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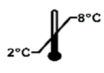
Please use only the valid version of the Instructions for Use provided with the kit

## 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

Cortisol Saliva ELISA Free is an enzyme immunoassay for the quantitative determination of active free cortisol in human saliva. The assay is intended for research use only. Not intended for diagnostic procedures.

Since the molecular structure of steroids is not species-specific, the Cortisol Saliva ELISA Free can also be used to determine cortisol in samples from other species, considering differences in sample matrices and any required pre-treatment or extraction step.

This 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) [1-3]. 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. 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 weeks).
Studies show that salivary cortisol concentration reflects the serum unbound cortisol concentration throughout the physiological concentration range [4-6]. In serum, 90 – 95% of cortisol is protein-bound while in saliva cortisol appears mainly in its free, metabolic active form.
Cortisol is a key focus of biomedical and psychological research, as it is the predominant stress hormone and has far-reaching effects on both body and mind. In research, it is primarily used as a biomarker for stress and to investigate metabolic processes [1-3].
Cortisol also plays an important role in animal research. It is used as a biomarker to measure animals' stress levels and monitor their well-being [7-12].

## 2. PRINCIPLE

The **Cortisol Saliva ELISA Free** 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. For professional use only.
2. Before starting the assay, read the instructions for use (IFU) completely and carefully. Use the valid version of the IFU provided with the kit. Be sure that everything is understood.
3. The microtiter plate 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 colored. Do not pour reagents back into vials as reagent contamination may occur.
6. Mix the contents of the microtiter plate wells thoroughly to ensure good test results. Do not reuse wells.
7. Do not let wells dry during assay; add reagents immediately after completing the washing 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 samples with skin and mucous membranes.
10. Do not smoke, eat, drink or apply cosmetics in areas where samples or kit reagents are handled.
11. Wear disposable latex gloves when handling samples and reagents. Microbial contamination of reagents or samples 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 microtiter plate readers.

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15. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different microtiter 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 lead to slightly different results.
16. Avoid contact with Stop Solution. It may cause skin irritation and burns.
17. Some reagents contain Proclin 300, CMIT and/or 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

**SA E-6031** **MTP** **96** **Microtiter Plate**

Content: 12x8 (break apart) strips, 96 wells; wells coated with polyclonal rabbit anti-cortisol antiserum.

**Standards and Controls** – ready to use

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

Conversion: 1 ng/ml = 2.76 nmol/l

Content: Buffer matrix spiked with defined concentration of cortisol.

**SA E-6040** **CONJUGATE** **Enzyme Conjugate** – ready to use

Content: Cortisol conjugated to horseradish peroxidase

Volume: 1 x 7.0 ml

Hazards identification:

H317 May cause an allergic skin reaction.

**AR E-0055** **SUBSTRATE** **Substrate Solution** – ready to use

Content: Tetramethylbenzidine (TMB)

Volume: 1 x 22 ml

**AR E-0080** **STOP-SOLN** **Stop Solution** – ready to use

Content: Contains 2 N hydrochlorid acid;

Avoid contact with the stop solution. It may cause skin irritations and burns.

Volume: 1 x 7.0 ml

**AR E-0030** **WASH-CONC 10x** **Wash Solution** – 10X concentrated

Volume: 1 x 50 ml;

See "Reagent Preparation".

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### 4.2 Material required but not provided

- Microcentrifuge
- A calibrated microtiter plate reader (450 nm)
- Microtiter plate 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 Reagent Preparation

Allow reagents and 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 **WASH-CONC** **10x** is stable for at least 12 weeks at room temperature. Precipitates may form when stored at 2 – 8 °C, which should dissolve again by swirling at room temperature. 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 has 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. SAMPLE 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. In case of visible blood contamination 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.

### 5.1 Sample 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 cap.
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 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 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. 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 sample collection. Furthermore, please avoid any strenuous physical exercises and intense stress situations.

### 5.2 Sample 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

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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. 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.

### 5.3 Sample Dilution

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

## 6. ASSAY PROCEDURE

### 6.1 General remarks

- All reagents and samples 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 to avoid cross contamination.
- OD 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 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 washing and aspiration. Wash and fill all reagents with care. While washing, 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.** | Prepare a sufficient number of microtiter plate wells to accommodate **Standards**, **Controls** and **Samples** in duplicates. |
| **2.** | Dispense **50 µl** of each **Standard**, **Control** and **Sample** in duplicates with new disposable tips into appropriate microtiter plate wells. |
| **3.** | Add **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.** | Discard the content of the wells and wash **4 times** with **300 µl** diluted **1x Wash Solution**. Remove as much as possible Wash Solution by beating the microtiter plate carefully on absorbent paper. **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 all wells. |
| **7.** | Incubate without shaking at room temperature (18 – 25 °C) for 30 minutes in the dark. |
| **8.** | Stop reaction by adding **50 µl** of **Stop Solution** to each well. Mix carefully. |
| **9.** | Determine the OD of each well at **450 nm**. It is recommended to read the wells within 15 minutes. |

### 6.3 Calculation of results

1. Calculate the average OD values for each set of Standards, Controls and Samples.
2. The obtained ODs 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 OD 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 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.

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## 7. QUALITY CONTROL

The kit control ranges and the mean results are stated in the QC-Report added to the kit. The values and ranges stated at the QC-Report always refer to the current kit lot and should be used for direct comparison of the results. For further internal quality control each laboratory is recommended to additionally use own controls.
If the results of the assay do not fit to the established acceptable ranges of controls, 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.

## 8. PERFORMANCE CHARACTERISTICS

### 8.1 Limit of Blank

The Limit of Blank of the Cortisol Saliva ELISA $^{Free}$ was found at 0.04 ng/ml.

### 8.2 Specificity (Cross-Reactivity)

The following materials have been evaluated for cross-reactivity.

| Compound | Crossreactivity at 50% binding |
|---|---|
| Cortisone | 29.3% |
| 11-Deoxycortisol | 16.4% |
| Prednisone | 18.7% |
| Prednisolone | 31.5% |

The following compounds show negligible reactivities (< 2% at 50% binding):
Corticosterone, 11-Deoxycorticosterone, Dexamethasone, Progesterone, 17-Hydroxyprogesterone, Danazole, Pregnenolone, Androstenedione.

The following compounds do not show any detectable cross-reactivity (< 0.004% at 50% binding):
Testosterone, DHEA, DHEA-S, Estriol, Estrone, 17ß-Estradiol.

### 8.3 Assay Range

The range of the assay is between 0.04 – 30 ng/ml.

### 8.4 Reproducibility

#### 8.4.1 Intra-Assay

The intra-assay variation was determined by 20 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)** | 4.32 | 1.07 | 12.86 |
| **SD (ng/ml)** | 0.24 | 0.07 | 0.98 |
| **CV (%)** | 5.6 | 6.2 | 7.6 |
| **n =** | 20 | 20 | 20 |

#### 8.4.2 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)** | 4.00 | 1.06 | 12.50 |
| **SD (ng/ml)** | 0.20 | 0.06 | 1.46 |
| **CV (%)** | 5.1 | 5.2 | 11.7 |
| **n =** | 10 | 10 | 10 |

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### 8.5 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 concentrations of the samples.

| Sample | Spiking | Measured Concentration (ng/ml) | Expected Concentration (ng/ml) | Recovery % |
|---|---|---|---|---|
| 1 | native | 1.76 | – | – |
| | 1 ng/ml | 3.03 | 2.76 | 110 |
| | 4 ng/ml | 6.08 | 5.76 | 106 |
| | 10 ng/ml | 12.67 | 11.76 | 108 |
| 2 | native | 1.97 | – | – |
| | 1 ng/ml | 3.14 | 2.97 | 106 |
| | 4 ng/ml | 6.21 | 5.97 | 104 |
| | 10 ng/ml | 12.29 | 11.97 | 103 |
| 3 | native | 1.74 | – | – |
| | 1 ng/ml | 2.99 | 2.74 | 109 |
| | 4 ng/ml | 6.02 | 5.74 | 105 |
| | 10 ng/ml | 12.06 | 11.74 | 103 |

### 8.6 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 concentrations for cortisol.

| Sample | Dilution | Measured Concentration (ng/ml) | Expected Concentration (ng/ml) | Linearity % |
|---|---|---|---|---|
| 1 | native | 12.97 | – | – |
| | 1:2 | 6.39 | 6.49 | 99 |
| | 1:4 | 3.34 | 3.24 | 103 |
| | 1:8 | 1.68 | 1.62 | 104 |
| 2 | native | 13.21 | – | – |
| | 1:2 | 6.05 | 6.61 | 92 |
| | 1:4 | 3.07 | 3.30 | 93 |
| | 1:8 | 1.52 | 1.65 | 92 |
| 3 | native | 14.92 | – | – |
| | 1:2 | 6.97 | 7.46 | 93 |
| | 1:4 | 3.46 | 3.73 | 93 |
| | 1:8 | 1.68 | 1.86 | 90 |

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## 9. LIMITATIONS OF PROCEDURE

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

### 9.1 Interfering Substances

- Blood contamination in saliva samples will affect results and usually can be seen by eye. In case of visible blood contamination, the collector should discard the sample, rinse the sampling device with water, wait for 10 minutes and take a new sample (see 5.1).
- 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 [13-15]. Therefore, interference with this immunoassay cannot be excluded. If unplausible results are suspected, they should be considered invalid and verified by further testing.

### 9.2 High Dose Hook Effect

Up to a tested concentration of 1500 ng/ml cortisol no High Dose Hook Effect could be observed for the Cortisol Saliva ELISA Free.

### 9.3 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 Prednisone, Prednisolone and Cortisone (see cross-reactivity in chapter 8.2).

## 10 LEGAL ASPECTS

### 10.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. The test results are only valid if all controls meet the specified ranges and all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact the manufacturer.

### 10.2 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

Please use only the valid version of the Instructions for Use provided with the kit

## 11 REFERENCES


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## 12. REVISION HISTORY OF INSTRUCTION FOR USE

Changes from the previous version 17.0-r to actual version 18.0-r

The IFU has been completely revised following a redevelopment of the assay.

## 13. SHORT INSTRUCTION

All steps at RT (18 – 25 °C).
All sample sizes given in µl.

| **Steps** | **Standard A – F** (0 – 30 ng/ml) | **Control 1 – 2** | **Sample** |
|---|---|---|---|
| Pipet **Standard A – F** | **50** | **–** | **–** |
| Pipet **Control 1 – 2** | **–** | **50** | **–** |
| Pipet **Samples** | **–** | **–** | **50** |
| Pipet **Enzyme Conjugate** | **50** | | |
| Incubate for **1 h** on a shaker (900 rpm). | | | |
| Decant<br>Wash **4x** with **Wash Solution** | **300** (4 times) | | |
| Pipet **Substrate Solution** | **200** | | |
| Incubate without shaking for **30 min** in the **dark**. | | | |
| Pipet **Stop Solution** | **50** | | |
| Read at **λ = 450 nm**. | | | |

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**Symbols:**

| | | | | | |
|---|---|---|---|---|---|
| | Storage temperature | | Manufacturer | | 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! |