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New Standard and Conjugate Storage Temperatures

> cyclic AMP Complete

Catalog # 900-163

96 Well Enzyme Immunoassay Kit
For use with cells, tissue, saliva, serum and culture supernatants

Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs

For proper performance, use the insert provided with each individual kit received

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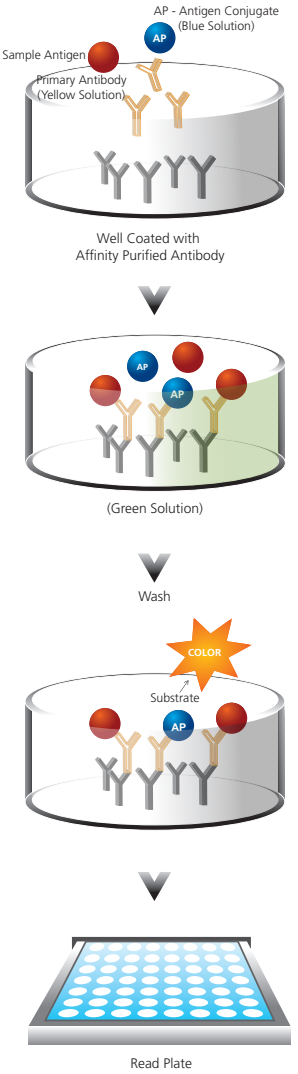
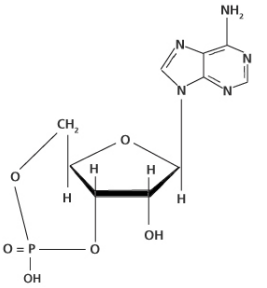
Introduction



Assay Designs' cyclic AMP Complete Enzyme Immunometric Assay (EIA) kit is a kit for the quantitative determination of cyclic AMP in cells and tissue treated with 0.1N HCl, in addition to culture supernates, saliva, and serum. The optional acetylated assay format provides an approximate 10-fold 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[1-5]. 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 a 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

1. **Assay Buffer 2**
   27 mL, Product No. 80-0055
   Sodium acetate buffer containing proteins and sodium azide
2. **0.1M HCl**
   27 mL, Product No. 80-0080
   0.1M hydrochloric acid in water
3. **cyclic AMP Standard**
   0.5 mL, Product No. 80-0056
   A solution of 2,000 pmol/mL cAMP
4. **Triethylamine**
   2 mL, Product No. 80-0063
5. **Acetic Anhydride**
   1 mL, Product No. 80-0064
6. **Goat anti-Rabbit IgG Microtiter Plate**
   One plate of 96 wells, Product No. 80-0060
   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody
7. **Neutralizing Reagent**
   5 mL, Product No. 80-1475
8. **cAMP Antibody**
   5 mL, Product No. 80-0604
   A yellow solution of rabbit polyclonal antibody to cAMP
9. **cAMP Conjugate**
   5 mL, Product No. 80-0053
   A blue solution of cAMP conjugated to alkaline phosphatase
10. **Wash Buffer Concentrate**
    27 mL, Product No. 80-1286
    Tris buffered saline containing detergents
11. **pNpp Substrate**
    20 mL, Product No. 80-0075
    A solution of p-nitrophenyl phosphate
12. **Stop Solution**
    5 mL, Product No. 80-0247
    A solution of trisodium phosphate in water
13. **cAMP Complete Assay Layout Sheet**
    1 each, Product No. 30-0243
14. **Plate Sealer**
    1 each, Product No. 30-0012

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

HCl is caustic. Keep tightly capped.

The standard should be handled with care due to the known and unknown effects of the antigen.

Triethylamine and acetic anhydride are lachrymators. Caution- corrosive, flammable, and harmful vapor.

The activity of the conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).

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

Stop solution is caustic. Keep tightly capped.

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Storage

Reagents require separate storage conditions.

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

Materials Needed but Not Supplied

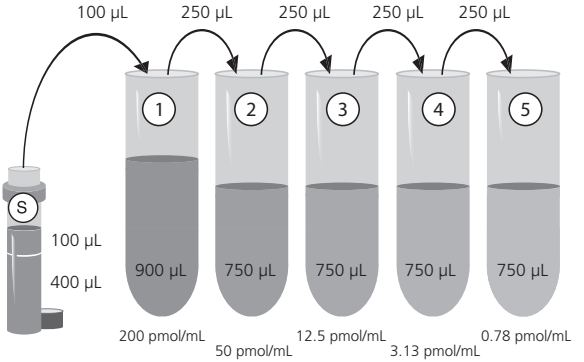
1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 50 µL and 200 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. Microplate shaker
7. Lint-free paper toweling for blotting
8. Microplate reader capable of reading at 405 nm

Reagent Preparation

1. **Wash Buffer**
   Prepare the wash buffer by diluting 5 mL of the supplied Wash Buffer Concentrate with 95 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**

   Three diluent options are available for the preparation of the standard curve, NSB, and Bo wells. For cell lysates and tissue samples prepared in 0.1M HCl, use the supplied 0.1M HCl as the standard diluent. Use Assay Buffer 2 for the standard diluent when the sample is serum or saliva. For culture supernate samples, use the same non-conditioned media for the standard diluent.



   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 the appropriate sample diluent into tube #1. Pipet 750 µL of the appropriate sample diluent 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.

Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

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Triethylamine and acetic anhydride are lachrymators. Caution- corrosive, flammable, and harmful vapor.

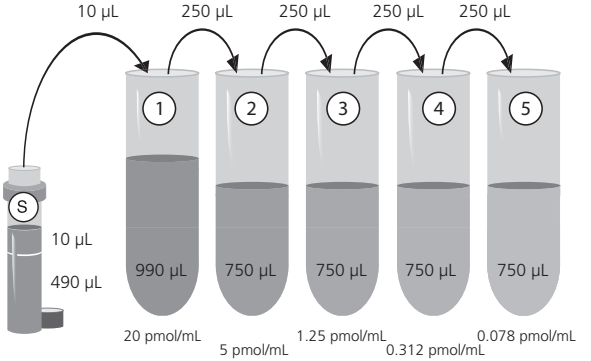
Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

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.

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

Three diluent options are available for the preparation of the standard curve, NSB, and Bo wells. For cell lysates and tissue samples prepared in 0.1M HCl, use the supplied 0.1M HCl as the standard diluent. Use Assay Buffer 2 for the standard diluent when the sample is serum or saliva. For culture supernate samples, use the same non-conditioned media for the standard diluent.



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 the appropriate sample diluent into tube #1. Pipet 750 µL of the appropriate sample diluent 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 the appropriate standard diluent 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.

Sample Handling

Treatment of cells and tissue with the supplied 0.1M 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 with the assay. EDTA plasma may precipitate during acetylation.

Biological fluids, such as serum and saliva, should be diluted in Assay Buffer 2 and run directly in the assay. A minimum 1:10 dilution is required for serum and a 1:4 dilution for saliva (see Sample Recoveries section). These are the minimum dilutions required to remove matrix interference of these samples.

Culture supernates may be run directly in the assay provided the same non-conditioned media is used as the standard diluent.

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.

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

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

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.

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Protocol for Tissue Samples

Two options are available for tissue samples. Protocol 1 is more straightforward and user-friendly. Protocol 2 is available if samples require concentration.

Protocol 1 : Treatment with 0.1 M HCl

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.

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

Protocol 2 : TCA / Ether Extraction

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 cold 5% TCA in a glass-Teflon tissue grinder.
4. Centrifuge at ≥600 x g to pellet the debris (~10 minutes).
5. Extract the supernatant with 3 volumes of water-saturated ether.
6. Dry the aqueous extracts and reconstitute in at least 250 µL Assay Buffer 2 (to allow for duplicate measures).

Note: standards must be diluted in Assay Buffer 2, no Neutralizing Reagent is used.

Assay Procedure

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 acetylated as per the instructions in the Reagent Preparation section. Acetylated standards and samples must be used within 30 minutes.

1. If using samples prepared in 0.1M HCl, pipet 50 µL of Neutralizing Reagent into each well except the Total Activity (TA) and Blank wells. Do not add Neutralizing Reagent for the other sample diluent options.
2. Pipet 100 µL of the appropriate standard diluent (Assay Buffer 2, 0.1M HCl, or non-conditioned culture media) into the NSB (non-specific binding) and Bo (0 pmol/mL standard) wells.
3. Add 50 µL of Assay Buffer 2 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 TA and Blank 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 5µL of the blue conjugate to the TA wells.
11. Add 200 µL of the substrate solution into each well.
12. Incubate for 1 hour at room temperature without 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.

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

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 in assay results.

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Calculation of Results

Several options are available for the calculation of the concentration of cAMP in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations 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. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

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

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

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

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

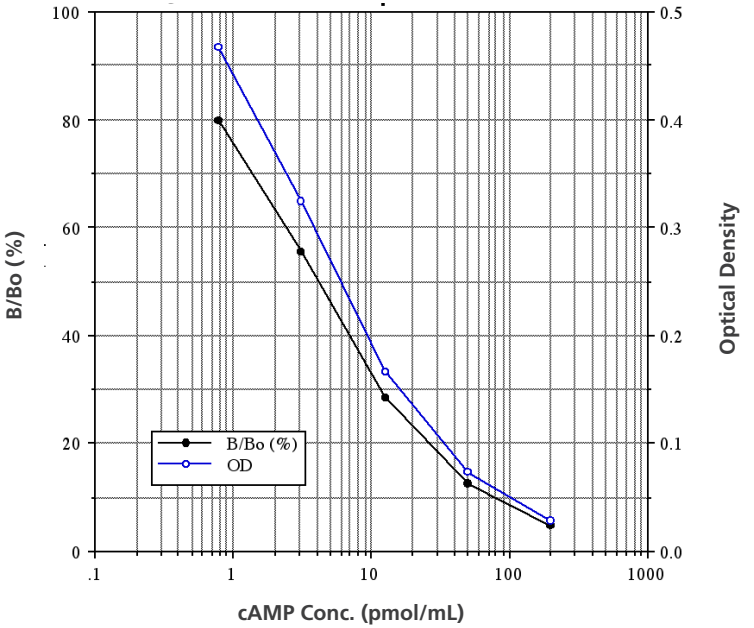
To normalize for protein content, divide the resulting picomole per mL determinations (pmol/mL) by the total protein concentration (mg/mL) in each sample. This is expressed as pmol cAMP per mg of total protein.

Typical Results

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

Non-acetylated assay format in Assay Buffer 2

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.085)	---	---
TA	0.494	---	---
NSB	0.000	0%	---
Bo	0.585	100%	**0**
S1	0.029	4.9%	**200**
S2	0.074	12.7%	**50**
S3	0.167	28.5%	**12.5**
S4	0.325	55.6%	**3.13**
S5	0.467	79.9%	**0.78**
Unknown 1	0.130	22.3%	**19.39**
Unknown 2	0.398	68.0%	**1.63**



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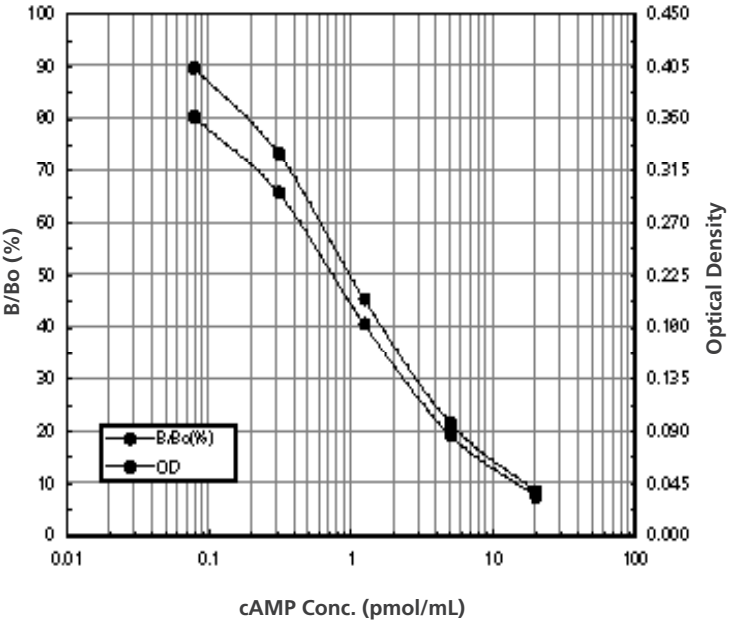
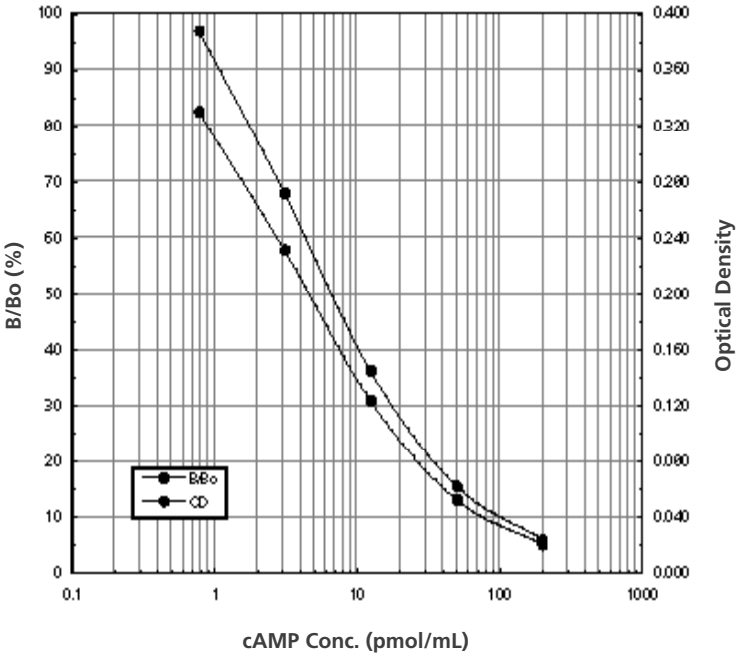
Acetylated assay format in Assay Buffer 2

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.086)	---	---
TA	0.435	---	---
NSB	0.002	0%	---
Bo	0.403	100%	**0**
S1	0.034	8.5%	**20**
S2	0.087	21.6%	**5**
S3	0.183	45.5%	**1.25**
S4	0.296	73.4%	**0.312**
S5	0.362	89.8%	**0.078**
Unknown 1	0.061	15.1%	**8.53**
Unknown 2	0.142	35.3%	**2.12**



Non-acetylated assay format in 0.1 M HCl

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.081)	---	---
TA	0.496	---	---
NSB	-0.001	0%	---
Bo	0.471	100%	**0**
S1	0.024	5.1%	**200**
S2	0.062	13.1%	**50**
S3	0.145	30.9%	**12.5**
S4	0.272	57.9%	**3.12**
S5	0.388	82.5%	**0.78**
Unknown 1	0.125	26.6%	**16.21**
Unknown 2	0.291	61.8%	**2.57**



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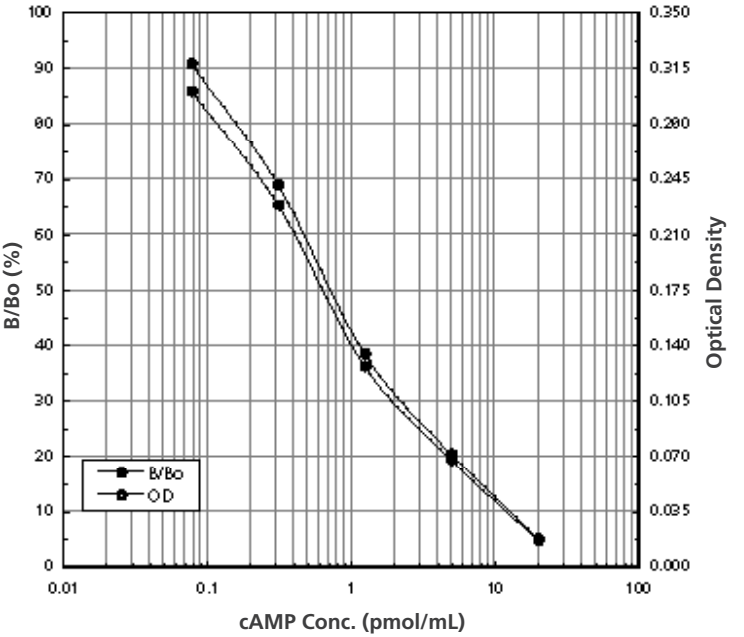
Acetylated assay format in 0.1 M HCl

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.083)	---	---
TA	0.437	---	---
NSB	0.003	0%	---
Bo	0.331	100%	**0**
S1	0.017	5.2%	**20**
S2	0.068	20.5%	**5**
S3	0.127	38.5%	**1.25**
S4	0.229	69.1%	**0.312**
S5	0.301	90.9%	**0.078**
Unknown 1	0.125	37.8%	**1.45**
Unknown 2	0.276	83.5%	**0.13**



Performance Characteristics

Specificity

The cross reactivities for a number of related compounds were determined by diluting the cross reactants in the kit assay buffer at a concentration of ten times the high standard. These samples were then measured in the assay.

For detailed cross-reactivity protocol see our website .

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%

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Sensitivity

Assay Buffer 2
The sensitivity of the assay, defined as the concentration of cAMP measured at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 0.30 pmol/mL in the non-acetylated assay format and 0.039 pmol/mL in the acetylated assay format.

0.1M HCl
The sensitivity of the assay, defined as the concentration of cAMP measured at 2 standard deviations from the mean of 16 zeros along the standard curve, was determined to be 0.39 pmol/mL in the non-acetylated assay format and 0.037 pmol/mL in the acetylated assay format.

Linearity

A buffer sample containing cAMP was serially diluted 1:2 in the kit assay buffer and measured in the assay. The results are shown in the table below.

Non-acetylated

Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	49.2	---
1:2	24.6	23.1	94%
1:4	12.3	13.7	112%
1:8	6.15	6.9	112%
1:16	3.07	3.4	111%

Acetylated

Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	5.42	---
1:2	2.71	2.86	106%
1:4	1.36	1.23	91%
1:8	0.68	0.51	75%
1:16	0.34	0.28	83%

A 0.1N HCl sample containing cAMP was serially diluted 1:2 in the 0.1N HCl diluent and measured in the assay. The results are shown in the table below.

Non-acetylated

Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	15.44	---
1:2	7.72	8.24	107%
1:4	3.86	3.67	95%
1:8	1.93	2.32	120%

Acetylated

Dilution	Expected (pmol/mL)	Observed (pmol/mL)	Recovery (%)
Neat	---	3.41	---
1:2	1.70	2.03	119%
1:4	0.85	0.95	111%
1:8	0.43	0.49	115%

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing cAMP in a single assay.

Non-Acetylated Format
In Assay Buffer 2

pmol/mL	%CV
1.18	10.5
5.96	2.5
18.6	2.9

In 0.1M HCl

pmol/mL	%CV
1.24	8.9
6.31	4.3
35.92	8.3

Acetylated Format
In Assay Buffer 2

pmol/mL	%CV
0.40	7.4
0.90	6.8
5.58	7.7

In 0.1M HCl

pmol/mL	%CV
0.679	4.6
3.58	8.4

Inter-assay precision was determined by measuring buffer controls of varying cAMP concentrations in multiple assays over several days.

Non-Acetylated Format

In Assay Buffer 2

pmol/mL	%CV
1.13	13.7
4.95	11.2
19.18	8.4

In 0.1M HCl

pmol/mL	%CV
1.18	13.1
5.53	4.2
30.36	11.6

Acetylated Format

In Assay Buffer 2

pmol/mL	%CV
0.46	11.2
0.98	11.2
4.75	7.9

In 0.1M HCl

pmol/mL	%CV
1.29	13.6
5.62	7.8

Sample Recoveries

cAMP standard was spiked into the following matrices diluted with Assay Buffer 2 and measured in the kit. The results were as follows:

Sample	% Recovery	Recommended Dilution, Non-Acetylated Format	% Recovery	Recommended Dilution, Acetylated Format
Tissue Culture Media	96.2%	None	101.2	None
Human Serum	101.5%	1:10	117.8	1:64
Human Saliva	103.2%	1:4	94.9	1:4

0.1 M HCl should not be used to dilute culture supernates, serum, or saliva samples.

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Limited Warranty

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MSDS (Material Safety Data Sheet) available online

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For more details concerning the information within this kit insert, or to order any of the Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

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