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Enzo® Life Sciences

Enabling Discovery in Life Science®

Direct cAMP ELISA kit

Catalog # ADI-900-066

96 Well Enzyme-linked Immunosorbent Assay Kit

For use with cells, tissue, 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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ALEXIS® assay designs® BIOMOL® Stressgen®

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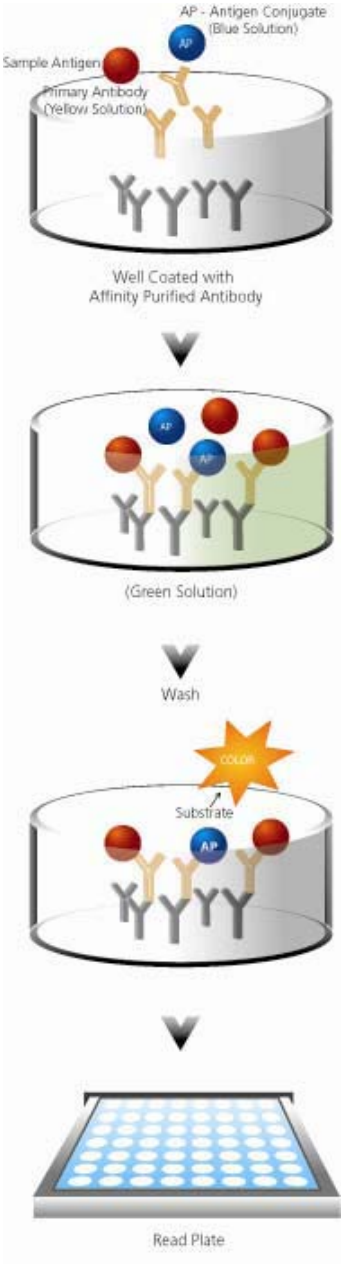
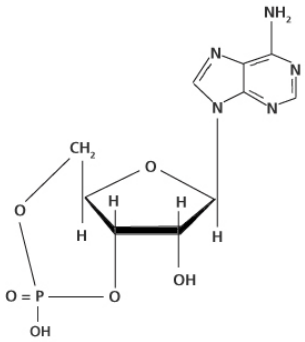


Introduction

The Direct cyclic AMP Enzyme-linked Immunosorbent Assay (ELISA) kit is a competitive immunoassay for the quantitative determination of cyclic AMP in cells, tissue, and culture supernatants treated with HCl. 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[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

HCl is caustic. Keep tightly capped.

1. 0.1M HCl

27 mL, Product No. 80-0080

0.1M hydrochloric acid in water

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

2. cyclic AMP Standard

0.5 mL, Product No. 80-0056

A solution of 2,000 pmol/mL cAMP

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

3. Triethylamine

2 mL, Product No. 80-0063

4. Acetic Anhydride

1 mL, Product No. 80-0064

5. 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

6. Neutralizing Reagent

5 mL, Product No. 80-1475

Avoid contamina-tion by endogenous alkaline phos-phatase. Do not expose reagents or supplies to bare skin.

7. cAMP ELISA Antibody

5 mL, Product No. 80-0604

A yellow solution of rabbit polyclonal antibody to cAMP

Activity of conju-gate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).

8. cAMP Conjugate

5 mL, Product No. 80-0053

A blue solution of cAMP conjugated to alkaline phosphatase

9. Wash Buffer Concentrate

27 mL, Product No. 80-1286

Tris buffered saline containing detergents

10. pNpp Substrate

20 mL, Product No. 80-0075

A solution of p-nitrophenyl phosphate

Stop solution is caustic. Keep tightly capped.

11. Stop Solution

5 mL, Product No. 80-0247

A solution of trisodium phosphate in water

12. cAMP Direct Assay Layout Sheet

1 each, Product No. 30-0101

13. Plate Sealer

1 each, Product No. 30-0012

Reagents require separate storage conditions.

Storage

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
9. Triton X-100 (optional for sample preparation)
10. Liquid nitrogen, mortar & pestle, and concentrated HCl (optional – for tissue samples)

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 frozen at or below -20° to avoid loss of bio-active analyte. Repeated freeze/thaw cycles should be avoided.

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 with the assay.

Culture supernatants may be run directly in the assay after treatment with concentrated HCl (**not** the 0.1M HCl supplied). Add 10 µL concentrated HCl to each 1 mL of culture media to be assayed. Centrifuge at ≥600 x g at room temperature. Culture supernatants may also be run directly in the cyclic AMP ELISA Kit (Cat. #900-067, 901-067) or the cyclic AMP Complete ELISA Kit (Cat. #900-163, 901-163) without pre-treatment with concentrated HCl.

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.

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

Sample Recoveries

cAMP standard was spiked into treated culture media, diluted with 0.1M HCl, and measured in the kit. The results were as follows:

	Non-Acetylated Format		Acetylated Format	
Sample	**% Recovery**	**Recommended Dilution**	**% Recovery**	**Recommended Dilution**
Tissue Culture Media	94.8%	1:4	95.2%	1:4

0.1 M HCl should not be used to dilute culture supernatants (without pre-treatment with concentrated HCl), serum, or saliva samples.

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.

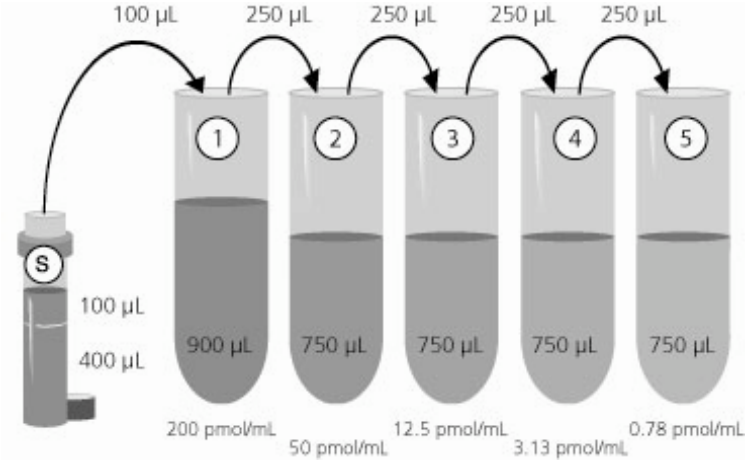
Note: Standards must be diluted in 0.1 M HCl and Neutralizing Reagent used.

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

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



   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 0.1 M HCl into tube #1. Pipet 750 µL of the 0.1 M 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.

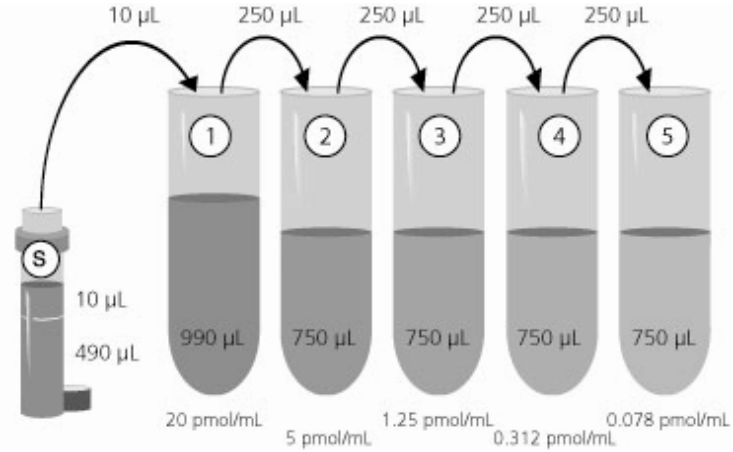
   Diluted standards should be used within 60 minutes of preparation. The concentrations of cAMP in the tubes are labeled above.

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

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.

Glass or polypropyl-ene tubes may be used for standard preparation. Avoid polystyrene.

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 the 0.1M HCl into tube #1. Pipet 750 µL of the 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 the 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 and samples should be used within 30 minutes of preparation. The concentrations of cAMP in the tubes are labeled above.

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.

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. Pipet 50 µL of Neutralizing Reagent into each well except the Total Activity (TA) and Blank wells.
2. Pipet 100 µL of the 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 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 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 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.

Calculation of Results

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

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. Assay Blaster! assay analysis software (Cat. #ADI-28-002) is an easy-to-use and cost effective program that provides the options of point-to-point, 4PL and 5PL curve fitting options.

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

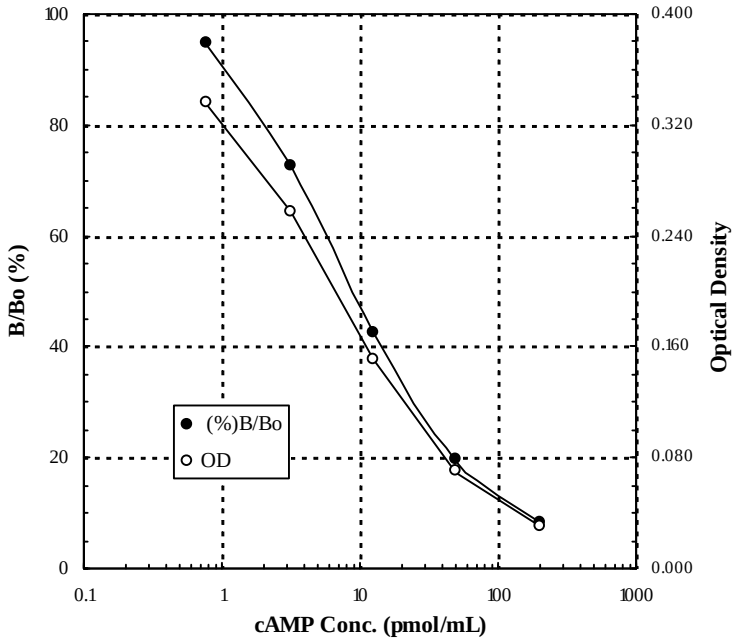
To normalize for protein content, divide the resulting picomole per mL determinations (pmol/mL) by the total protein concentration (mg/mL) in each sample. This is expressed as pmol cAMP per mg of total protein.

Typical Results

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

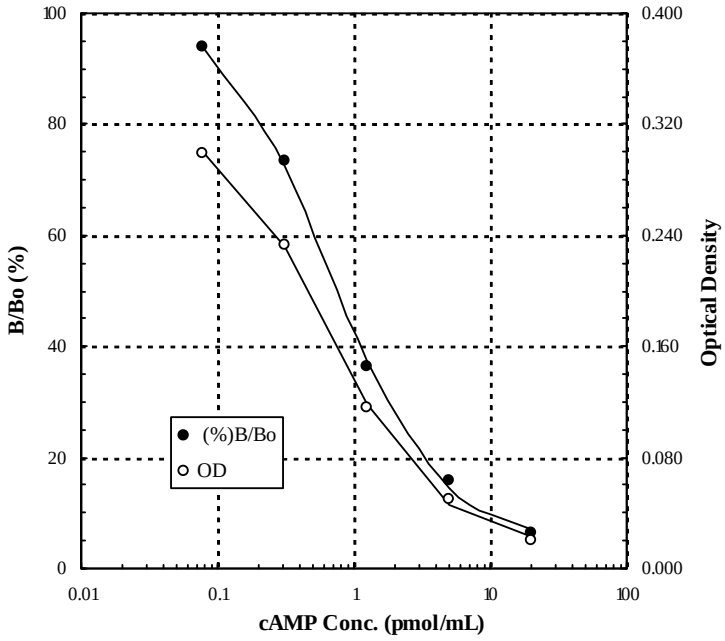
Non-acetylated assay format

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.088)	---	---
TA	0.506	---	---
NSB	-0.001	-0.35%	---
Bo	0.354	100%	0
S1	0.030	8.4%	200
S2	0.069	19.5%	50
S3	0.151	42.6%	12.5
S4	0.258	72.8%	3.125
S5	0.335	94.6%	0.781
Unknown 1	0.119	33.6%	19.68
Unknown 2	0.264	74.5%	2.86



Acetylated assay format

Sample	Average Net OD	Percent Bound	cAMP (pmol/mL)
Blank (mean)	(0.097)	---	---
TA	0.495	---	---
NSB	-0.001	-0.36%	---
Bo	0.331	100%	0
S1	0.021	6.5%	20
S2	0.050	15.8%	5
S3	0.117	36.6%	1.25
S4	0.233	73.1%	0.312
S5	0.300	94.0%	0.078
Unknown 1	0.047	14.8%	4.90
Unknown 2	0.103	32.4%	1.56



For detailed cross-reactivity protocol see our website

Performance Characteristics

Specificity

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

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%

Sensitivity

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

0.1M HCl sample containing cAMP was serially diluted 1:2 in the 0.1M 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 0.1M HCl controls containing cAMP in a single assay.

Non-Acetylated Format		Acetylated Format	
pmol/mL	**%CV**	**pmol/mL**	**%CV**
1.24	8.9	0.679	4.6
6.31	4.3	3.58	8.4
35.92	8.3		

Inter-assay precision 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**
1.18	13.1	1.29	13.6
5.53	4.2	5.62	7.8
30.36	11.6		

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Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

Enzo Life Sciences International, Inc. makes no warranty of any kind, expressed or implied, which extends beyond the description of the product in this brochure, except that the material will meet our specifications at the time of delivery. Enzo Life Sciences International, Inc. makes no guarantee of results and assumes no liability for injuries, damages or penalties resulting from product use, since the conditions of handling and use are beyond our control.

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