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ab133039 – Cyclic AMP ELISA Kit

Instructions for Use

For quantitative detection of Cyclic AMP in Saliva, Serum and Cell Culture Supernatants.

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 11 January 2019

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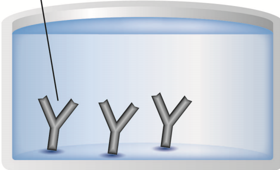
1. BACKGROUND

Abcam's Cyclic AMP *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) is a colorimetric competitive enzyme immunoassay kit for the quantitative determination of extracellular cAMP.

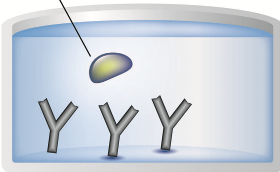
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-cAMP antigen and a polyclonal rabbit antibody specific to cAMP. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of cAMP captured in the plate.

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

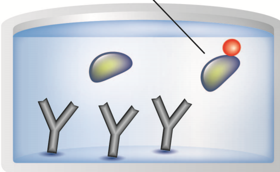
2. ASSAY SUMMARY



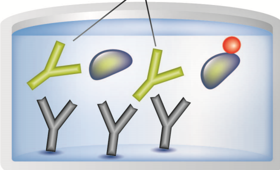
Prepare all reagents and samples as instructed.



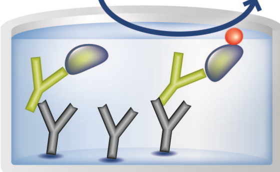
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add Cyclic AMP antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance
- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results

4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the AP Conjugate & Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4ºC
Cyclic AMP Alkaline Phosphatase Conjugate	5 mL	-20ºC
Cyclic AMP Antibody	5 mL	+4ºC
Cyclic AMP Standard	500 µL	-20ºC
20X Wash Buffer Concentrate	27 mL	+4ºC
pNpp Substrate	20 mL	+4ºC
Stop Solution	5 mL	+4ºC
Acetylation kit - Triethylamine	2 mL	+4ºC
Acetylation kit - Acetic Anhydride	1 mL	+4ºC
Assay Buffer 2	27 mL	+4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- Triton X-100 (optional for sample preparation)
- Optional (for tissue samples): Liquid nitrogen, mortar & pestle, and concentrated HCl

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- 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
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, 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. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 **Cyclic AMP Alkaline Phosphatase Conjugate**

Allow the Cyclic AMP Alkaline Phosphatase Conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C.

9.2 **1X Wash Buffer**

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently.

9.3 **Acetylation Reagent (optional)**

Prepare the Acetylating Reagent by adding 500 µL 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.

10. STANDARD PREPARATIONS – NON-ACETYLATED FORMAT

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 **For:**

10.1.1 **Serum/saliva** samples: dilute the Cyclic AMP standards Assay Buffer 2.

10.1.2 **Culture Supernatants** samples: dilute the Cyclic AMP standards with the same non-conditioned media as the samples

10.2 Allow the 2,000 pmol/mL Cyclic AMP **Stock Standard** solution to warm to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

10.3 Label five tubes with numbers #1 – #5.

10.4 Add 900 µL appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1.

10.5 Add 750 µL appropriate diluent to tubes #2 through #7

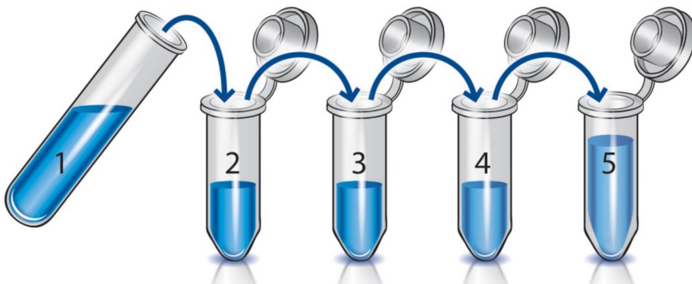
10.6 Prepare a 200 pmol/mL **Standard 1** by adding 100 µL of 2,000 pmol/mL Stock Standard into tube 1. Vortex thoroughly.

10.7 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube 2. Vortex thoroughly.

10.8 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Vortex thoroughly.

10.9 Using the table below as a guide, repeat for tubes 4 and 5.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pmol/mL)	Final Conc. (pmol/mL)
1	Stock	100	900	2,000	200
2	Standard 1	250	750	200	50
3	Standard 2	250	750	50	12.5
4	Standard 3	250	750	12.5	3.13
5	Standard 4	250	750	3.13	0.78



11. STANDARD PREPARATIONS –ACETYLATED FORMAT (optional)

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

11.1 **For:**

11.1.1 **Serum/saliva** samples: dilute the Cyclic AMP standards Assay Buffer 2.

11.1.2 **Culture Supernatants** samples: dilute the Cyclic AMP standards with the same non-conditioned media as the samples

11.2 Allow the 2,000 pmol/mL Cyclic AMP **Stock Standard** solution to warm to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

11.3 Label five tubes with numbers #1 – #5.

11.4 Add 990 µL of appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1.

11.5 Add 750 µL of appropriate diluent to tubes #2 through #7.

11.6 Prepare a 20 pmol/mL **Standard 1** by adding 10 µL of 2,000 pmol/mL Stock Standard tube 1. Vortex thoroughly.

11.7 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube 2. Vortex thoroughly.

11.8 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Vortex thoroughly.

11.9 Using the table below as a guide, repeat for tubes 4 and 5.

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

NOTE: If acetylating standards, then samples must be acetylated in the same format by adding 10 µL of the Acetylating Reagent for each 200 µL of the sample.

11.11 Add 1 mL of 0.1M HCl into the B_0 tube, followed by 50 µL of the Acetylating Reagent. Use in steps 13.3 and 13.4 of the Assay Procedure.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pmol/mL)	Final Conc. (pmol/mL)
1	Standard	10	990	2,000	20
2	Standard 1	250	750	20	5
3	Standard 2	250	750	5	1.25
4	Standard 3	250	750	1.25	0.312
5	Standard 4	250	750	0.312	0.078

12. SAMPLE COLLECTION AND STORAGE

- Samples must be stored frozen or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze-thaw cycles
- 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. These are the minimum dilutions required to remove matrix interference of these samples
- Culture supernatants 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 Cyclic AMP and additional dilution may be required. Samples with low levels of Cyclic AMP may be assayed in the acetylated format or the samples may be concentrated
- If acetylating standards, then samples must be acetylated in the same format by adding 10 µL of the Acetylating Reagent for each 200 µL of the sample

13. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at +4°C
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay

	1	**2**	**3**	**4**
A	B_s	Std 1	Std 5	Sample 4
B	B_s	Std 1	Std 5	Sample 4
C	TA	Std 2	Sample 1	etc
D	TA	Std 2	Sample 1	etc
E	NSB	Std 3	Sample 2	
F	NSB	Std 3	Sample 2	
G	B_0	Std 4	Sample 3	
H	B_0	Std 4	Sample 3	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:
$\mathbf{B_s}$ = Blank; contains substrate only.
TA = Total Activity; contains conjugate (5 µL) and substrate.
NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

$\mathbf{B_0}$ = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

14. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**
- **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.**

13.1 Add 150 µL appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells

13.3 Add 100 µL of prepared standards and 100 µL diluted samples to appropriate wells.

13.4 Add 50 µL of the Cyclic AMP Alkaline Phosphatase Conjugate (blue) into NSB, B0, standard and sample wells, i.e. not the Total Activity (TA) and Bs wells.

13.5 Add 50 µL of the Cyclic AMP Antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells *NOTE:* Every well used should be green in color except the NSB wells which should be blue. The B_0 and TA wells are empty at this point and have no color.

13.6 Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided.

13.7 Empty the contents of the wells and wash by adding 200 µL of 1X Wash Buffer to every well. Repeat the wash

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.

13.8 Add 5 µL of the Cyclic AMP Alkaline Phosphatase Conjugate to the TA wells.

13.9 Add 200 µL of the pNpp Substrate solution to every well. Incubate at room temperature for 1 hour without shaking.

13.10 Add 50 µL Stop Solution into each well. The plate should be read immediately.

13.11 Blank the plate reader against the blank wells, read the O.D. absorbance 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.

15. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

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

14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of Cyclic AMP for the standards. The concentration of Cyclic AMP in the unknowns can be determined by interpolation of net OD values.

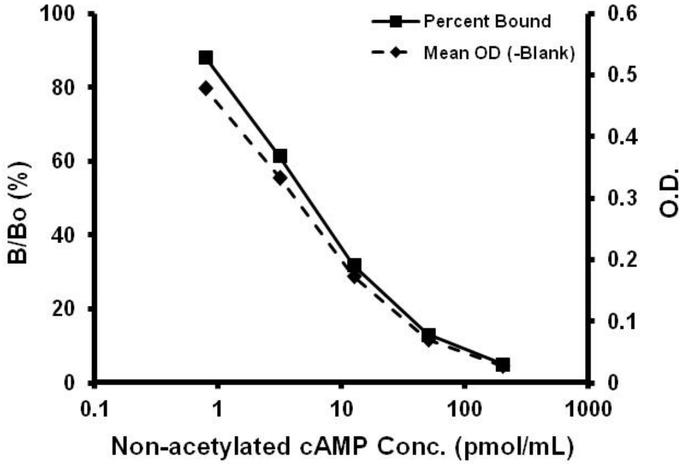
A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

16. TYPICAL DATA

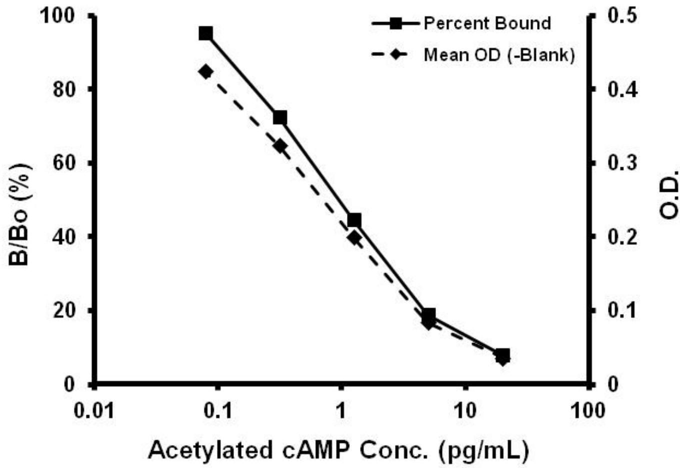
TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

Non-Acetylated:



Sample	Mean OD (B_s)	Percent Bound (%)	cAMP (pmol/mL)
B_s	(0.086)		
TA	0.454		
NSB	0.001	0.18	
Standard 1	0.028	5.1	200
Standard 2	0.071	13.1	50
Standard 3	0.173	31.8	12.5
Standard 4	0.334	61.5	3.125
Standard 5	0.479	88.2	0.781
B_0	0.543	100	0
Unknown 1	0.135	24.8	18.93
Unknown 2	0.357	65.8	2.57

Acetylated:



Sample	Mean OD ($-B_s$)	Percent Bound (%)	cAMP (pmol/mL)
B_s	(0.093)		
TA	0.463		
NSB	-0.003	-0.87	
Standard 1	0.036	8	20
Standard 2	0.084	18.9	5
Standard 3	0.199	44.7	1.25
Standard 4	0.323	72.5	0.3125
Standard 5	0.424	95.3	0.0781
B_0	0.445	100	0
Unknown 1	0.059	13.3	9.2
Unknown 2	0.143	32	2.27

17. TYPICAL SAMPLE VALUES

SENSITIVITY –

The sensitivity, minimum detectable dose of Cyclic AMP using this Abcam ELISA kit was measured at 2 standard deviations from the mean of 16 zeros along the standard curve, and was determined to be 0.30 pmol/mL in the non-acetylated assay format and 0.039 pmol/mL in the acetylated assay format.

SAMPLE RECOVERY –

Recovery was determined by Cyclic AMP into tissue culture media, human saliva, and serum. Mean recoveries are as follows:

Non-Acetylated Format:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	96.2	None
Human Serum	101.5	1:10
Human Saliva	103.2	1:4

Acetylated Format:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	101.2	None
Human Serum	117.8	1:64
Human Saliva	94.9	1:4

LINEARITY OF DILUTION –

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

PRECISION –

Intra-assay precision was determined by assaying 20 replicates of three 0.1M HCl controls containing Cyclic AMP in a single assay.
Inter-assay precision was determined by measuring 0.1M HCl controls of varying Cyclic AMP concentrations in multiple assays over several days.

Non-Acetylated Format:

Intra-Assay

pmol/mL	%CV
1.18	10.5
5.96	2.5
18.6	2.9

Inter-Assay

pmol/mL	%CV
1.13	13.7
4.95	11.2
19.18	8.4

Acetylated Format:

Intra-Assay

pmol/mL	%CV
0.40	7.4
0.90	6.8
5.58	7.7

Inter-Assay

pmol/mL	%CV
0.46	11.2
0.98	11.2
4.75	7.9

18. ASSAY SPECIFICITY

CROSS REACTIVITY –

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:

Cyclic AMP	100 %
AMP	<0.33 %
ATP	<0.12 %
cGMP	<0.001 %
GMP	<0.001 %
GTP	<0.001 %
cUMP	<0.001 %
CTP	<0.001 %

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

20. NOTES

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