



EAGLE
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Cortisol Saliva ELISA Assay Kit

Catalog Number:

CRT32-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

v. 1.0

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INTENDED USE

The Eagle Biosciences Cortisol Saliva ELISA Assay Kit (enzyme-linked immunoassay kit) is intended for the quantitative determination of Cortisol in human saliva by an enzyme immunoassay. The Eagle Biosciences Cortisol Saliva ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

INTRODUCTION

Cortisol is the most abundant circulating steroid and the major glucocorticoid secreted by the adrenal cortex. Cortisol is physiologically effective in blood pressure maintenance and anti-inflammatory activity. It is also involved in calcium absorption, gluconeogenesis as well as the secretion of gastric acid and pepsin. It is increased under stress situations, physical exercise and external administration of ACTH. Measurement of cortisol levels in general can be used as an indicator of adrenal function and the differential diagnosis of Addison's and Cushing's diseases as well as adrenal hyperplasia and carcinoma.

Most circulating cortisol is bound to cortisol binding globulin or transcortin and albumin. The free cortisol, which is considered the active part of blood, is about 1–2%. In the absence of appreciable amounts of the cortisol binding proteins in saliva, salivary cortisol is considered to be free and shows a diurnal rhythm with the highest levels in the morning and the lowest levels at night.

PRINCIPLE OF THE ASSAY

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of cortisol in the sample. A set of standards is used to plot a standard curve from which the amount of cortisol in patient samples and controls can be directly read.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.



10. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
11. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of cortisol in human saliva. The kit is not calibrated for the determination of cortisol in serum, plasma or other specimens of human or animal origin.
2. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
3. Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
4. This kit is intended for research use only and should not be used as a diagnostic tool.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and controls has been tested and found to be nonreactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. No test method however, can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 1 mL of saliva is required per duplicate determination. Collect 4–5 mL of saliva into a clean glass tube (Salivette by Sarstedt may be used) without force or inducement and before eating, drinking or brushing the teeth. Simply rinse the mouth with water before collection. Do not use blood-contaminated specimens. Store samples at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

Specimen tubes are to be placed into a freezer and allowed to freeze. When ready to use, the specimens are to be thawed and centrifuged. The supernatants are to be collected and poured into freshly labelled tubes.



REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 50, 100, 150 and 300 μ L
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Benchtop centrifuge
6. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10)

REAGENTS PROVIDED

1. **Rabbit Anti-Cortisol Antibody-Coated Break-Apart Well Microplate** — Ready To Use
Contents: One 96-well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
2. **Cortisol Peroxidase (HRP) Conjugate Concentrate** — Requires Preparation X50
Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 300 μ L/vial
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:50 in assay buffer before use (eg. 40 μ L of HRP in 2 mL of assay buffer). If the whole plate is to be used dilute 240 μ L of HRP in 12 mL of assay buffer. Discard any that is left over.
3. **Cortisol Saliva Calibrators** — Ready To Use
Contents: Six vials containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of cortisol.

* Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
A	0 ng/mL	2.0 mL
B	1 ng/mL	0.6 mL
C	3 ng/mL	0.6 mL
D	10 ng/mL	0.6 mL
E	30 ng/mL	0.6 mL
F	100 ng/mL	0.6 mL

Storage: Refrigerate at 2–8°C.
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. **Controls** — Ready to Use
Contents: Two vials containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of cortisol. Refer to vial labels for the acceptable range.
Volume: 0.6 mL/vial



Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the controls should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. **Wash Buffer Concentrate** — Requires Preparation x10

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute the wash buffer concentrate 1:10 in distilled or deionized water to prepare the working wash buffer. If one whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. **Assay Buffer** – Ready to Use

Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 15 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

7. **TMB Substrate** — Ready To Use

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

8. **Stopping Solution** — Ready To Use

Contents: One bottle containing 1M sulfuric acid.
Volume: 6 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

ASSAY PROCEDURE

Specimen Pretreatment: Freezing and Centrifugation

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the Cortisol-HRP conjugate and wash buffer.
2. Remove the required number of well strips from the microplate and assemble into a plate frame. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 μ L of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.



- Pipette 100 μ L of the conjugate working solution into each well. (We recommend using a multichannel pipette.)
- Incubate on a plate shaker (\sim 200 rpm) for 45 minutes at room temperature.
- Wash the wells 3 times each time with 300 μ L/well of diluted wash buffer per well. After washing tap the plate firmly against absorbent paper to remove any residual liquid (the use of a washer is strongly recommended).
- Pipette 150 μ L of the TMB substrate into each well at timed intervals.
- Incubate on a plate shaker for 15-20 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
- Pipette 50 μ L of stopping solution into each well at the same timed intervals as in step 7.
- Measure the absorbance at 450 nm in all wells with a microplate reader, within 20 minutes after addition of the stopping solution.

* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

CALCULATIONS

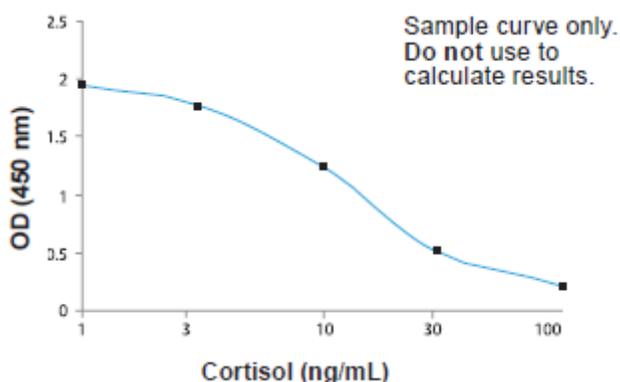
- Calculate the mean optical density of each calibrator duplicate.
- Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
- Calculate the mean optical density of each unknown duplicate.
- Read the values of the unknowns directly off the calibrator curve.
- If a sample reads more than 100 ng/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA

Sample data only. Do not use to calculate results.

Calibrator	OD 1	OD 2	Mean OD	Value (μ g/dL)
A	2.241	2.133	2.187	0
B	1.965	1.914	1.940	1
C	1.757	1.799	1.778	3
D	1.221	1.254	1.238	10
E	0.540	0.502	0.521	30
F	0.222	0.216	0.219	100
Unknown	0.287	0.283	0.285	63

TYPICAL CALIBRATOR CURVE





PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Cortisol Saliva ELISA kit is **1.0 ng/mL**.

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the Direct Cortisol Saliva ELISA kit with cortisol cross-reacting at 100%.

Steroid	% Cross-Reactivity
Cortisol	100
Prednisolone	13.6
Corticosterone	7.6
Deoxycorticosterone	7.2
Progesterone	7.2
Cortisone	6.2
Deoxycortisol	5.6
Pednisone	5.6
Dexamethasone	1.6

No cross reaction was detected with DHEAS and Tetrahydrocortisone.

Please note that there is an observed cross-reactivity of 13.6% with prednisolone. Since prednisone is converted to prednisolone in vivo, caution must be exercised when assaying the cortisol levels of patients undergoing either therapy.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in ng/mL) are tabulated below:

Sample	Mean	SD	CV %
1	6.6	0.68	10.3
2	24.8	1.98	8.0
3	52.4	3.40	6.5

INTER-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in ng/mL) are tabulated below:

Sample	Mean	SD	CV %
1	6.3	0.63	9.8
2	23.7	2.06	8.7
3	51.8	3.37	6.5



RECOVERY

Spiked samples were prepared by adding defined amounts of cortisol to three patient saliva samples (1:1). The results (in ng/mL) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1 Unspiked	6.28	-	-
+ 1.0	4.14	3.64	113.7
+ 10.0	9.05	8.14	111.2
+ 100.0	61.85	53.14	116.4
2 Unspiked	8.03	-	-
+ 3.0	6.05	5.52	109.6
+ 30.0	20.64	19.02	108.5
+ 100.0	52.20	54.02	96.6
3 Unspiked	6.98	-	-
+ 3.0	5.38	4.99	107.8
+ 10	8.76	8.49	103.2
+ 30	19.00	18.49	102.8

LINEARITY

Three patient saliva samples were diluted with calibrator A. The results (in ng/mL) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1	18.18	-	-
1:2	10.32	9.09	113.5
1:4	5.09	4.55	112.1
1:8	2.20	2.27	96.7
2	49.89	-	-
1:2	28.03	24.95	112.3
1:4	13.29	12.47	106.6
1:8	7.97	7.24	110.1
3	68.53	-	-
1:2	34.27	31.49	91.9
1:4	17.13	13.81	80.6
1:8	8.57	7.48	87.3

EXPECTED VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Random male and female samples were taken in the early morning and had an absolute range of: 5 – 21.6 ng/mL.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.