



High Sensitivity

SALIVARY CORTISOL

ENZYME IMMUNOASSAY KIT

For Diagnostic In-Vitro Use

CE

Item No. 1-3102, (Single) 96-Well Kit;

1-3102-5, (5-Pack) 480 Wells

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Must use IFU that is shipped with product.

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HS SALIVARY CORTISOL EIA KIT

Intended Use

The Salimetrics™ cortisol kit is a competitive immunoassay specifically designed and validated for the quantitative *in vitro* diagnostic measurement of salivary cortisol. This kit may be used to measure adrenal cortical function and as a screen for Cushing's and Addison's disease. (1,2) Saliva cortisol accurately reflects the amount of serum cortisol in the circulation. Salimetrics has not validated this kit for use with serum or plasma samples.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in false values.

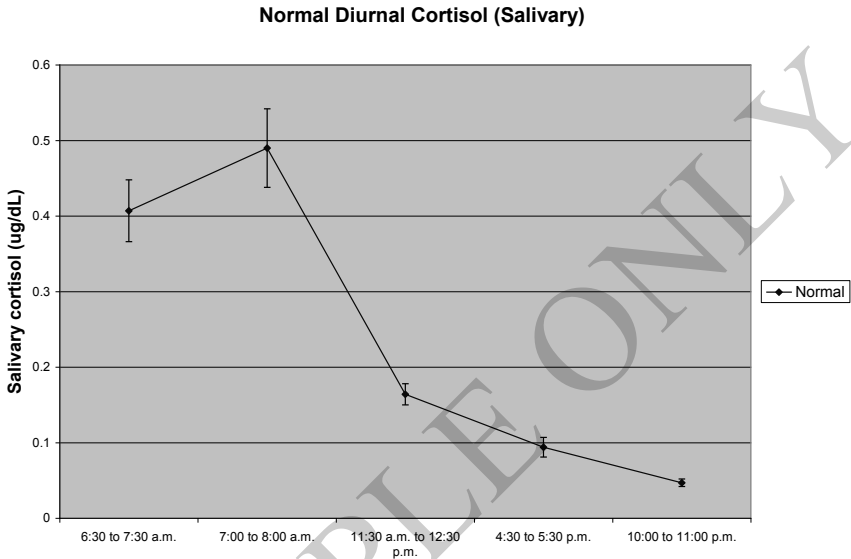
For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

Cortisol (hydrocortisone, Compound F) is the major glucocorticoid produced in the adrenal cortex. Cortisol production has a circadian rhythm. (3) Levels peak in the early morning and drop to the lowest concentration at night. (4) Levels rise independently of circadian rhythm in response to stress. (5) Increased cortisol production is associated with Cushing's syndrome, while decreased cortisol production is associated with adrenal insufficiency (e.g., Addison's disease). (6)

In blood, only about 5-10% of cortisol is in its unbound or biologically active form. The remaining cortisol is bound to serum proteins. (7) Unbound serum cortisol enters the saliva via intracellular mechanisms; in saliva, the majority of cortisol remains unbound to protein. (8) Salivary cortisol levels are unaffected by salivary flow rate and are relatively resistant to degradation from enzymes or freeze-thaw cycles. (8,9)

Studies consistently report high correlations between serum and saliva cortisol, indicating that salivary cortisol levels reliably estimate serum cortisol levels. (10-12)



(Internal Salimetrics Data, n=26. Time of cortisol peak will vary in individuals relative to their normal wake-up time.)

Test Principle

A microtitre plate is coated with monoclonal antibodies to cortisol. Cortisol in standards and unknowns competes with cortisol linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound cortisol peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of cortisol peroxidase detected, as measured by the intensity of color, is inversely proportional to the amount of cortisol present. (13)

pH Indicator

A pH indicator in the assay diluent alerts the user to samples with high or low pH values. Acidic samples will turn the diluent yellow. Alkaline samples will turn the diluent purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Cortisol values from samples with a pH ≤ 3.5 or ≥ 9.0 may be artificially inflated or lowered. (14)

Storage

All components of this kit are stable at 2-8°C until the kit's expiration date.

Safety Precautions

- Liquid stop is a 3-molar solution of sulfuric acid. This solution is caustic; use with care.
- See “Material Safety Information” at the end of procedure.

Materials Needed But Not Supplied

- Precision pipette to deliver 15 and 25 μL
- Precision multichannel pipette to deliver 50 μL and 200 μL
- Vortex
- Plate rotator with 0.08–0.17 inch orbit (if unavailable, tap plate to mix)
- Plate reader with a 450 nm filter
- Log-linear graph paper or computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable tube capable of holding 24 mL
- Pipette tips
- Serological pipette to deliver up to 24 mL

Materials Supplied with Single Kit

	Item	Quantity/Size
1	Microtitre Plate Coated with monoclonal anti-cortisol antibodies.	1/96-well
2	Cortisol Standards Ready to use, traceable to NIST standard: 3.0, 1.0, 0.333, 0.111, 0.037, 0.012 µg/dL (82.77, 27.59, 9.19, 3.06, 1.02, 0.33 nmol/L). Contain: cortisol, buffer, preservative.	6 vials/500 µL each
3	Cortisol Controls High, Low. Ready to use. Contain: cortisol, buffer, preservative.	2 vials/500 µL each
4	Wash Buffer Concentrate (10x) Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle/100 mL
5	Assay Diluent Contains: phosphate buffer, pH indicator, preservative.	1 bottle/60 mL
6	Cortisol Enzyme Conjugate Concentrate. Dilute before use with assay diluent. (See step 5 of Procedure.) Contains: cortisol conjugated to HRP, preservative.	1 vial/50 µL
7	TMB Substrate Solution Non-toxic, ready to use.	1 bottle/25 mL
8	3 M Stop Solution Contains: sulfuric acid.	1 bottle/12.5 mL
9	Non-Specific Binding (NSB) Wells Do not contain anti-cortisol antibody. Break off and insert as blanks (optional) where needed.	1 strip

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Bovine hormones normally present in dairy products can cross-react with anti-cortisol antibodies and cause false results. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Donors may collect whole saliva by tilting the head forward, allowing the saliva to pool on the floor of the mouth, then passing the saliva through a short straw into a polypropylene vial. Samples from adults and from children ages 6 and above may also be collected using the Salimetrics Oral Swab (SOS), Item No. 5001.02. Samples from children under the age of 6 may be collected with the Salimetrics Children's Swab (SCS), Item No. 5001.06. The Salimetrics Infant's Swab (SIS), Item No. 5001.08, is available for use with children under the age of 6 months.

Samples visibly contaminated with blood should be recollected. Samples collected from populations that have little or no dental care, or known oral health problems, may be screened for possible blood contamination (15,16) using our Blood Contamination EIA Kit (Item No.1-1302;1-3102-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

It is important to record the time and date of specimen collection when samples are obtained due to the diurnal variation in cortisol levels. Samples for Cushing's diagnosis should be collected at 11:00 pm.

Sample Handling and Preparation

After collection it is important to keep samples cold, in order to avoid bacterial growth in the specimen. Refrigerate samples within 30 minutes, and freeze at or below -20°C within 4 hours after collection. (Samples may be stored at -20°C or lower for long-term storage.) ***Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.***

Freezing saliva samples will precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Samples

should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Be careful not to disrupt the pellet, since particulate matter may interfere with antibody binding, leading to falsely elevated results.

Avoid multiple freeze-thaw cycles. However, if samples have been refrozen, centrifuge again prior to assaying.

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3° C). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- Routine calibration of pipettes is critical for the best possible assay performance.

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is necessary for the 24 mL of assay diluent used in Step 5 (conjugate dilution) to come to room temperature.
- Bring microtitre plate to room temperature before use. ***It is important to keep the pouch with the plate strips closed until warmed to room temperature as humidity may have an effect on the coated wells.***
- Prepare 1X wash buffer by diluting wash buffer concentrate 10-fold with room-temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H₂O). ***Dilute only enough for current day's use, and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)

Procedure

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.000 Std	3.000 Std	Control High	Control High								
B	1.000 Std	1.000 Std	Control Low	Control Low								
C	0.333 Std	0.333 Std	Unknown 1	Unknown 1								
D	0.111 Std	0.111 Std	Unknown 2	Unknown 2								
E	0.037 Std	0.037 Std	Unknown 3	Unknown 3								
F	0.012 Std	0.012 Std	Unknown 4	Unknown 4								
G	Zero	Zero	Unknown 5	Unknown 5								
H	NSB	NSB	Unknown 6	Unknown 6								

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place

the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSBs included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch containing unused wells and desiccant. Store at 2-8°C.

Cautions: 1. Extra NSB wells should not be used for determination of standards, controls or unknowns.

2. Do not insert wells from one plate into a different plate.

Step 3: Pipette 24 mL of assay diluent into a disposable tube. Set aside for Step 5.

Step 4:

- Pipette 25 μ L of standards, controls, and unknowns into appropriate wells. Standards, controls, and unknowns should be assayed in duplicate.
- Pipette 25 μ L of assay diluent into 2 wells to serve as the zero value.
- Pipette 25 μ L of assay diluent into each NSB well.

Step 5: Make a 1:1600 dilution of the conjugate by adding 15 μ L of the conjugate to the 24 mL of assay diluent prepared in Step 3. (Scale down proportionally if not using the entire plate.) Immediately mix the diluted conjugate solution and pipette 200 μ L into each well using a multichannel pipette.

Step 6: Mix plate on rotator for 5 minutes at 500 rpm (or tap to mix) and incubate at room temperature for an additional 55 minutes.

Step 7: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 μ L of wash buffer into each well, and then discarding the liquid by inverting the plate over a sink. After each wash, the plate should be thoroughly blotted on paper towels before being turned upright. ***If using a plate washer, blotting is still recommended after the last wash.***

Step 8: Add 200 μ L of TMB solution to each well with a multichannel pipette.

Step 9: Mix on a plate rotator for 5 minutes at 500 rpm (or tap to mix) and incubate the plate in the dark at room temperature for an additional 25 minutes.

Step 10: Add 50 μL of stop solution with a multichannel pipette.

Step 11:

- Mix on a plate rotator for 3 minutes at 500 rpm (or tap to mix).

Caution: *Spillage may occur if mixing speed exceeds 600 rpm.*

- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding stop solution. (For best results, a secondary filter correction at 490 to 630 nm is recommended.)

Limitations

- Diagnosis of Cushing's syndrome should be confirmed by additional diagnostic tests for the disease, such as low-dose dexamethasone suppression testing.
- Cortisol levels are elevated during the later stages of pregnancy and in women on oral contraceptives or after long-term use of oral contraceptives. (12,17)
- Some studies show developmental differences in cortisol as well as an association between cortisol and weight. (18)
- Elevated cortisol levels can be found in conditions of sepsis, infection, chronic liver disease, and renal failure. Low cortisol levels result from liver disease, pituitary hyposecretion, hypothyroidism, or steroid therapy.
- See "Specimen Collection" recommendations to insure proper collection of saliva specimens and to avoid interfering substances.

Assay Summary

1. Bring all reagents to room temperature and mix before use.
2. Bring plate to room temperature and prepare for use with NSB wells. (Use of NSB wells is optional.)
3. Prepare 1X wash buffer.
4. Prepare tube with 24 mL of assay diluent for conjugate dilution, which will be made later.
5. Pipette 25 μ L of standards, controls, and unknowns into appropriate wells.
6. Pipette 25 μ L of assay diluent into zero and NSB wells.
7. Make final 1:1600 dilution of conjugate (15 μ L into 24 mL assay diluent), mix, and immediately pipette 200 μ L into each well.
8. Mix plate for 5 minutes at 500 rpm. Incubate for an additional 55 minutes at room temperature.
9. Wash plate 4 times with 1X wash buffer. Blot.
10. Add 200 μ L TMB solution to each well.
11. Mix plate for 5 minutes at 500 rpm. Incubate in dark at room temperature for 25 additional minutes.
12. Add 50 μ L stop solution to each well. Mix for 3 minutes at 500 rpm.
13. Wipe plate bottom clean and read within 10 minutes of adding stop.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns.
3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the controls and unknowns by interpolation using software capable of logistics. We recommend using a 4-parameter sigmoid minus curve fit.
5. If a dilution of the sample is used, multiply the results by the dilution factor. Samples with cortisol values greater than 3.0 $\mu\text{g/dL}$ (82.77 nmol/L) should be diluted with assay diluent and rerun for accurate results.

When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.

Quality Control

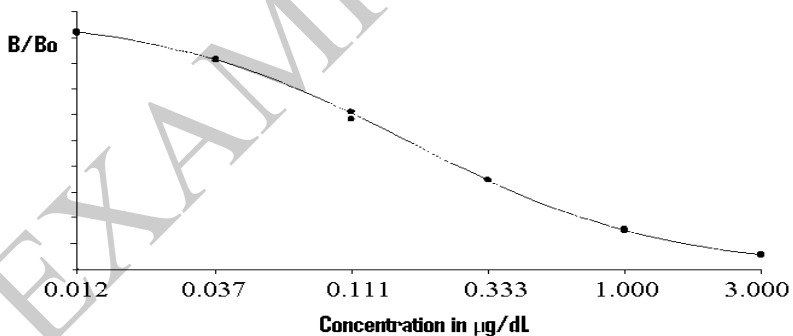
The Salimetrics high and low salivary cortisol controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

The following chart and graph are for illustration only and should not be used to calculate results from another assay.

Well	Sample	Average OD	B	B/Bo	Cortisol ($\mu\text{g/dL}$)
A1,A2	S1	0.094	0.071	0.048	3.000
B1,B2	S2	0.236	0.213	0.145	1.000
C1,C2	S3	0.524	0.501	0.340	0.333
D1,D2	S4	0.897	0.874	0.593	0.111
E1,E2	S5	1.219	1.196	0.812	0.037
F1,F2	S6	1.379	1.356	0.921	0.012
G1,G2	Bo	1.496	1.473	NA	NA
H1,H2	NSB	0.023	NA	NA	NA

Example: HS Cortisol 4-Parameter Sigmoid Minus Curve Fit



Material Safety Information*

Hazardous Ingredients

Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. Specific kit component MSDS sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

HS Salivary Cortisol EIA Kit Performance Characteristics

Correlation with Serum

The correlation between serum and saliva cortisol was determined by assaying 49 matched samples using the Diagnostic Systems Laboratories serum Cortisol EIA and the Salimetrics HS Salivary Cortisol EIA.

The correlation between saliva and serum was highly significant, $r(47) = 0.91$, $p < 0.0001$.

Linearity of Dilution

Two saliva samples were diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1			2.176	
	1:2	1.088	1.065	97.9
	1:4	0.544	0.503	92.5
	1:8	0.272	0.233	85.7
	1:16	0.136	0.109	80.1
2			0.508	
	1:2	0.254	0.247	97.2
	1:4	0.127	0.118	92.9
	1:8	0.064	0.058	90.6
	1:16	0.032	0.031	96.9

Recovery

Six saliva samples containing different levels of endogenous cortisol were spiked with known quantities of cortisol and assayed.

Sample	Endogenous (µg/dL)	Added (µg/dL)	Expected (µg/dL)	Observed (µg/dL)	Recovery (%)
1	0.088	2.000	2.088	2.176	104.2
2	0.077	0.300	0.377	0.380	100.8
3	0.062	0.011	0.073	0.071	97.3
4	0.066	2.500	2.566	2.723	106.1
5	0.210	0.300	0.510	0.508	99.6
6	0.086	0.011	0.097	0.094	96.9

Specificity of Antiserum

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS Salivary Cortisol EIA
Prednisolone	100	0.568
Prednisone	1000	ND
Cortisone	1000	0.130
11-Deoxycortisol	500	0.156
21-Deoxycortisol	1000	0.041
17 α -Hydroxy-progesterone	1000	ND
Dexamethasone	1000	19.2
Triamcinolone	1000	0.086
Corticosterone	10,000	0.214
Progesterone	1000	0.015
17 β -Estradiol	10	ND
DHEA	10,000	ND
Testosterone	10,000	0.006
Transferrin	66,000	ND
Aldosterone	10,000	ND

ND = None detected (<0.004)

Precision

1. The intra-assay precision was determined from the mean of 14 (low) and 18 (high) replicates each.

Sample	N	Mean (µg/dL)	Standard Deviation (µg/dL)	Coefficient of Variation (%)
Level 1	18	0.999	0.033	3.35
Level 2	14	0.097	0.004	3.65

2. The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

Sample	N	Mean (µg/dL)	Standard Deviation (µg/dL)	Coefficient of Variation (%)
Level 1	12	1.020	0.038	3.75
Level 2	12	0.101	0.006	6.41

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 µg/dL level. The minimal concentration of cortisol that can be distinguished from 0 is < 0.003 µg/dL.

Salivary Cortisol Expected Ranges

Each laboratory should establish its own range of expected values. The following values have been reported for salivary cortisol.

Group	Number	Overall Range (µg/dL)
Children, neonatal	275	ND - 3.417
Children, age 6 months	165	ND - 2.734

Group	Number	AM Range (µg/dL)	PM Range (µg/dL)
Children, ages 2.5-5.5	112	0.034 - 0.645	0.053 - 0.607
Children, ages 8-11	285	0.084 - 0.839	ND - 0.215
Adolescents, ages 12-18	403	0.021 - 0.883	ND - 0.259
Adult males, ages 21-30	26	0.112 - 0.743	ND - 0.308
Adult females, ages 21-30	20	0.272 - 1.348	ND - 0.359
Adult males, ages 31-50	67	0.122 - 1.551	ND - 0.359
Adult females, ages 31-50	31	0.094 - 1.515	ND - 0.181
Adult males, ages 51-70	28	0.112 - 0.812	ND - 0.228
Adult females, ages 51-70	23	0.149 - 0.739	0.022 - 0.254
All adults	192	0.094 - 1.551	ND - 0.359

Group	Number	2300h (ug/dL)
Normal subjects	19	0.007 – 0.115
Cushing's subjects	21	0.130 – 2.972

ND = None detected

Expected ranges for neonates to 5.5 years were derived using the Salimetrics Salivary Cortisol Immunoassay Kit.

Expected ranges for 8 to 18 years were reported from an unpublished manuscript, Pennsylvania State University's Behavioral Endocrinology Laboratory. Adult ranges were obtained from published literature. (7)

References

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Seller's Limited Warranty

“Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in writing. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties.”

“European Authorized Representative”

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