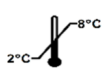
LDN®
IMMUNOASSAYS AND SERVICES
BIOGENIC AMINES & NEUROSCIENCE | ENDOCRINOLOGY | FOOD SAFETY
LABOR DIAGNOSTIKA NORD GmbH & Co.KG | Am Eichenhain 1 | 48531 Nordhorn | Germany | Tel. +49 5921 8197-0 | Fax +49 5921 8197-222 | info@ldn.de | www.ldn.de

Instructions for use

Cortisol Saliva ELISA Free

REF SA E-6000R



RUO For research use only – Not for use in diagnostic procedures

Cortisol Saliva ELISA Free

1 INTRODUCTION

1.1 Intended Use

Enzyme immunoassay for the quantitative determination of active free cortisol in human saliva.
The assay is intended for research use only. Manual processing is recommended. The usage of laboratory automats is the user's sole responsibility. The kit is intended for single use only.

1.2 Description of the analyte

Cortisol (hydrocortisone) is the major glucocorticoid produced in the adrenal cortex. Cortisol is a potent stress hormone and the secretion is regulated by the Hypothalamic-Pituitary-Adrenal-axis (HPA-axis).
The secretion of cortisol has a specific circadian rhythm with a curve presenting a sharp peak in the early morning and a gradually decrease over the day with a nadir in the evening (7). The position of this peak-value is strongly influenced by the average wake-up time during the past weeks. It is not dependent on the actual wake-up time of the specific day of sample collection (if different from the average wake-up time of the past week).

2 PRINCIPLE

The **Cortisol Saliva ELISA Free** Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a polyclonal rabbit antibody directed against the cortisol molecule. The samples are dispensed in the coated wells and incubated with the enzyme conjugate (cortisol conjugated to horseradish peroxidase). During incubation endogenous cortisol of a sample competes with the enzyme conjugate for binding to the coated antibody. The unbound conjugate is removed by washing the wells.
Subsequently, the substrate solution is added and the color development is stopped after a defined time. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample. The optical density (OD) is measured at 450 nm with a microtiter plate reader. A standard curve is constructed by plotting OD values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

3 WARNINGS AND PRECAUTIONS

1. This kit is for research use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. The microplate contains break apart strips. Unused wells must be stored at 2 – 8 °C in the sealed foil pouch and used in the frame provided.
4. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
5. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution coloured. Do not pour reagents back into vials as reagent contamination may occur.
6. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
7. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
8. Allow the reagents to reach room temperature (18 – 25 °C) before starting the test. Temperature will affect the absorbance readings of the assay.
9. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
10. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
11. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
12. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
13. Do not use reagents beyond expiry date as shown on the kit labels.
14. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
15. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
16. Avoid contact with Stop Solution. It may cause skin irritation and burns.
17. Some reagents contain Proclin 300, CMIT and MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
18. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
19. For information please refer to Safety Data Sheets. Safety Data Sheets for this product are available upon request directly from the manufacturer.
20. If product information, including labeling, is incorrect or inaccurate, please contact the kit manufacturer or supplier.

4 REAGENTS

4.1 Reagents provided

1. **96** **SA E-6031 Microtiterwells**
12x8 (break apart) strips, 96 wells; wells coated with rabbit anti-cortisol antibody (polyclonal).

2. **Standards and Controls** – ready to use

	Cat. no.	Standard	Concentration	Volume/Vial
STANDARD A	**SA E-6001**	Standard A	0 ng/ml	2.0 ml
STANDARD B	**SA E-6002**	Standard B	0.1 ng/ml	0.5 ml
STANDARD C	**SA E-6003**	Standard C	0.4 ng/ml	0.5 ml
STANDARD D	**SA E-6004**	Standard D	1.7 ng/ml	0.5 ml
STANDARD E	**SA E-6005**	Standard E	7.0 ng/ml	0.5 ml
STANDARD F	**SA E-6006**	Standard F	30 ng/ml	0.5 ml
CONTROL 1	**SA E-6051**	Control 1	For control values and ranges please refer to QC-Report.	0.5 ml
CONTROL 2	**SA E-6052**	Control 2		0.5 ml

Buffer matrix spiked with defined concentration of cortisol.
Conversion factor: 1 ng/ml = 2.76 nmol/l

3. **CONJUGATE** **SA E-6040 Enzyme Conjugate** – ready to use
1 vial, 7.0 ml; cortisol conjugated to horseradish peroxidase
Hazards identification:

H317 May cause an allergic skin reaction.

4. **SUBSTRATE** **AR E-0055 Substrate Solution** – ready to use
1 vial, 22 ml; Tetramethylbenzidine (TMB)

5. **STOP-SOLN** **AR E-0080 Stop Solution** – ready to use
1 vial, 7.0 ml; contains 2 N hydrochlorid acid.
Avoid contact with the stop solution. It may cause skin irritations and burns.
Hazards identification:

H290 May be corrosive to metals.
H314 Causes severe skin burns and eye damage.
H335 May cause respiratory irritation.

6. **WASH-CONC 10x** **AR E-0030 Wash Solution**
1 vial, 50 ml (**10x** concentrated); see „Preparation of Reagents".

4.2 Material required but not provided

- Microcentrifuge
- A calibrated microtiter plate reader (450 nm)
- Microplate mixer operating at 900 rpm
- Manual or automatic equipment for microtiter plate washing
- Vortex mixer
- Calibrated variable precision micropipettes and multichannel pipettes with disposable pipette tips
- Absorbent paper
- Distilled or deionized water
- Timer
- Semi logarithmic graph paper or software for data reduction

4.3 Storage conditions

When stored at 2 – 8 °C unopened reagents will be stable until expiration date. Do not use reagents beyond this date. Opened reagents must be stored at 2 – 8 °C. After first opening the reagents are stable for 30 days if used and stored properly. Keep away from heat and direct sunlight.
Microtiter wells must be stored at 2 – 8 °C. Take care that the foil bag is sealed tightly.

4.4 Preparation of reagents

Allow the reagents and the required number of wells to reach room temperature (18 – 25 °C) before starting the test.

Wash Solution
Dilute 50 ml of 10X concentrated *Wash Solution* with 450 ml deionized water to a final volume of 500 ml. The diluted Wash Solution is stable for at least 12 weeks at room temperature (18 – 25 °C). Precipitates may form when stored at 2 – 8 °C, which should dissolve again by swirling at room temperature (18 – 25 °C). The Wash Solution should only be used when the precipitates have completely dissolved.

4.5 Disposal of the kits

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Safety Data Sheet.

4.6 Damaged test kits

In case of any severe damage of the test kit or components, the manufacturer have to be informed written, latest one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN COLLECTION AND PREPARATION

Samples containing sodium azide must not be used in the assay. The saliva samples should be completely colorless. Even the slightest red color shows blood contamination. Such blood contamination will result in falsely elevated concentration values. In case of visible blood contamination the donor should discard the sample, rinse the collection device with water, also rinse the mouth with (preferably) cold water, wait for 10 minutes and take a new sample. Chewing anything during the sampling period must be avoided. Any pressure on the teeth may result in falsely elevated measurements due to an elevated content of gingival liquid in the saliva sample.

5.1 Specimen Collection

For the correct collection of saliva we recommend to only use appropriate devices made from ultra-pure polypropylene (PP). Do not use any PE devices or Salivettes for sampling; in most cases this will result in significant interferences. Glass tubes can be used as well, but in this case special attention is necessary for excluding any interference caused by the stopper.
As the Cortisol secretion in saliva as well as in serum shows an obvious secretion pattern throughout the day, it is important to care for a proper sample timing of the sampling. In order to avoid arbitrary results it is recommended to always collect five samples within a period of two hours (multiple sampling) preferably before a meal. The morning peak normally appears during the first two hours after the average wake-up time. Therefore it is recommended to take five separate samples within a period of two hours (multiple sampling) directly after the usual wake-up time (e.g. 1 min, 30 min, 60 min, 90 min and 120 min). It is important to know that the timing of the morning peak is not related to the absolute time or day light. It is just related to the wake-up habits of the donor. If possible the volume of each single sample should be a minimum of 0.5 ml (better 1 ml).
As food might contain significant amounts of steroid hormones samples should be taken preferably while fasting. Do not collect samples within 60 minutes after eating a major meal, 12 hours after consuming alcohol or 60 minutes after brushing teeth. Rinse mouth with water 10 minutes prior to specimen collection. Furthermore please avoid any strenuous physical exercises and intense stress situations.

5.2 Specimen Storage and Preparation

Saliva samples may be stored at 2 – 8 °C for up to one week. For longer storage, it is recommended to store the samples at ≤ -20 °C. Whenever possible samples preferably should be kept at a temperature of ≤ -20 °C. Avoid multiple freeze-thaw cycles.
Each sample has to be frozen, thawed, and centrifuged at least once in order to precipitate and separate the mucins. Upon arrival of the samples in the lab the samples have to be stored frozen at least overnight. In the next morning the frozen samples are thawed, brought to room temperature and mixed carefully. Then the samples have to be centrifuged for five to ten minutes. Now the clear colorless supernatant is easy to pipette. If the sample should show even a slight reddish tinge it should be discarded. Otherwise the concentration value most probably will be falsely elevated. Due to the episodic variations of the cortisol secretion the strategy of multiple sampling is highly recommended. If such a set of multiple samples has to be tested the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the aliquots of the five single samples in a separate sampling device and perform the testing from this mixture. If the shape of the morning peak has to be determined, all five morning samples have to be tested separately.

5.3 Specimen Dilution

If in an initial assay a specimen is found to contain more cortisol than the highest standard, the specimens must be diluted with *Standard A* and re-assayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.

6 ASSAY PROCEDURE

6.1 General remarks

- All reagents and specimens must be allowed to come to room temperature (18 – 25 °C) before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control, or sample in order to avoid cross contamination.
- Absorbance is a function of incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equally elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.
- Respect the incubation times as stated in this instructions for use.
- Duplicate determination of standards, controls and samples is recommended in order to identify potential pipetting errors.
- Microtiter plate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or a multistepper, or an automatic microtiter plate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with wash solution, and that there are no residues in the wells.
- A standard curve must be established for every run.

6.2 Assay procedure

1.	Secure the desired number of coated strips in the frame holder.
2.	Dispense **50 µl** of each **Standard, Control** and **Sample** in duplicates with new disposable tips into appropriate wells.
3.	Dispense **50 µl** of **Enzyme Conjugate** into each well.
4.	Incubate for **60 minutes** at room temperature (18 – 25 °C) on a plate shaker (900 rpm).
5.	Briskly empty the contents of the wells by aspiration or by decanting. Rinse the wells 4 times with **diluted Wash Solution (300 µl per well)**. Strike the wells sharply on absorbent paper to remove residual droplets. **Important note:** The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6.	Add **200 µl** of **Substrate Solution** to each well.
7.	Incubate for **30 minutes** without shaking in the dark at room temperature (18 – 25 °C).
8.	Stop the enzymatic reaction by adding **50 µl** of **Stop Solution** to each well.
9.	Determine the optical density (OD) of each well at **450 nm** within 15 minutes after adding the Stop Solution.

6.3 Calculation of results

1. Calculate the average optical density values for each set of standards, controls, and samples.
2. The obtained OD of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic paper or using an automated method.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in this instruction for use have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred calculation method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations this dilution factor has to be taken into account.

6.3.1 Example of typical standard curve

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.

Standard		Optical Density (450 nm)
Standard A	0.0 ng/ml	3.099
Standard B	0.1 ng/ml	2.647
Standard C	0.4 ng/ml	1.932
Standard D	1.7 ng/ml	0.856
Standard E	7 ng/ml	0.357
Standard F	30 ng/ml	0.172

7 EXPECTED NORMAL VALUES

Because of differences, which may exist between laboratories and location with respect to population, laboratory technique and selection of reference group, it is important for each laboratory to determine its own normal and pathological values and to establish the appropriateness of adopting the reference range suggested here.

Time of day	5 – 95. percentile (ng/ml)	n
Morning	1.6 – 9.2	234
Midday	0.9 – 6.9	427
Afternoon	0.6 – 3.6	129
Evening	0.4 – 3.9	419
Midnight	< 1.2	26

Since cortisol levels show diurnal cycles, we recommend to always collect a series of samples in the morning and another sample in the evening. The difference between morning and evening is an important parameter.

8 QUALITY CONTROL

Good laboratory practice requires that controls are run with each standard curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.
It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day-to-day validity of results. Use controls at both normal and pathological levels.
The kit control values and the corresponding results are stated in the QC certificate added to the kit. The values and ranges stated at the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results. Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices, microtiter plate reader, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods. After checking the above mentioned items without finding any error contact your distributor or the manufacturer directly.

9 PERFORMANCE CHARACTERISTICS

9.1 Analytical Sensitivity

The analytical sensitivity of the Cortisol Saliva ELISA ^{Free} was calculated by subtracting two standard deviations from the mean of twenty (20) replicate analyses of *Standard A*. The analytical sensitivity of the assay is 0.019 ng/ml.

9.2 Specificity (Cross Reactivity)

The following materials have been evaluated for cross reactivity.

Steroids	% Crossreactivity
Testosterone	< 0.1
Corticosterone	6.2
Cortisone	0.8
11-Deoxycorticosterone	2.6
11-Deoxycortisol	50
Dexamethasone	< 0.1
Estriol	< 0.1
Estrone	< 0.1
Prednisolone	100
Prednisone	0.9
Progesterone	< 0.1
17-Hydroxyprogesterone	1.3
Danazole	< 0.1
Pregnenolone	< 0.1
Estradiol	< 0.1
Androstenedione	< 0.1

9.3 Assay Dynamic Range

The range of the assay is between 0.1 – 30 ng/ml.

9.4 Reproducibility

9.4.1 Intra-Assay

The intra-assay variation was determined by replicate measurements of three saliva samples within one run using the Cortisol Saliva ELISA ^{Free}. The intra-assay variation is shown below:

	Sample 1	Sample 2	Sample 3
Mean (ng/ml)	0.64	2.01	4.87
SD (ng/ml)	0.05	0.08	0.21
CV (%)	7.1	4.1	4.3
n =	20	20	20

9.4.2 Inter-Assay

The inter-assay variation was determined by duplicate measurements of three saliva samples in ten different runs using the Cortisol Saliva ELISA ^{Free}. The inter-assay variation is shown below:

	Sample 1	Sample 2	Sample 3
Mean (ng/ml)	0.65	2.05	5.31
SD (ng/ml)	0.03	0.15	0.48
CV (%)	4.2	7.5	9.1
n =	10	10	10

9.5 Recovery

Recovery of the Cortisol Saliva ELISA Free was determined by adding increasing amounts of the analyte to three different saliva samples containing different amounts of endogenous analyte. Each sample (non-spiked and spiked) was assayed and analyte concentrations of the samples were calculated from the standard curve. The percentage recoveries were determined by comparing expected and measured values of the samples.

Saliva	Spiking	Measured Concentration (ng/ml)	Expected Concentration (ng/ml)	Recovery %
1	native	0.53	–	–
	3 ng/ml	3.35	3.53	95%
	5 ng/ml	6.57	5.53	119%
	7 ng/ml	8.31	7.53	110%
2	native	0.54	–	–
	3 ng/ml	3.52	3.54	99%
	5 ng/ml	7.02	5.54	126%
	7 ng/ml	8.61	7.54	114%
3	native	0.82	–	–
	3 ng/ml	3.51	3.82	92%
	5 ng/ml	6.32	5.82	108%
	7 ng/ml	9.15	7.82	117%

9.6 Linearity

Three saliva samples containing different amounts of analyte were serially diluted with Standard A and assayed with the Cortisol Saliva ELISA Free. The percentage linearity was calculated by comparing the expected and measured values for cortisol.

Saliva	Dilution	Measured Concentration (ng/ml)	Expected Concentration (ng/ml)	Linearity %
1	native	4.13	–	–
	1 in 2	2.04	2.07	99%
	1 in 4	1.07	1.03	104%
	1 in 8	0.60	0.52	115%
2	native	4.13	–	–
	1 in 2	2.26	2.07	109%
	1 in 4	1.24	1.03	120%
	1 in 8	0.66	0.52	127%
3	native	4.48	–	–
	1 in 2	2.32	2.24	104%
	1 in 4	1.33	1.12	119%
	1 in 8	0.65	0.56	116%

10 LIMITATIONS OF PROCEDURE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to GLP (Good Laboratory Practice). Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

- Blood contamination in saliva samples will affect results, and usually can be seen by eye. In case of visible blood contamination, the donor should discard the sample, rinse the sampling device with water, wait for 10 minutes and take a new sample.
- Samples containing sodium azide should not be used in the assay. This can cause false results.
- The result of any immunological test system may be affected by heterophilic antibodies, anti-species antibodies or rheumatoid factors present in human samples (11 – 13). For example, the presence of heterophilic antibodies in donors who are regularly exposed to animals or animal products may interfere with immunological tests. Therefore, interference with this immunoassay cannot be excluded.

10.2 High-Dose Hook Effect

A High-Dose-Hook Effect is not known for competitive assays.

10.3 Drug Interferences

Any medication (cream, oil, pill etc) containing Cortisol of course will significantly influence the measurement of this analyte in saliva. The same is true for any medication containing Prednisolone.

11 REVISION HISTORY OF INSTRUCTION FOR USE

Changes from the previous version 16.0a-r to actual version 17.0-r

General	Editorial changes
Chapter 10.1 & 12	added

Updated information in chapter 1.1, 3, 4.2, 4.4, 5.1, 5.2, 6.1, 10.3, 13

12 REFERENCES

1. Fleiner T., Zijlstra W, Dauth H., Haussermann P.: Evaluation of a hospital-based day-structuring exercise programme on exacerbated behavioural and psychological symptoms in dementia – the exercise carrousel: study protocol for a randomised controlled trial, BioMedCentral **2015** 16:228
2. De Steenwinkel F.D.O, Hokken-Koelega A.C.S, Hazes J.M.W, Dolhain R.J.E.M.: The influence of foetal prednisone exposure on the cortisol level in the offspring
   Clinical Endocrinology **2014,** 80, 804-810
3. Balsalobre-Fernández C., Tjero-González C.M., del Campo-Vecino J.: Relationships between Training Load, Salivary Cortisol Responses and Performance during Season Training in Middle and Long Distance Runners; PLOS ONE **2014**, Vol. 9, Issue 8
4. LK Nieman, BMK Biller, JW Findling, J Newell-Price, VM Montori: The diagnosis of Cushing's syndrome: An Endocrine Society Clinical Practice Guideline
   J. Clin. Endocrin. & Metabol. **2008**, Vol.93, No.5, pages 1526-1540
5. Kirschbaum C., Hellhammer DH: Salivary cortisol in psychobiological Research: An overview, Neuropsychobiology, **1989**, 22, 150-169
6. Kirschbaum C, Hellhammer Dh.: Salivary cortisol in psychoneuroendocrine Research: Recent developments and applications, Psychoneuroendocrinology **1994**, 19, pp 313-333
7. Vining RF, et al., Salivary cortisol: a better measure of adrenal cortical function than serum cortisol, Ann Clin Biochem, **1983**, 20, 329-335
8. L.D. Dorn, J.F. Lucke, T.L. Loucks, S.L. Berga: Salivary Cortisol reflects serum Cortisol: Analysis of circadian profiles.
   Ann Clin Biochem **2007** Vol. 44, pages 281-284
9. Aardal E. and Holm AC: Cortisol in Saliva – Reference Ranges and Relation to Cortisol in Serum
   Eur. J. Clin. Chem. Clin. Biochem. (**1995**), 33:927-932
10. Garde A.H. and Hansen A.M.: Long-term stability of salivary cortisol
    Scand J Clin Lab Invest **2005**; 65:433-436
11. Marks V.: False-Positive Immunoassay Results: A Multicenter Survey of Erroneous Immunoassay Results from Assays of 74 Analytes in 10 Donors from 66 Laboratories in Seven Countries
    Clinical Chemistry **2002**, 48:11: 2008-2016
12. Tate J. & Ward G.: Interferences in Immunoassays,
    Clin. Biochem Rev Vol 25, May **2004**
13. Selby C.: Interference in Immunoassays;
    Ann. Clin. Biochem **1999**, 36: 704-721

Symbols:

+8 +2 °C	Storage temperature		Manufacturer	Σ	Contains sufficient for <n> tests
	Use-by date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number		Distributor
	Date of manufacture			RUO	For research use only!