ATHENS RESEARCH & TECHNOLOGY

ARBOR ASSAYS™

N-CAL™ KIT

NCal™ International Standard Kit

Human C-Reactive Protein (CRP)
Enzyme Immunoassay Kit

1 Plate Kit Catalog Number K069-H1
5 Plate Kit Catalog Number K069-H5

Sample Types Validated:

Serum and Plasma

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.
Not for human diagnostic use.

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BACKGROUND

C-reactive protein (CRP) is a member of the pentraxin family of proteins, which also includes serum amyloid P component (SAP). Human CRP is comprised of five noncovalently associated subunits and forms a homopentameric structure[1]. It is a widely used biomarker for systemic inflammation and tissue injury[2]. CRP specifically binds to phosphocholine (PCh) residues of polysaccharides on many microbial pathogens and on apoptotic or necrotic cell membranes in a Ca^{2+}-dependent manner. The PCh-bound CRP can be recognized by the C1q complex and efficiently initiate the activation of the complement system, which leads to the elimination of foreign pathogens[1-2]. The binding of CRP to PCh on damaged cells can facilitate the clearance of apoptotic or necrotic host cells, contributing to restoration of normal structure and function of injured tissue[2]. During myocardial infarction and ischemia/reperfusion injury, the same response can lead to additional tissue damage resulting from complement activation[3]. CRP also binds some nuclear constituents which do not contain PCh, such as histones and small nuclear ribonucleoproteins[1-2]. The binding of CRP to Fc receptors FcγRI and FcγRIIa mediates the interaction of damaged cells or particles with phagocytic cells leading to phagocytosis of the cells or particles[4]. The function of CRP in eliminating foreign pathogens and damaged cells through recruitment of the complement system and phagocytic cells makes CRP a critical molecule in the frontline of innate host defense. In response to infection, cell damage or tissue injury, the serum CRP concentration may increase by up to 1000 fold. Elevated CRP levels have been reported in patients with infection[2], chronic inflammatory disorders[5], myocardial infarction[3,6], ischemia/reperfusion injury[3,6], atherosclerosis[7], cancer[8], pulmonary disorders[9], metabolic syndrome[10] and depression[11].

1. Volanakis, J. E. (2001). Human C-reactive protein: expression, structure, and function. *Molecular Immunology*, 38(2–3), 189–197.
2. Pepys, M. B., & Hirschfield, G. M. (2003). C-reactive protein: A critical update. T*he Journal of Clinical Investigation*, 111(12), 1805–1812.
3. Agrawal, A., et al. (2014). Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators of Inflammation*, 2014(319214).
4. Mold, C., et al. (2002). C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. *Journal of Immunology*, 169(12), 7019–7025.
5. Du Clos, T. W. (2003). C-reactive protein as a regulator of autoimmunity and inflammation. *Arthritis and Rheumatology*, 48(6), 1475–¬1477.
6. Pepys, M. B., et al. (2006). Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature*, 440(7088), 1217–1221.
7. Singh, S. K., et al, (2008). The connection between C-reactive protein and atherosclerosis. *Annals of Medicine*, 40(2), 110–120.
8. Allin, K. H., & Nordestgaard, B. G. (2011). Elevated C-reactive protein in the diagnosis, prognosis, and cause of cancer. *Critical Reviews in Clinical Laboratory Science*, 48(4), 155–157.
9. Agassandian, M., et al. (2014). C-reactive protein and lung diseases. *The International Journal of Biochemistry and Cell Biology*, 53, 77–88.
10. Deetman, P. E., et al. (2013). High sensitive C-reactive protein and serum amyloid A are inversely related to serum bilirubin: Effect-modification by metabolic syndrome. *Cardiovascular Diabetology*, 12, 166.

ASSAY PRINCIPLE

The human C-Reactive Protein (CRP) ELISA Kit is designed to quantitatively measure CRP present in a variety of samples. Please read the complete kit insert before performing this assay. A C-Reactive Protein standard, calibrated to the WHO 1st International Reference Preparation 85/506, is provided to generate a standard curve for the assay and all samples should be read off the standard curve. A peroxidase-conjugated CRP monoclonal antibody is pipetted into a clear microtiter plate coated with a monoclonal antibody to capture CRP present in the sample. Samples or standards are added and the plate is incubated for 2 hours and washed. Substrate is then added to the plate, which reacts with the bound CRP conjugated antibody. After incubation, the substrate reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of CRP in the sample is calculated, after making suitable correction for dilution, using software available with most plate readers.

RELATED PRODUCTS

Arbor Assay Kits	Catalog No.
Allopregnanolone ELISA Kits	K061-H1/H5
Atrial Natriuretic Peptide (ANP) ELISA Kits	K026-H1/H5
Endothelin-1 (ET-1) ELISA Kit	K045-H1
Hemoglobin High Sensitivity Colorimetric Detection Kits	K013-HX1/HX5
Myeloperoxidase (MPO) ELISA Kit	K060-H1
Nitric Oxide (NO) Colorimetric Detection Kit	K023-H1
Prostaglandin E_2 (PGE_2) Multi-Format ELISA Kits	K051-H1/H5
Protein Kinase A (PKA) Activity Kit	K027-H1
ST2 Human ELISA Kit	K055-H1

Athens Research and Technology Reagents	Catalog No.
Alpha-1-Acid Glycoprotein (AGP)	16-16-010700
Alpha-2-Macroglobulin	16-16-012013
Ceruloplasmin	16-16-030518
Complement C3c	16-16-030303
Complement C4c	16-16-030304-85
Haptoglobin Mixed Type	16-16-080116
Haptoglobin Mixed Type, Low Endotoxin	16-16-0116-LEL
Haptoglobin Phenotype 1-1, Low Endotoxin Level	16-16-080116-1/1-LEL
Haptoglobin Phenotype 1-1	16-16-080116-1/1
Haptoglobin Phenotype 2-2	16-16-080116-2/2

SUPPLIED COMPONENTS

Mouse anti-C-Reactive Protein Clear Coated 96 Well Plate

Clear plastic microplate with break-apart strips coated with mouse anti-human C-Reactive Protein.

Kit K069-H1 **or** -H5	1 **or** 5 Each	Catalog Number C261-1EA

C-Reactive Protein Standard

A stock solution of native human CRP at 400 ng/mL.

Kit K069-H1 **or** -H5	40 µL **or** 200 µL	Catalog Number C260-40UL **or** -200UL

Calibrated to the 1st WHO International Standard, NIBSC code: 85/506

C-Reactive Protein Conjugate

An antibody to human C-Reactive Protein labeled with peroxidase.

Kit K069-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number C259-5ML **or** -25ML

Assay Buffer Concentrate

A green-colored 5X concentrate that should be diluted with deionized or distilled water.

Kit K069-H1 **or** -H5	28 mL **or** 80 mL	Catalog Number X142-28ML **or** -80ML

Wash Buffer Concentrate

A 20X concentrate that should be diluted with deionized or distilled water.

Kit K069-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K069-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

Stop Solution

A 1M hydrochloric acid solution. **CAUSTIC**.

Kit K069-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K069-H1 **or** -H5	1 **or** 5 each	Catalog Number X002-1EA **or** -5EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Polypropylene or glass test tubes.

Repeater pipet and disposable tips capable of dispensing 100 µL and 50 µL.

A microplate washer.

Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

The CRP Standard is purified from a human source and as such should be treated as potentially hazardous. Proper safety procedures must be followed.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers' wash buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on page 8.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

SAMPLE TYPES

This assay has been validated for human serum and plasma samples only. Samples containing visible particulate should be centrifuged prior to using. This assay has low or no reactivity to rodent CRP. The end user should test this kit for application in their samples.

SAMPLE PREPARATION

Serum and Plasma Samples

Serum and plasma samples must be diluted a **minimum** of ≥ 1:10 in diluted Assay Buffer to remove interference of the matrix.

Typical CRP concentrations in human serum and plasma can be as high as mg/mL levels depending on disease state. We recommend diluting all samples **1:500 or 1:1,000 fold** in diluted Assay Buffer.

Any samples with concentrations outside the standard curve range should be diluted further with Assay Buffer, as appropriate, to obtain readings within the standard curve range.

Use all samples within 2 hours of dilution.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer

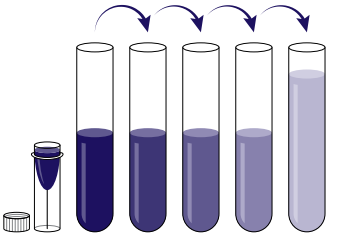
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable at 4°C for 3 months. The buffer contains green food coloring to aid in pipetting samples and standards to the plate wells.

Wash Buffer

Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable at 4°C for 3 months.

Standard Preparation

Label test tubes as 1 through 7. Pipet 60 µL of Assay Buffer into tube 1. Pipet 40 µL of Assay Buffer into tubes 2 to 7. Carefully add 15 µL of the 400 ng/mL CRP standard to tube 1, and vortex completely. Take 40 µL of the CRP solution in tube 1, add it to tube 2, and vortex completely. Repeat the serial dilutions for tubes 3 through 7. The concentration of C-Reactive Protein in the tubes 1 through 7 will be 80, 40, 20, 10, 5, 2.5, and 1.25 ng/mL.



Use all Standards within 2 hours of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
Assay Buffer Volume (µL)	**60**	40	40	40	40	40	40
Addition	**Stock**	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	**15**	40	40	40	40	40	40
Final Conc (ng/mL)	80	40	20	10	5	2.5	1.25

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine CRP concentrations.

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziplock plate bag and store at 4˚C.
2. Pipet standards or samples down the plate strip columns (A to H) to ensure maximum use of the strip wells.
3. Add 50 µL of the C-Reactive Protein Conjugate to each well, using a repeater pipet.
4. **Assay binding reaction starts as soon as the first sample or standard is added.** Immediately pipet 10 µL of samples or standards into wells in the plate. Pipet 10 µL of diluted Assay Buffer into the zero standard wells. Pipet samples and standards accurately but rapidly.
5. Cover the plate with the plate sealer and shake at room temperature for 2 hours. *NOTE: Incubation without shaking reduces overall signal by approximately 20%.*
6. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
7. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
8. Incubate the plate at room temperature for 30 minutes, without shaking.
9. Add 50 µL of the Stop Solution to each well, using a repeater pipet.
10. Read the optical density generated from each well at 450 nm.
11. Use the plate reader's built-in 4PLC software capabilities to calculate C-Reactive Protein concentration for each sample.

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

CALCULATION OF RESULTS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using computer software capable of generating a four-parameter logistic curve (4PLC) fit. The sample concentrations should be multiplied by the dilution factor to obtain neat sample values.

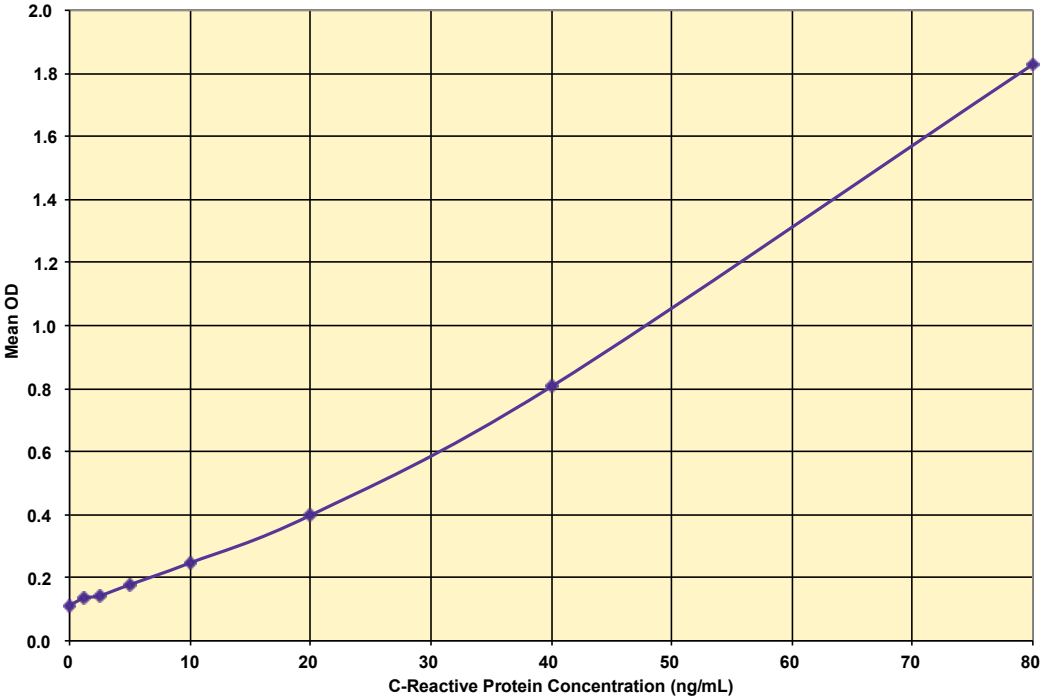
TYPICAL DATA

Sample	Mean OD	C-Reactive Protein Conc. (ng/mL)
Standard 1	1.830	80
Standard 2	0.806	40
Standard 3	0.398	20
Standard 4	0.247	10
Standard 5	0.178	5
Standard 6	0.143	2.5
Standard 7	0.136	1.25
Zero	0.113	0
Sample 1	0.388	19.25
Sample 2	0.924	44.96

Always run your own standard curve for calculation of results. Do not use this data.

The assay standard has been calibrated to the WHO 1st International CRP Standard, NIBSC code: 85/506 1.02 µg of human CRP is equivalent to 1.0 milli-International Unit.

Typical Standard Curve



Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection

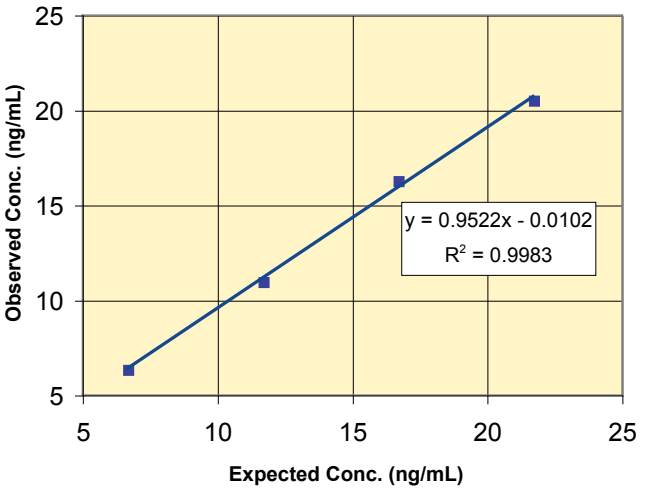
Sensitivity was calculated by comparing the OD's for nineteen wells run for each of the zero and standard 7. The detection limit was determined at two (2) standard deviations from the zero along the standard curve. **Sensitivity was determined as 0.616 ng/mL.**

The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty replicates for each of the zero standard and a low concentration serum sample. **Limit of Detection was determined as 0.932 ng/mL.**

Linearity

Linearity was determined by taking two diluted samples, one with a low C-Reactive Protein level and one with a higher level, and mixing them in the ratios given below. The measured concentrations were compared to the values determined for each diluted sample.

High Sample (26.75 ng/mL)	Low sample (1.67 ng/mL)	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
80%	20%	21.74	20.50	94.3%
60%	40%	16.72	16.27	97.3%
40%	60%	11.70	10.96	93.7%
20%	80%	6.68	6.35	95.0%
			Mean Recovery	**95.1%**



High Dose Hook

The assay was tested to evaluate the effect of high CRP values on signal. Samples with very high CRP levels were tested. hCRP up to 100,000 ng/mL was tested in the assay and read at 80% of the highest standard.

Intra Assay Precision

A serum sample was diluted with diluted Assay Buffer and run in replicates of twenty in an assay. The mean and precision of the calculated CRP concentrations were:

Sample	C-Reactive Protein Conc. (ng/mL)	%CV
1	40.65	7.8
2	31.17	8.0
3	19.37	7.0

Inter Assay Precision

A serum sample was diluted with Assay Buffer and run in duplicates in thirty assays run over multiple days by five operators. The mean and precision of the calculated CRP concentrations were:

Sample	C-Reactive Protein Conc. (ng/mL)	%CV
1	41.09	9.9
2	29.72	11.5
3	16.92	9.7

SAMPLE VALUES

Six human plasma samples were tested in the kit. Normal plasma levels ranged from 0.036 to 6.28 µg/mL. Disease state CRP plasma values read from 27.3 to 147 µg/mL. Numerous human serum samples were also tested and levels ranged from 0.036 to over 80 µg/mL.

The Mayo Clinic Laboratories states normal CRP levels in serum are ≤ 8 µg/mL.

CROSS REACTIVITY

The following cross reactants were tested in the assay and cross reactivity calculated within the standard curve.

Steroid	Cross Reactivity (%)
human CRP	100%
recombinant mouse CRP	0%
recombinant rat CRP	0%
recombinant porcine CRP	0%
recombinant Pentraxin 2/SAP	0%

LIMITED WARRANTY

Arbor Assays and Athens Research and Technology warrant that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 5 days of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

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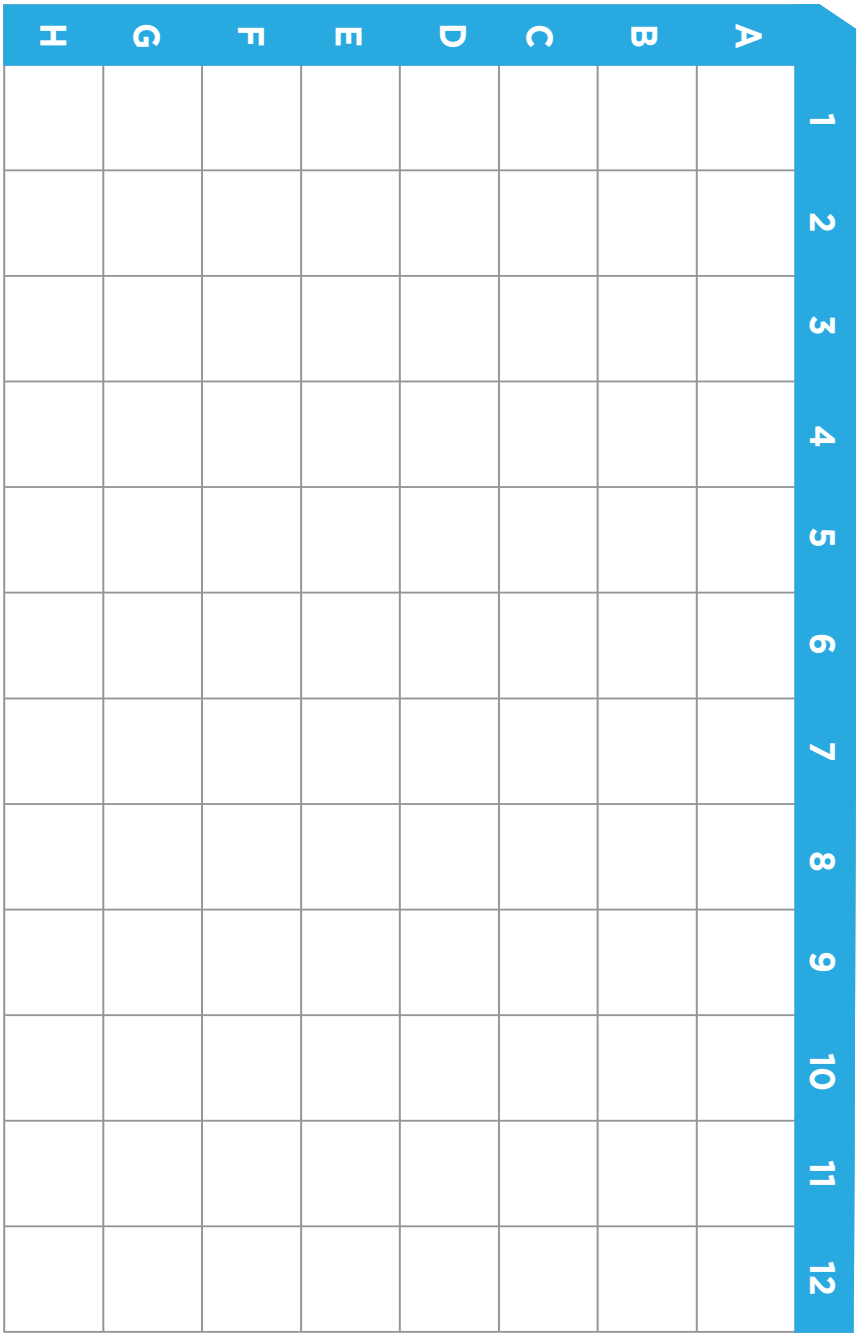

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