



Diagnostics Biochem Canada Inc.

Manufacturer of In Vitro Diagnostic Test Kits Since 1973

ELISA Kit...The EiAsy™ Way

CORTISOL SALIVA

REF CAN-C-290

Version: 3.0

Effective: October 15, 2003



INTENDED USE

For the quantitative determination of Cortisol by enzyme immunoassay in human saliva. For *in vitro* diagnostic use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. 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.

CLINICAL APPLICATIONS

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.

PROCEDURAL CAUTIONS AND WARNINGS

- 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.
- Control materials should be included in every run at a high and low level for assessing the reliability of results.
- When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
- In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.

- 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.
- A calibrator curve must be established for every run.
- The control should be included in every run and fall within established confidence limits.
- Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the control do not reflect established ranges.
- When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
- 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.
- When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
- To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
- 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.
- Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

- 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.
- Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
- Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
- The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient's background including the frequency of exposure to animals/products if false results are suspected.

SAFETY CAUTIONS AND WARNINGS POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and control has been tested and found to be non-reactive 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. However no test method 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

- Precision pipettes to dispense 50, 100, 150 and 300 µl
- Disposable pipette tips
- Distilled or deionized water
- Plate shaker
- Benchtop centrifuge
- Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10).

REAGENTS PROVIDED

1. Rabbit Anti-Cortisol Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.

Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant. Storage: Refrigerate at 2-8°C. Stability: 12 months or as indicated on label.

2. Cortisol-Horseradish Peroxidase (HRP) Conjugate Concentrate – [X50]

Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative. Volume: 300 µl/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 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12ml 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
Calibrator A	0 ng/ml	2.0 ml
Calibrator B	1 ng/ml	0.6 ml
Calibrator C	3 ng/ml	0.6 ml
Calibrator D	10 ng/ml	0.6 ml
Calibrator E	30 ng/ml	0.6 ml
Calibrator 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. Control - Ready To Use.

Contents: One vial containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of cortisol. Refer to vial label for expected value and acceptable range.

Volume: 0.6 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - [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 1:10 in distilled or deionized water before use. If the 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 vial containing a protein-based buffer with a non-mercury preservative.

Volume: 15 ml/vial

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 vial containing 1M sulfuric acid.

Volume: 6 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.



European Conformity



Consult Instructions For Use



In Vitro Diagnostic Use



Lot Number



Temperature Limit



Use By



Manufacturer



Catalogue Number



Dilute 1:# Before Use



Reconstitute With # mL H₂O



Authorized Representative



Authorized Representative

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 microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µl of the conjugate working solution into each well (We recommend using a multichannel pipette).
5. Incubate on a plate shaker (approximately 200 rpm) for 45 minutes at room temperature.
6. Wash the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).
7. Pipette 150 µl of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 15-20 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
9. Pipette 50 µl of stopping solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

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

CALCULATIONS

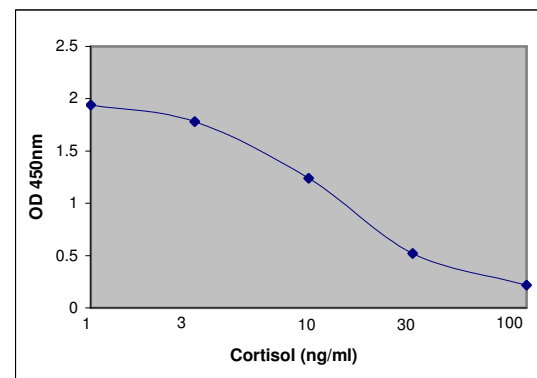
1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. 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

Calibrator	OD 1	OD 2	Mean OD	Value (ng/ml)
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

Sample curve only. **Do not** use to calculate results.



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 dbc 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 over a period of four weeks. 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	9.05	8.14	111.2
+ 100	61.85	53.14	116.4
2 Unspiked	8.03	-	-
+ 3.0	6.05	5.52	109.6
+ 30	20.64	19.02	108.5
+ 100	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 NORMAL 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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CONTACT INFORMATION

GENERAL:



dbc-Diagnostics Biochem Canada Inc.
1020 Hargrieve Road
London, Ontario, Canada, N6E 1P5
Tel/Fax: (519) 681 8731, E-mail: dbc@dbc-labs.com
Internet: www.dbc-labs.com

ISO 9001:2000 and ISO 13485 Certified

IN EUROPE:



Emergo Europe
Molenstraat 15
2513 BH, The Hague
The Netherlands
Tel: (+31) 70 345 8570
Fax: (+31) 70 346 7299