Alpha Amylase Saliva ELISA
Test instruction

ORDER NO.	ANTIGEN	SUBSTRATE	FORMAT
EQ 6231-9601 S	Alpha amylase	Ab-coated microplate wells	96 x 01 (96)

Indication: Test system for the in vitro determination of alpha amylase in human saliva for the diagnosis of conditions associated with overproduction of alpha-amylase, such as suspected mental and physical stress, stress-related psychosomatic diseases, pain syndrome (fibromyalgia), and monitoring of the disease course and therapy in stress-related conditions.

Principles of the test: The ELISA test kit provides a quantitative in vitro assay for free alpha amylase in human saliva. The test kit contains microtiter strips each with 8 break-off reagent wells coated with anti-rabit antibodies. In the first reaction step, diluted patient samples are pipetted into the reagent wells together with peroxidase-labelled alpha-amylase and a specific rabbit anti-alpha amylase antibody. Alpha amylase from the patient sample and the labelled alpha amylase in the conjugate compete for the free binding sites of the specific antibody. In the third incubation step, the bound peroxidase catalyses a colour reaction with the peroxidase substrate tetramethyl benzidine (TMB). The intensity of the colour formed is inversely proportional to the concentration of alpha amylase in the sample. The results for the samples are determined using the standard curve.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells coated with antibodies 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1, 0 U/ml, ready for use	light red to dark red	1 x 1.0 ml	CAL 1
3. Calibrator 2, 10 U/ml, ready for use		1 x 1.0 ml	CAL 2
4. Calibrator 3, 30 U/ml, ready for use		1 x 1.0 ml	CAL 3
5. Calibrator 4, 80 U/ml, ready for use		1 x 1.0 ml	CAL 4
6. Calibrator 5, 200 U/ml, ready for use		1 x 1.0 ml	CAL 5
7. Calibrator 6, 500 U/ml, ready for use		1 x 1.0 ml	CAL 6
8. Control 1, ready for use	green	1 x 1.0 ml	CONTROL 1
9. Control 2, ready for use	blue	1 x 1.0 ml	CONTROL 2
10. Antiserum polyclonal anti-alpha amylase antibody (rabbit), ready for use	blue	1 x 12 ml	ANTIBODY SOLUTION
11. Enzyme conjugate peroxidase-labelled alpha amylase, ready for use	orange	1 x 12 ml	CONJUGATE
12. Sample buffer, ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
13. Wash buffer, 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
14. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
15. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
16. Protective foil	---	3 pieces	
17. Test instruction	---	1 booklet	
18. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostics
CE
Storage temperature
Unopened usable until

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antibodies can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.
- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.
- **Antiserum:** Ready for use. The antiserum must be mixed thoroughly before use.
- **Sample buffer:** Ready for use.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready for use.

Warning: Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Preparation and stability of the patient samples

Sample material: Human saliva (total saliva).
EUROIMMUN recommends collecting saliva samples with blue cortisol Salivette® (Sarstedt AG & Co, Germany).

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:201** sample buffer.
For example: 5 µl sample to 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

Sample incubation: (1. step)

Transfer **20 µl of the calibrators, controls and diluted patient samples** into the individual microplate wells according to the pipetting protocol.

Pipette **100 µl of enzyme conjugate solution** (peroxidase-labelled alpha-amylase) into each of the microplate wells.

Pipette **100 µl of antiserum solution** (polyclonal anti-alpha amylase antibody) into each of the microplate wells.

Cover the microplate wells with the protective foil provided and incubate for **60 minutes** on a **microplate shaker (400 U/min)** at room temperature (+18 °C to +25 °C).

Washing:

Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells 3 times with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Modus”).

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to false high extinction values.

Substrate incubation: (2. step)

Pipette **100 µl of chromogen/substrate solution** into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C). Protect from direct sunlight.

Stopping the reaction:

Pipette **100 µl of stop solution** into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:

Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Pipetting protocol

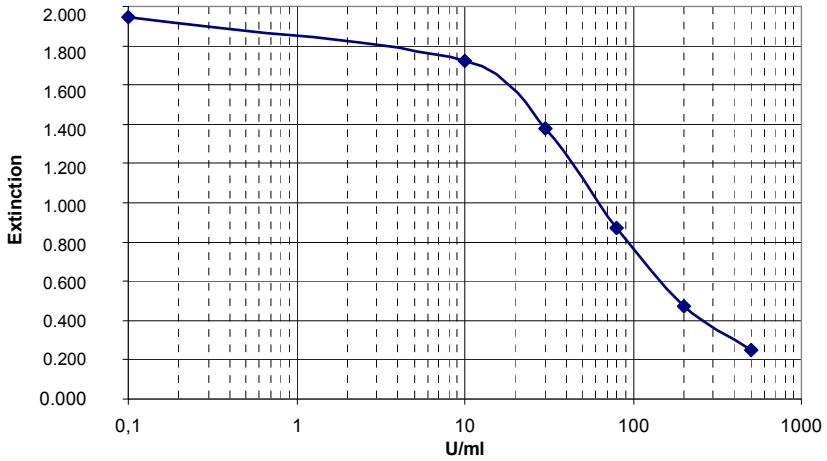
	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 1**	**P 1**	**P 9**	**P 17**								
B	**C 2**	**P 2**	**P 10**	**P 18**								
C	**C 3**	**P 3**	**P 11**	**P 19**								
D	**C 4**	**P 4**	**P 12**	**P 20**								
E	**C 5**	**P 5**	**P 13**	**P 21**								
F	**C 6**	**P 6**	**P 14**	**P 22**								
G	**Co1**	**P 7**	**P 15**	**P 23**								
H	**Co2**	**P 8**	**P 16**	**P 24**								

The pipetting protocol for microtiter strips 1-4 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 6), the controls 1+2 (Co1 + Co2), and the patient samples have each been incubated in one well. The reliability can be improved by duplicate determinations for each sample.
The controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Quantitative: The standard curve from which the alpha-amylase concentration in the unknown saliva samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 6 calibration sera against the corresponding units (linear/log). Use "4-parameter logistics" plotting for calculation of the standard curve by computer. For correct logarithmic representation it might be necessary to set the concentration of calibrator 1 to e.g. 0.1 U/ml. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of alpha-amylase concentrations in patient samples.



If the extinction of a patient sample lies above the value of calibrator 6 (500 U/ml), the result should be given as “>500 U/ml”. It is recommended that the sample be re-tested at a dilution of e.g. 1:401 instead of 1:201. The result in U/ml read from the calibration curve for this sample must then be multiplied by a factor of 2.

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

Therapeutic decisions should not be made on the basis of results from this test, but only under consideration of clinical findings and further diagnostic values.

For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

Test characteristics

Calibration: The standards and controls are calibrated gravimetrically.

For every group of tests performed, the values of the concentrations must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antibodies: A polyclonal anti-alpha amylase antibody is used, which specifically detects alpha amylase in human saliva.

Linearity: The linearity of the ELISA was determined by assaying 4 serial dilutions of 3 saliva samples. The linear regression was calculated and R^2 amounts to > 0.95 in all samples. The Alpha Amylase Saliva ELISA is linear at least in the tested concentration range (12 to 500 U/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable alpha amylase concentration. The detection limit of the Alpha Amylase Saliva ELISA is 3.6 U/ml.

Cross reactivity: This ELISA specifically detects alpha amylase in human saliva. Cross reactions with other amylases are listed in the table below:

Cross reactant	%
Alpha amylase in human saliva	100
Porcine pancreatic alpha amylase	< 0.23
Alpha amylase from Bacillus sp.	< 0.01

Interference: Contamination with blood up to a concentration of 4.0 % (v/v) did not cause interference with the ELISA. Red tint of the alpha-amylase sample indicates significant contamination with blood. The sample should not be used. We recommend taking a new sample at a later stage instead. Sodium azide can be added as preservative agent. A concentration of up to 0.9% has no effect on the measurement result.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation using 3 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on duplicate determinations performed in 5 different runs.

Intra-assay variation, n = 20		
Saliva	**Mean value (U/ml)**	**CV (%)**
1	100	4.9
2	148	3.6
3	242	5.5

Inter-assay variation, n = 2 x 5		
Saliva	**Mean value (U/ml)**	**CV (%)**
1	23	9.6
2	105	4.2
3	234	4.8

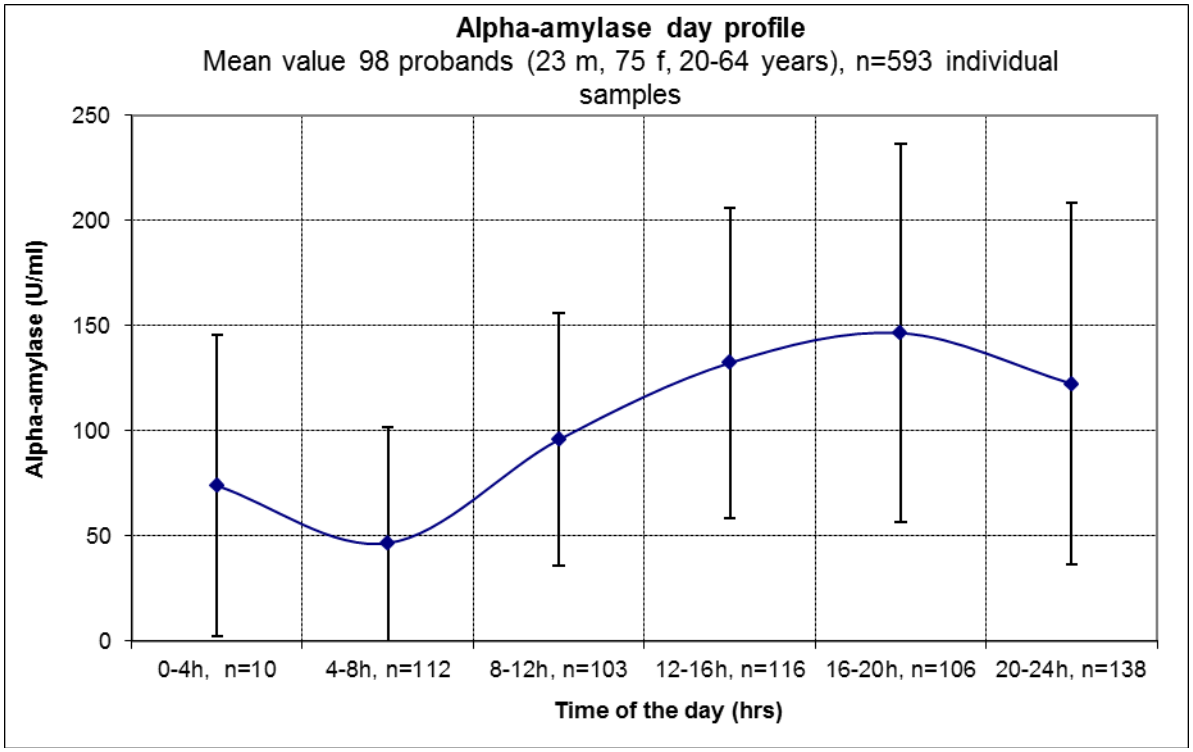
Correlation: A comparison of the EUROIMMUN assay with several reference tests yielded the following correlation values (sample range of 0-958 U/ml):

Salimeterics, Alpha amylase enzymatic test	**EI = 1.04 x Salimetrics – 1.56 U/ml** **n = 38; R^2 = 0.982**

Reference range: 593 saliva samples from healthy adults between 20 and 64 years of age (75 women, 23 men) were analysed using the EUROIMMUN Alpha Amylase Saliva ELISA.

Alpha amylase concentration (n = 98 test persons, measured between 0 a.m. and 12 p.m.)			
		Mean value	**Standard deviation**
Time of day	**n=**	**U/ml**	
0-4 a.m.	9	73.8	71.4
4-8 a.m.	113	46.5	54.8
8-12 a.m.	104	95.7	60.2
12-4 p.m.	119	132.2	73.8
4-8 p.m.	108	146.3	89.9
8-12 p.m.	140	122.2	86.2
n total=	593		

Standard values can be determined from the measured average diurnal profile. They agree with data from the literature (Rohleder, 2004, Ann. N.Y. Acad. Sci. 1032: 258-263).



Every laboratory should use their own normal values established under specific ambient conditions.

Clinical significance

The Alpha Amylase Saliva ELISA (sAA ELISA) is designed for the determination of the alpha amylase concentration in saliva depending on the mental burden, particularly stress, of the patient [1, 2].

The enzyme alpha amylase is a digestive ferment which cleaves polysaccharides (long carbohydrate chains) such as “vegetable starch” (amylose and amylopectin, which are found for example in flour) and "animal starch" (glycogen, which occurs in the liver and muscles) into smaller sugar molecules (e.g. malt sugar and other so-called disaccharides) via hydrolysis of 1,4-α-D-glycoside bonds. Three isoforms of alpha amylase are produced in the salivary glands, especially of the ear, and two in the pancreas. They are released into the mouth or duodenum. Some smaller amounts also enter the blood stream.

The relationship between mental/psychiatric stress and physical health was not well researched or measured using scientific methods until the end of the 20th century, and particularly the beginning of the 21st century [2, 3]. Significantly, autoimmune diseases, tumours (particularly carcinoma) and possibly also allergies are often caused by acute or long duration of mental stress in addition to genetic components (gene mutation) and noxious substances (e.g. virus infections).

Today it is possible to measure mental or psychosocial stress, in addition to physical stress, by objective and reproducible determination of antibody and enzyme concentrations (biological stress markers) in saliva using immunological test methods. The effect of stress sensors on the immune system can be assessed reliably, easily and economically without the need to carry out complicated tests such as the investigation of catecholamines, cortisol, lactoferrin, IL-2, IL-6 or IL-12 or heart frequency analysis [1, 2, 3, 4, 6].

Like the Secretory IgA ELISA (sIgA ELISA), the Alpha Amylase Saliva ELISA (sAA ELISA) is a test for measuring the degree of the individual physiopathological effect of stress (stress reactivity) in saliva samples [1, 2, 3, 4, 5, 7]. The concentration of sAA is independent of the amount of saliva produced [8].
The effect of stress on the functioning of inner organs via the autonomic nervous system is explained by the association of sAA secretion with the activity of the autonomic (sympathetic and parasympathetic) nervous system [2, 4]. Thus, stress (e.g. school stress, harassment, etc.), which can be measured indirectly using sAA ELISA, can cause psychosomatic diseases such as cardiovascular symptoms [2, 3].
It is significant that the enzyme level (alpha amylase) in saliva is directly proportional to the immunoglobulin level (IgA) and is direct proportional to the degree of stress [3, 9, 10]. An increased sAA titer can also occur in parotitis, independently of mental stress.

In adults, the sAA peak is achieved 10 to 20 minutes after the onset of physical stress, in children much earlier (after approx. 5 minutes) [9]. With psychosocial stress the sAA titer generally increases later [6, 9]. The sAA level starts to decrease approx. 10 minutes after the stress has subsided [3].

Since stress is a multidimensional condition and sAA results may be affected by nicotine or caffeine intake, different physiological systems should be analysed for diagnosis using saliva tests, e.g. sAA ELISA combined with sIgA ELISA [1, 3]. As an additional test, the concentration of cortisol, the most potent human corticoid, can be determined in saliva. The production of sAA is independent of pancreatic alpha amylase synthesis. Furthermore, the serum alpha amylase level is not directly linked to the sAA concentration.

The effectiveness of psychotherapeutic stress therapy and drug-therapy for the treatment of autonomic stress responses (e.g. using beta blockers) can be monitored by investigation of the sAA titer [11].
Indication for the use of the Alpha Amylase Saliva ELISA (non-invasive measuring of the stress psychobiology in saliva) [2, 3]:

1. Suspected clinical cases of
   - stress (physical and/or mental/psychosocial)
   - stress-related psychosomatic disease
   - pain syndrome (e.g. fibromyalgia)
2. Monitoring of disease course and therapy success in stress-related diseases

Literature references

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