Cortisol ELISA Kit

Enzyme Immunoassay for the quantitative determination of human free Cortisol in saliva

Catalog number: ARG80859

Package: 96 wells

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INTRODUCTION

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. 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).

The loss of circadian rhythm with absence of a late-night cortisol nadir is a consistent abnormality in patients with Cushing´s syndrome. This difference forms the basis for measurement of late-night salivary cortisol.

Studies show that salivary cortisol concentration reflects the serum unbound cortisol concentration throughout the physiological concentration range. In serum, 90-95% of cortisol is bound to protein while in saliva cortisol appears mainly in its free, metabolic active form. The salivary cortisol concentration is independent of saliva flow rate as well as of the serous and mucous content.

Spontaneous increases in cortisol concentration during the day may occur commonly due to stress or food intake. Changed patterns of Cortisol levels have been observed in connection with abnormal ACTH levels, clinical depression, psychological stress, and various physiological stressors as hypoglycemia, illness, fever, trauma, surgery, or pain.

PRINCIPLE OF THE ASSAY

This assay employs the competitive enzyme immunoassay technique. A highly specific Cortisol antibody has been pre-coated onto a microtiter plate. Cortisol containing samples or standards and a Cortisol -HRP conjugate are given into the wells of the microtiter plate. Enzyme labeled and free Cortisol compete for the antibody binding sites. After incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated, resulting in the development of a blue color. The color development is inhibited by the addition of a stop solution, and the color turns yellow. The yellow color is measured at 450 nm. The concentration of Cortisol is indirectly proportional to the color intensity of the test sample.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	12 strips x 8-well	4°C
HRP-Cortisol Conjugate	7 ml (ready to use)	4°C
Standard 0	2 ml (ready to use)	4°C
Standards 1-5 (0.1, 0.4, 1.7, 7.0, 30 ng/ml)	5 X 0.5 ml (ready to use)	4°C
Control Low (0.35 ng/ml; acc. Range: 0.2-0.5 ng/ml)	0.5 ml (ready to use)	4°C
Control High (2.15 ng/ml; acc. Range:1.4-2.9 ng/ml)	0.5 ml (ready to use)	4°C

10X Wash Buffer	50 ml	4°C
TMB substrate	22 ml (ready to use)	4°C (Protect from light)
STOP solution	7 ml (ready to use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Microplate shaker at 600-900 rpm (optional)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times. Opened reagents must be stored at 2°-8°C. After first opening the reagents are stable for 30 days if used and stored properly.
- 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.
- Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
- 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.

- Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Allow the reagents to reach room temperature (18-25°C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the samples will not be affected.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Once the test has been started, all steps should be completed without interruption.
- 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.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

Samples containing sodium azide should 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 give falsely elevated concentration values. In case of visible blood contamination the patient should discard the sample, rinse the sampling device with tap water, also rinse the mouth with (preferably) cold water, wait for 10 minutes and take a new sample. Do not chew anything during the sampling period. Any pressure on the teeth may result in falsely elevated measurements due to an elevated content of gingival liquid in the saliva sample.

Collection –

1. For the correct collection of saliva we are recommending to only use appropriate devices made from ultrapure polypropylene (PP). Do not use any PE devices or Salivates 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.
2. As the Cortisol secretion in saliva as well in serum shows an obvious secretion pattern throughout the day, it is important to care for a proper timing of the sampling. The morning peak normally appears during the first 2 hours after the average wake-up time. But also during the day there might be smaller peaks in the Cortisol secretion. Therefore we recommend taking 5 separate samples within a period of 2 hours (multiple

sampling) directly after the usual wake-up time (e.g. 1 min, 30 min, 60 min, 90 min, 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 patient. If possible the volume of each single sample should be a minimum of 0.5 ml (better 1 ml).

3. As food might contain significant amounts of steroid hormones samples preferably should be taken while fasting. If fasting should be a problem it is allowed to eat small amount of vegetarian food directly after collecting one sample. After eating such a small amount the patient should clean his mouth by washing it with water. If possible the volume of each single sample should be a minimum of 0.5 ml (better 1 ml).
4. As food might contain significant amounts of steroid hormones samples preferably should be taken 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.
5. It is important to know that the timing of the morning peak is not related to the absolute time or the day light. It is just related to the wakeup habits of the patient. In order to catch the peak we recommend collecting the samples at approximately 1 min, 30 min, 60 min, 90 min, and 120 minutes after the usual weak-up time of the last 10 days. The typical timing for a morning collection period would be as follows. Wake-up at 6:00 AM, 1st sample at 6:01 AM, drinking water and brushing teeth, followed by samples at 6:30 AM, 7:00 AM, 7:30 AM, and 8:00 AM, followed by breakfast at maybe 8:15 AM. Modest variation in the collection timing will

not be critical, and the collection time-frame can be extended up to 3 hours. Special care has to be taken in case the patient recently has done trip over several time zones.

6. Furthermore please avoid any strenuous physical exercises and intense stress situations.
7. The collection for the evening sample (e.g. midnight Cortisol for the detection of Morbus Cushing) has to be done during the late evening (at best between 10 and 12 PM). Also in this case we recommend collecting 5 samples in intervals of at least 30 minutes. If only 5 sampling devices are available for the collection of a day profile, sampling also can be done as follows. 30 min, 60 min, and 90 minutes after the usual wake-up time for the morning value, followed by 2 samples in the late evening collected during the last hour prior to regular bed time.

Storage and Preparation-

- Saliva samples in general are stable at ambient temperature for several days. Therefore mailing of such samples by ordinary mail without cooling will not create a problem. Whenever possible samples preferable should be kept at a temperature of -20°C. We recommend avoiding multiple freeze-thaw cycles.

- Each sample has to be frozen, thawed, and centrifuged at least once anyhow 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. Now the clear colorless supernatant is easy to pipette. If the sample should show even a slightly reddish color it should be discarded. Otherwise the

concentration value most probably will be falsely elevated. Due to the episodic variation of steroid secretion we highly recommend the strategy of multiple sampling. If such a set of multiple samples have to be tested the lab (after at least one freezing, thawing, and centrifugation cycle) has to mix the aliquots of the 5 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 5 morning samples have to be tested separately.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 10X Wash buffer into distilled water to yield 1X Wash buffer. (E.g. 50 ml of 10X wash buffer + 450 ml of distilled water) The diluted Wash buffer is stable for at least 12 weeks at room temperature (18-25°C).
- Sample: 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).

ASSAY PROCEDURE

All materials and samples should be equilibrated to room temperature (RT, 18-25°C) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl** of each **Standard, control and sample** in duplicate into the appropriate wells.
3. Add **50 µl** of **HRP-Cortisol conjugate** into each well. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step. Incubate for **60 minutes at RT**. Shaking on a horizontal shaker during incubation improves the sensitivity of the test.
4. Aspirate each well and wash, repeating the process 3 times for a total **4 washes**. Wash by filling each well with **1X Wash Buffer (300 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
5. Add **200 µl** of **TMB substrate** to each well. Incubate for **30 minutes at room temperature** in dark.
6. Add **50 µl** of **Stop Solution** to each well.
7. Read the OD with a microplate reader at **450 nm** immediately. It is recommended to read the absorbance within 15 minutes after adding stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using log-log, semi-log or linear graph paper, 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 IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results. The diluted samples must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)

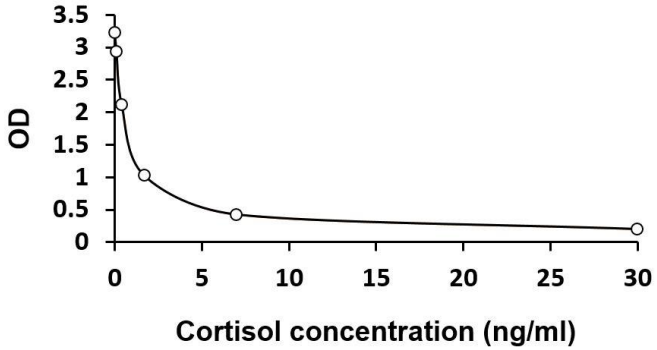
6. The concentration of the samples can be determined directly from this calibrator curve. Samples with concentrations higher than that of the highest calibrator have to be further diluted by standard 0. For the calculation of the concentrations, this dilution factor has to be taken into account.

7. Refer to the table below for molar conversion:

	Concentration of standards					
Standard	0	1	2	3	4	5
Cortisol (ng/ml)	0	0.1	0.4	1.7	7.0	30
Cortisol (nmol/L)	0	0.2762	1.1048	4.6954	19.334	82.86
Conversion	Cortisol (ng/ml) x 2.762 = Cortisol (nmol/L)					

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.



QUALITY ASSURANCE

Sensitivity

The analytical sensitivity was calculated from the mean plus two standard deviations of twenty (20) replicate analyses of Standard 0 is 0.019 ng/ml.

Specificity

The following substances were tested for cross reactivity of the assay:

Steroid	Cross Reaction (%)
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%
Danazole	< 0.1%
Pregnenolone	< 0.1%
Estradiol	< 0.1%
17-Hydroxyprogesterone	0.3%
Androstenedione	< 0.1%

Intra-assay and inter-assay precision

The CV value of intra-assay precision was 5.17% and the CV value of inter-assay precision was 6.9%.

Recovery

92-126%

Linearity

99-127%