Product information

Useŕs Manual

Salivary Cortisol ELISA

Enzyme Immunoassay for the quantitative measurement of active free cortisol (hydrocortisone and hydroxycorticosterone) in saliva

IVD

REF **IB79305**

96 wells

Contents

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1 INTRODUCTION

1.1 Intended Use

An enzyme immunoassay for the quantitative in vitro diagnostic measurement of active free cortisol (hydrocortisone and hydroxycorticosterone) in saliva. Measurements of cortisol are used in the diagnosis and treatment of disorders of the adrenal gland. **For in vitro diagnostic use only.**

1.2 Summary and Explanation

Cortisol, the most potent glucocorticoid, is produced by the zona fasciculate of the human adrenal cortex (1-3). It is synthesized from cholesterol and its production is stimulated by pituitary adrenocorticotropic hormone (ACTH) in response to corticotropin-releasing hormone (CRH). ACTH and CRH secretions are inhibited by high cortisol levels in a negative feedback loop. Cortisol acts through specific intracellular receptors and affects numerous physiologic systems including immune function, glucose counter regulation, vascular tone, and bone metabolism.

Cortisol release follows a diurnal rhythm with highest concentrations in the morning (about 1 hour after wakening). Thereafter, Cortisol concentration steadily decreases to a very low level 12 hours later (4-7). Cortisol secretion increases in response to any stress in the body, whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological (8-14). After secretion, cortisol causes a breakdown of muscle protein, leading to release of amino acids into the bloodstream. These amino acids are then used by the liver to synthesize glucose for the brain, a process called gluconeogenesis. Cortisol also leads to the release of fatty acids from fat cells and its utilization in muscle cells. Taken together, these energy-directing processes prepare the individual to deal with stressors and ensure that the brain receives adequate energy sources.

Elevated cortisol levels and lack of diurnal variation have been identified with Cushing's disease (ACTH hypersecretion) (7). CRH is released in a cyclic fashion by the hypothalamus, resulting in diurnal peaks (elevated in the morning) and nadirs (low in the evening) for plasma ACTH and cortisol levels. The diurnal variation is lost in patients with Cushing and these patients have elevated levels of evening plasma cortisol. The measurement of late-night salivary cortisol is an effective and convenient screening test for Cushing syndrome (15). Elevated circulating cortisol levels have also been identified in patients with adrenal tumors (16). Low cortisol levels are found in primary adrenal insufficiency (e.g. adrenal hypoplasia, Addison's disease) and in ACTH deficiency (17). Due to the normal circadian variation in cortisol levels, distinguishing normal from abnormally low cortisol levels can be difficult, therefore several daily collections are recommended.

Saliva is an excellent medium to measure steroids because it is a natural ultra-filtrate of blood. 90-99% of steroid hormones in the blood are bound to carrier proteins (corticoid-binding globulin, sex-hormone binding globulin and albumin) and are unavailable to target tissues. Only about 1-10% of the steroids in blood are in the unbound or free fraction, and can diffuse into target tissues of the body and into saliva. The process of passive diffusion of non-bound steroid hormones is supported by their low molecular weight (less than 400 daltons) and relative lipophilicity, thus enabling them to freely diffuse from blood to saliva (18-21).

2 PRINCIPLE

The **Salivary Cortisol ELISA** Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.

The microtiter wells are coated with a monoclonal (mouse) antibody directed towards an antigenic site on the cortisol molecule.

Endogenous cortisol of a patient sample competes with a cortisol-horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off.

The amount of bound peroxidase conjugate is inversely proportional to the concentration of cortisol in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of cortisol in the patient sample.

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3 WARNINGS AND PRECAUTIONS

1. This kit is for in vitro diagnostic use only. For professional use only.
2. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/11, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
3. 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.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
6. 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.
7. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
8. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
9. Allow the reagents to reach room temperature (21 °C to 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
10. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
11. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
12. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
13. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
14. Do not use reagents beyond expiry date as shown on the kit labels.
15. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiterplate readers.
16. 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.
17. Avoid contact with *Stop Solution* containing 0.5 M H_2SO4. It may cause skin irritation and burns.
18. Some reagents contain Proclin, BND and/or MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
19. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
20. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
21. For information on hazardous substances included in the kit please refer to Safety Data Sheets. Safety Data Sheets for this product are available upon request directly from IBL-America.

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4 REAGENTS

4.1 Reagents provided

1. **SORB MT Microtiterwells,** 12 x 8 (break apart) strips, 96 wells; Wells coated with a anti-cortisol antibody (monoclonal).
2. **CAL 0 — 6 Standard (Standard 0-6),** 7 vials, 1 mL each, ready to use;
   Concentrations: 0 - 0.1 0.5 - 1.5 — 4 —10 - 30 nglmL,
   Standards are calibrated against mass spectrometry.
   Conversion factor: 1 nglmL = 2.76 nmollL;
   Contain non-mercury preservative.
3. **CONTROL low & high Control Low & High,** 2 vials, 1 mL each, ready to use; For control values and ranges please refer to vial label or QC-Datasheet.
   Contain non-mercury preservative.
4. **ENZ CONJ Enzyme Conjugate,** 1 vial, 26 mL, ready to use; Cortisol conjugated to horseradish peroxidase;
   Contain non-mercury preservative.
5. **SUB TMB Substrate Solution,** 1 vial, 25 mL, ready to use; Tetramethylbenzidine (TMB).
6. **STOP SOLN Stop Solution,** 1 vial, 14 mL, ready to use; contains 0.5M H_2SO_4.Avoid contact with the stop solution.
   It may cause skin irritations and burns.
7. **WASH SOLN 40x Wash Solution, 1** vial, 30 mL (40X concentrated);
   see „Preparation of Reagents".

Note: Additional *Standard 0* for sample dilution is available upon request.

4.2 Materials required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm)
- Calibrated variable precision micropipettes (100 pL, 200 pL).
- 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 retain reactivity until expiration date. Do not use reagents beyond this date. Opened reagents must be stored at 2 °C to 8 °C. Microtiter wells must be stored at 2 - 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again.

4.4 Reagent Preparation

Bring all reagents to room temperature before use.

Wash Solution

Add deionized water to the 40X concentrated *Wash Solution.* Dilute 30 mL of concentrated *Wash Solution* with 1170 mL deionized water to a final volume of 1200 mL. *The diluted Wash Solution is stable for 2 weeks at room temperature.*

4.5 Disposal of the Kit

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, LBL-AMERICA 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.

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5 SPECIMEN COLLECTION AND PREPARATION

Eating, drinking, chewing gums or brushing teeth should be avoided for 30 minutes before sampling. Otherwise, it is recommended to rinse mouth thoroughly with cold water 5 minutes prior to sampling. Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination). If there is visible blood contamination the patient specimen, it should be discarded, rinse the sampling device with water, wait for 10 minutes and take a new sample.

Note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

For the correct collection of saliva, we are recommending to only use appropriate devices made from ultra-pure polypropylene. Do not use any PE devices or Salivettes for sampling; this in most cases 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. Please contact IBL-America Diagnostics for more details.

Due to the cyclic secretion pattern of steroid hormones it is important to care for a proper timing of the sampling. In order to avoid arbitrary results, we recommend that 5 samples always be taken within a period of 2 – 3 hours (multiple sampling) preferably before a meal. As food might contain significant amounts of steroid hormones samples preferably should be taken while fasting. If fasting should be a problem the collection period should be timed just before lunch or before dinner.

5.2 Specimen Storage and Preparation

The saliva samples may be stored at 2 °C to 8 °C up to one week and should be frozen at -20 °C for longer periods; repeated thawing and freezing is no problem.

Each sample has to be frozen, thawed, and centrifuged at least once in order to separate the mucins by centrifugation.
Upon arrival of the samples in the lab the samples have to stay in the deep freeze at least overnight.
Next morning the frozen samples are warmed up to room temperature and mixed carefully.
Then the samples have to be centrifuged for 5 to 10 minutes (at 2000 - 3000 x g).
Now the clear colorless supernatant is easy to pipette.

If a set of multiple samples is to be tested, the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the 5 single samples in a separate sampling device and perform the testing from this mixture.

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Standard 0* and re-assayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.
Example:

a) Dilution 1:10: 10 µL saliva + 90 µL *Standard 0* (mix thoroughly)
b) Dilution 1:100: 10 µL of dilution a) + 90 µL *Standard 0* (mix thoroughly).

6 ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature 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 the 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 equal 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.

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6.2 Assay Procedure

Each run must include a standard curve.

1. Secure the desired number of coated strips in the frame holder.
2. Dispense **100 µL** of each ***Standard, Control*** **and samples** with new disposable tips into appropriate wells.
3. Dispense **200 µL** ***Enzyme Conjugate*** into each well. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4. Incubate for **60 minutes** at room temperature. **Note:** Incubation on a shaker at 300 rpm is recommended.
5. Briskly shake out the contents of the wells. Rinse the wells 5 times with diluted Wash Solution (400 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** at room temperature.
8. Stop the enzymatic reaction by adding **100 µL** of ***Stop Solution*** to each well.
9. Determine the absorbance of each well at **450 ±10 nm.** It is recommended that the wells be read within 10 minutes.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the Instructions for Use have been calculated automatically using a 4Parameter curve fit. (4 Parameter Rodbard or 4 Parameter Marquardt are the preferred methods.) 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 Units (450 nm)
Standard 0	0 ng/mL	2.00
Standard 1	0.1 ng/mL	1.89
Standard 2	0.5 ng/mL	1.62
Standard 3	1.5 ng/mL	1.22
Standard 4	4.0 ng/mL	0.75
Standard 5	10 nq/mL	0.40
Standard 6	30 nglmL	0.18

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7 EXPECTED NORMAL VALUES

In order to determine the normal range of Salivary Cortisol ELISA, samples from adult male and female apparently healthy subjects, were collected in the morning, at noon, and in the evening and analyzed using the IBL-AMERICA ELISA kit. The following range was calculated from this study.

	Morning	Noon	Evening
n	73	73	73
Range (ng/mL)	0.94 - 19.80	0.32 - 12.70	0.20 - 4.00
Mean (ng/mL)	3.02	1.52	0.88
2.5t" — 97.5`h Percentile (ng/mL)	1.19 - 7.21	0.66 - 3.72	0.33 - 2.23
Median (ng/mL)	2.44	1.25	0.77

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

Since cortisol levels show diurnal cycles, we recommend that the samples be obtained the same hour each day. Furthermore, we recommend that each laboratory determine its own range for the population tested.

8 QUALITY CONTROL

Good laboratory practice requires that controls be run with each calibration 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 controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values and ranges stated on 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 analyzing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices; photometer, 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 IBL-AMERICA directly.

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9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range
The range of the assay is between 0.09 — 30 ng/mL.

9.2 Specificity (Cross Reactivity)
The following materials have been evaluated for cross reactivity.

Steroids	% Cross reactivity
Progesterone	23.40
Androstenedione	1.97
Estriol (E3)	1.47
Testosterone	0.04
DHEA	< 0.001
DHEA-S	< 0.001
Estrone (E1)	< 0.001
Estradiol (E2)	< 0.001

9.3 Analytical Sensitivity
The analytical sensitivity of the IBL-AMERICA ELISA was calculated by subtracting 2 standard deviations from the mean of twenty (20) replicate analyses of *Standard 0 (S_O).* The analytical sensitivity of the assay is 0.09 ng/mL.

9.4 Reproducibility

9.4.1 Intra-Assay
The intra-assay variation was determined by replicate measurements of 3 saliva samples within one run using the IBL-AMERICA ELISA. The within-assay variability is shown below:

Sample	n	Mean (ng/mL)	CV (%)
1	20	3.08	6.1
2	20	8.18	3.0
3	20	20.14	2.6

9.4.2 Inter-Assay
The inter-assay variation was determined by duplicate measurements of 3 saliva samples in 20 different runs using the IBL-AMERICA ELISA. The inter-assay variability is shown below:

Sample	n	Mean (ng/mL)	CV (%)
1	40	0.64	13.6
2	40	7.37	5.6
3	40	11.76	6.2
4	40	19.78	4.3

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9.5 Recovery

Recovery of the IBL-AMERICA ELISA was determined by adding increasing amounts of the analyte to 3 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 1	Saliva 2	Saliva 3
Concentration	**(ng/mL)**	8.60	11.62	20.33
Average % recovery		103.1	97.4	102.2
Range of % recovery	**from**	98.9	96.5	98.2
	to	107.1	98.8	108.3

9.6 Linearity

Three saliva samples containing different amounts of analyte were serially diluted with Standard 0 and assayed with the IBL-AMERICA ELISA. The percentage recovery was calculated by comparing the expected and measured values for cortisol.

		Saliva 1	Saliva 2	Saliva 3
Concentration	**(ng/mL)**	7.91	13.84	19.20
Average % recovery		104.9	109.7	96.5
Range of % recovery	**from**	97.1	105.5	87.8
	to	111.3	114.5	102.2

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 good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 High-Dose-Hook Effect

No hook effect was observed in this test

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of Cortisol in a sample.

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11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test. The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact IBL-AMERICA.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient. Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived. The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 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. Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2 are also invalid. 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.

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SYMBOLS USED WITH IBL-AMERICA ASSAYS

Symbol	English	Deutsch	Italiano	Español	Français
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes
	Consult instructions for use *	Gebrauchsanweisung beachten *	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation
IVD	*In vitro* diagnostic medical device *	*In-vitro*-Diagnostikum *	Diagnostica in vitro	Diagnóstico in vitro	Diagnostic in vitro
REF	Catalogue number *	Artikelnummer *	No. di Cat.	No de catálogo	Référence
LOT	Batch code *	Chargencode *	Lotto no	Número de lote	No. de lot
Σ	Contains sufficient for <n> tests *	Ausreichend für <n> Prüfungen *	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos	Contenu suffisant pour "n" tests
	Temperature limit *	Temperaturbegrenzung *	Temperatura di conservazione	Temperatura de conservacion	Température de conservation
	Use-by date *	Verwendbar bis *	Data di scadenza	Fecha de caducidad	Date limite d'utilisation
	Legal Manufacturer *	Hersteller *	Fabbricante	Fabricante	Fabricant
	Caution *	Achtung *			
RUO	For research use only	Nur für Forschungszwecke	Solo a scopo di ricerca	Sólo para uso en investigación	Seulement dans le cadre de recherches
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