cellular debris.
5. The supernatant may be assayed immediately or stored frozen for later analysis.

   Note: Standards must be diluted in 0.1 M HCl and Neutralizing Reagent used.

PROTOCOL FOR TISSUE SAMPLES

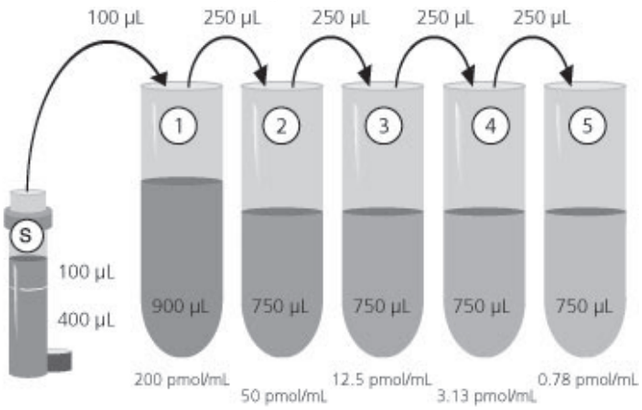
1. After collection, tissue samples should be flash frozen in liquid nitrogen. If analysis cannot be carried out immediately, store tissue at -80˚C.
2. Grind frozen tissue to a fine powder under liquid nitrogen in a stainless steel mortar.
3. When liquid nitrogen has evaporated, weigh the frozen tissue and homogenize in 10 volumes of 0.1M HCl (e.g., 0.1 g of tissue should be homogenized in 1 mL of 0.1M HCl).
4. Centrifuge ≥600 x g to pellet the debris (~10 minutes).
5. The supernatant may be further diluted in the 0.1M HCl provided and run directly in the assay or stored frozen for later analysis.

REAGENT PREPARATION

1. **Wash Buffer**

   Prepare the wash buffer by diluting 10 mL of the supplied Wash Buffer Concentrate with 190 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **cAMP Standard, non-acetylated format**



   Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm x 75mm tubes #1 through #5. Pipet 900 µL of 0.1M HCl into tube #1. Pipet 750 µL of 0.1M HCl into tubes #2 through #5. Add 100 µL of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

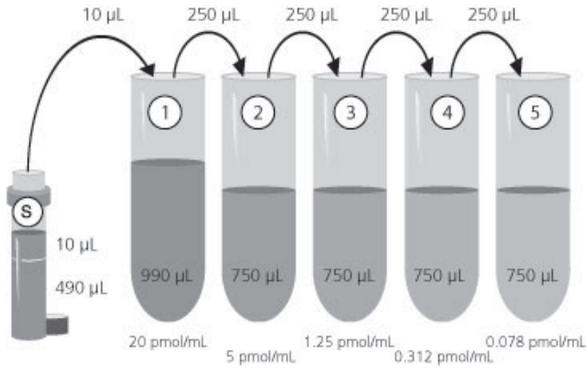
   Diluted standards should be used within 60 minutes of preparation. The concentrations of cAMP in the tubes are labeled above.

3. **Acetylation Reagent (optional)**

   Prepare the Acetylating Reagent by adding 0.5 mL of Acetic Anhydride to 1 mL of Triethylamine. Note that this volume is sufficient to add to 30 mL of diluted standards and samples. Use the prepared reagent within 60 minutes of preparation. Discard any unused portion of the Acetylating Reagent.

Triethylamine and acetic anhydride are lachrymators. Caution - corrosive, flammable, and harmful vapor. **Must not use plastic consumables other than polypropylene; other plastics such as polystyrene will corrode.**

4. **cAMP Standard, acetylated format (optional)**



Allow the 2,000 pmol/mL standard stock to warm to room temperature. Label five 12mm x 75mm tubes #1 through #5. Pipet 990 µL of 0.1M HCl into tube #1. Pipet 750 µL of 0.1M HCl into tubes #2 through #5. Add 10 µL of the 2,000 pmol/mL standard stock into tube #1 and vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #5.

Acetylate all **standards and samples** by adding 10 µL of the Acetylating Reagent for each 200 µL of the standard or sample. Add the Acetylating Reagent directly to the diluted standard or sample and vortex immediately after the addition of the Acetylating Reagent.

Label one 12mm x 75mm tube as the Bo/NSB tube. Pipet 1 mL of 0.1M HCl into this tube. Add 50 µL of the Acetylating Reagent to the Bo/NSB tube and use in Steps 2 and 3 of the Assay Procedure.

The acetylated standards should be used within 30 minutes of preparation. The concentrations of cAMP in the tubes are labeled above.

ASSAY PROTOCOL

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

Note: If the acetylated format of the assay is to be run, all standards, samples, and the diluent for the NSB and Bo wells must be section. Acetylated standards and samples must be used within 30 minutes.

Bring all reagents to room temperature for at least 1 hour prior to use.

1. Pipet 50 µL of Neutralizing Reagent into each well except the Total Activity (TA) and Blank wells.
2. Pipet 100 µL of 0.1M HCl into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells.
3. Add 50 µL of 0.1M HCl to the NSB wells.
4. Pipet 100 µL of Standards #1 through #5 to the bottom of the appropriate wells.
5. Pipet 100 µL of the samples to the bottom of the appropriate wells.
6. Pipet 50 µL of the blue conjugate into each well except the Blank and TA wells.
7. Pipet 50 µL of the 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.
8. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature.
9. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 2 more times for a total of 3 washes. 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.
10. Pipet 5 µL of the blue conjugate to the TA wells.
11. Add 200 µL of the substrate solution into each well.
12. Incubate for 1.5 hours at room temperature with shaking.
13. Pipet 50 µL stop solution into each well.
14. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

All standards and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation on assay results

CALCULATION OF RESULTS

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 4 parameter logistic (4PL) curve fitting program. The concentration of cAMP can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

2. Using data analysis software, plot the Average Net OD for each standard versus cAMP concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalyzed using alternative dilution(s).

To normalize for protein content, divide the resulting picomole per mL determinations (pmol/mL) by the total protein concentration (mg/mL) in each sample. This is expressed as pmol cAMP per mg of total protein.

TYPICAL RESULTS

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

Non-acetylated assay format

Sample	Mean OD	Net OD	%B/Bo	cAMP (pmol/mL)
TA	--	0.161	--	--
NSB	0.104	--	--	---
Bo	1.004	0.900	--	0
S1	0.238	0.134	14.90	200
S2	0.403	0.299	33.22	50
S3	0.638	0.534	59.37	12.5
S4	0.853	0.749	83.39	3.125
S5	0.958	0.854	95.16	0.781

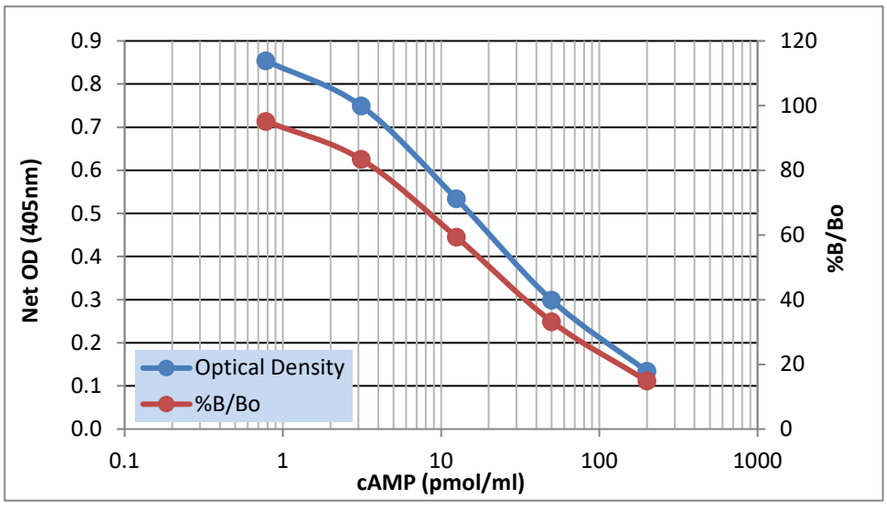
Acetylated assay format

Sample	Mean OD	Net OD	%B/Bo	cAMP (pmol/mL)
TA	--	0.162	--	--
NSB	0.104	--	--	---
Bo	0.660	0.556	--	0
S1	0.129	0.025	4.45	20
S2	0.172	0.068	12.18	5
S3	0.273	0.169	30.12	1.25
S4	0.438	0.334	59.97	0.3125
S5	0.580	0.476	85.69	0.0781

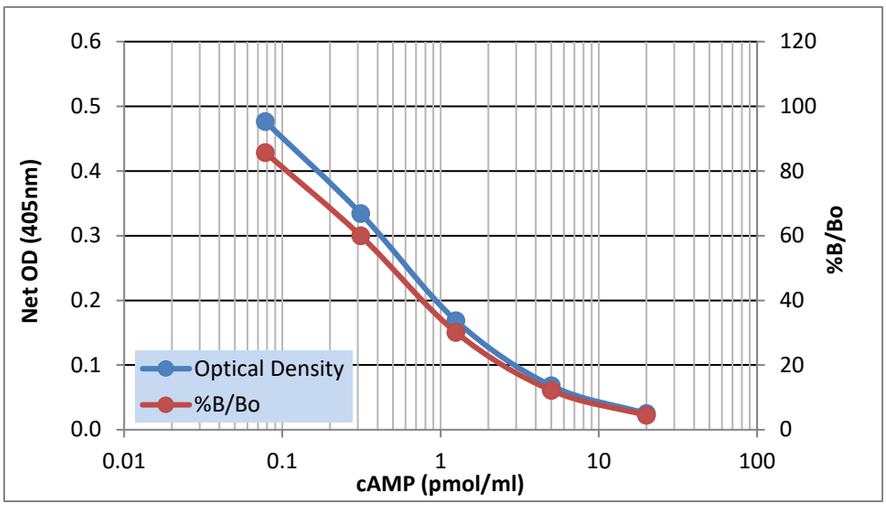
TYPICAL STANDARD CURVES

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 assay.

Non-acetylated assay format



Acetylated assay format



PERFORMANCE CHARACTERISTICS

Specificity

The specificity of the assay was determined by running serial dilutions of the analytes, including the cross-reactants, in the assay, fitting the resulting dose response curves to a 4PL curve-fit and determining the ED_{50}. The ED_{50} of each cross-reactant was then divided by the determined ED_{50} of the cAMP standard curve and multiplied by 100.

Analyte	Cross Reactivity, %
cAMP	100
AMP	<0.001
ATP	<0.001
cGMP	<0.001
GMP	<0.001
GTP	<0.001
cUMP	<0.001
CTP	<0.001

Sensitivity

The sensitivity of the assay, defined as the concentration of cAMP measured at 2 standard deviations from the mean of 18 zeros along the standard curve, was determined to be 1.18 pmol/mL in the non-acetylated assay format and 0.006 pmol/mL in the acetylated assay format.

Precision

Intra-assay was determined by assaying 20 replicates of three 0.1M HCl controls containing cAMP in a single assay.

Non-Acetylated Format		Acetylated Format	
pmol/mL	%CV	pmol/mL	%CV
20	3.08	2.0	4.09
5	11.59	0.5	4.41
2	6.13	0.2	6.14

Inter-assay was determined by measuring 0.1M HCl controls of varying cAMP concentrations in multiple assays over several days.

Non-Acetylated Format		Acetylated Format	
pmol/mL	**%CV**	**pmol/mL**	**%CV**
20	10.68	2.0	18.69
5	11.94	0.5	13.29
2	13.95	0.2	12.92

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Catalog Number: ADI-900-066A
Document Number: PM-PL0075
Rev. 022624