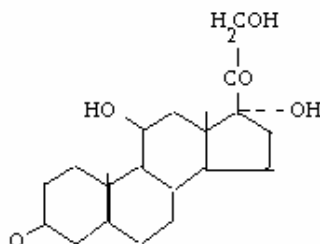


Cortisol ELISA

Catalog # CO078S

1.0 INTRODUCTION

Cortisol /hydrocortisone, compound F) is the main corticosteroid secreted in humans by the adrenal cortex. This steroid hormone, of molecular weight 363.5, has the following formula:



In most physiological conditions, only about 10% of plasma cortisol circulates unbound from transcortin and albumin. Among the products of the human adrenal cortex, only cortisol is involved in the regulation of ACTH secretion. As the level of free (non-protein bound) cortisol in blood rises, the release of ACTH is inhibited by the negative feedback effect. Conversely, if cortisol levels are subnormal, the negative feedback decreases, ACTH levels rise, and the adrenal cortex secretes cortisol until normal blood levels are restored. The release of ACTH is under control of hypothalamic corticotrophin-releasing hormone (CRH); the negative feedback system involving cortisol has been identified at both hypothalamic and pituitary levels. (1). Normally during the day there is a fluctuation of cortisol achieving the highest level in the morning and the lowest in the night. Useful information is given when cortisol measurement is done in samples withdrawn at a fixed hour (8.00 a.m.).

The main biological effects of cortisol are: promotion of gluconeogenesis, deposition of liver glycogen, increase in blood glucose concentration when the carbohydrate utilization is reduced, effect on fat metabolism and anti-inflammatory action.

Cortisol measurement is a powerful tool for the evaluation of suspected abnormalities in glucocorticoid production: Cushing's Syndrome (hypercortisolism), Addison's disease or secondary adrenal insufficiency (hypocortisolism).

In many cases, it is necessary to perform dynamic tests (suppression or stimulation) in order to localize the defect at one of the three main levels (i.e. adrenal, pituitary, hypothalamus).

2.0 PRINCIPLE OF THE TEST

The solid phase enzyme immunoassay for cortisol is a competitive type immunoassay wherein horse radish peroxidase-labeled cortisol (HRP-cortisol) competes with cortisol present in the patient sample for a fixed and limited number of antibody sites immobilized on the wells of the microstrips.

Once the competitive immunoreaction has occurred, the wells are rinsed, and the HRP -cortisol fraction bound to the antibody in the solid phase is measured by adding a chromogen/substrate solution which is converted to a blue compound.

After 15 minutes of incubation, the enzymatic reaction is stopped with hydrochloride acid

3.0 WARNING AND PRECAUTIONS

3.1 ALL THE MATERIAL SUPPLIED IS FOR IN VITRO DIAGNOSTIC USE ONLY:

3.2 Potentially Infectious Material

The human blood products supplied as components of this kit have been obtained from donors who were tested individually and found to be negative for the presence of Human Immunodeficiency Virus antibodies (HIV-ab) as well as for Hepatitis B surface Antigen (HBsAg) using reliable methods.

Since no test method can offer complete assurance that Hepatitis B Virus, Human Immunodeficiency Virus (HIV) or other infectious agents are absent, all human blood products should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists, (i.e. USA Center for Disease Control/National Institutes of Health Manual "Biosafety in Microbiological and Biomedical Laboratories", 1984).

3.3 Precautions

The kit requires the use of sulfuric acid 0.5 M (corrosive) and 3,3',-5,5'-Tetramethylbenzidine. Avoid contact of these reagents with skin and mucous membranes. Should this occur, wash thoroughly with cold tap water.

DO NOT PIPETTE BY MOUTH.

4.0 KIT COMPONENTS AND PREPARATION OF THE WORKING SOLUTIONS

Coated Microplate:	12 Strips x 8 Wells	1 bag
Cortisol Calibrators		7 vials
HRP-Cortisol Conjugate*		1 vial
Washing Solution		1 bottle
Chromogen/Substrate solution		1 vial
Stop Solution		1 vial

The expiration date of the kit is printed on the external label.

Upon receipt, store the kit at 2-8°C.

*The Enzyme Conjugate may contain black particles. These don't influence the kit performance.

4.1 Coated Microplate

The bag contains a microplate of 12 strips x 8 wells. Each well is coated with monoclonal anti-cortisol antibody

(mouse). After the first opening the strips are stable for 8 weeks when stored at 2-8°C in the plastic zip pouch with the desiccant.

4.2 Cortisol Standards

Ready to use reagent.

The vials contain standard cortisol in stripped human serum. Cortisol concentrations are the following: 0, 20, 50, 100, 200, 400, 800 ng/ml, thus corresponding to 0, 55.2, 138, 276, 552, 1104, 2208 nmol/l (conversion factor:

1 ng/ml = 2.76 nmol/l).

The volume of all calibrators is 1 ml.

Store at 2-8°C. Stable for 8 weeks after first opening.

4.3 HRP-Cortisol Conjugate

Ready to use reagent.

The vial contains 25 ml of HRP-Cortisol conjugate in buffer supplemented by enzyme stabilizers and cortisol binding protein displacers.

Store at 2-8°C. Stable for 8 weeks after first opening.

Note: The Enzyme Conjugate may contain black particles. This does not influence the assay performance.

4.4 Washing Solution.

The bottle contains 30 ml of washing solution (**Concentrate 40 X**).

The working solution is to be prepared by **mixing the content of the bottle with 1170 ml of distilled water**. Stable for 8 weeks when stored at room temperature.

4.5 Chromogen (TMB) / Substrate (H₂O₂) Solution - Ready -to-use Reagent.

The vial contains 14 ml of a stabilized mixture of TMB (3,3' - 5,5' Tetramethylbenzidine) and H₂O₂ (Hydrogen Peroxide). Store at 2-8°C. After the first opening the solution is stable for 8 weeks at 4°C and 2 weeks at room temperature

The TMB/H₂O₂ single solution is colourless or slightly yellow-blue. If accidental contamination occurs, the solution starts to develop a blue colour and, therefore, it must be discarded.

In order to avoid contamination, should one part of the vial content be used, transfer the volume needed into a clean plastic container previously washed with ethanol and rinsed with high-quality distilled water.

The TMB/H₂O₂ single solution is not sensitive to the light. However, direct sunlight can oxidize the solution to a blue colour. Such a colour disappears after 4 hours storage in the dark and the solution becomes again usable.

4.6 Stop Solution

Ready to use.

The vial contains 14 ml of 0.5 M H₂SO₄.

Store at 2-8°C.

5.0 EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 5.1 Precision pipettes with disposable tips (20µl).
- 5.2 Semi-automatic pipette for the repetitive dispensing of 100 and 200 µl volumes.
- 5.3 High quality distilled water.
- 5.4 A microtiter plate reader equipped for the measurement of absorbance at 450 nm. It is advised to use a bichromatic reader capable of measuring the absorbance at 450 and 620 nm at the same time.
- 5.5 Automatic plate washer (optional).

6.0 SAMPLE STORAGE

Serum or plasma samples can be stored overnight at 2-8°C. For a longer storage, the samples should be frozed at - 20°C.

7.0 ASSAY PROCEDURE

- 7.1 Bring all reagents to room temperature.
- 7.2 Leave sufficient strips in the strip holder to enable the running of calibrators, controls, and samples in duplicate, plus one well for chromogen blank.
Place the exceeding strips and the desiccant into the transparent plastic pouch and seal it properly.
- 7.3 Pipette 20 µl of calibrators and samples into the appropriate wells of the strips.
- 7.4 Add 200 µl of HRP.cortisol conjugate to each well in sequence.
- 7.5 Incubate for 60 minutes at room temperature without covering the plate.
- 7.6 Washing: discard the incubation solution, rinse the wells with the washing solution three times, and remove any residual (see point 8.2).
- 7.7 Promptly pipette 100 µl of the chromogen/substrate mixture into the rinsed wells.
- 7.8 Incubate for 15 minutes at room temperature.

- 7.9 Stop the reaction by pipetting 100 µl of stop solution into the wells with the same sequence adopted to dispense the chromogen/substrate mixture.
- 7.10 Shake gently the microplate being careful not to let the content come out from the wells and read at 450 nm within 1 hour from the stopping.

8.0 PROCEDURAL NOTES

8.1 The total dispensing time of calibrators, controls and specimens for a whole plate should not exceed 15 minutes.

8.2 Rinsing Protocol

For the rinsing procedure, the use of an automatic plate wash equipment is recommended. After the last washing, tap the inverted plate on absorbent paper to remove any residual from the wells.

Three washings are required.

If an automatic plate washer is not available, the washing procedure can be carried out manually using a simple wash-bottle filled with the washing solution:

Empty the content of the wells by keeping the plate tight in the middle and turning it firmly upside-down.

Fill the wells with the washing solution contained in the wash-bottle and empty them as described above.

Repeat this procedure twice more.

After the third washing, firmly tap the inverted plate on absorbent paper to remove any residual liquid from the wells.

8.3 Dispensation of the Chromogen/Substrate Solution.

In order to obtain precise and accurate results it is necessary to dispense the chromogen/substrate solution immediately after the washing.

It is recommended to time the addition of the chromogen/substrate solution and stop solution until a good familiarity with the method is acquired, (i.e. if the chromogen/substrate solution is dispensed into the wells every 3 seconds one from the other, also the stop solution should be dispensed in the same order and with the same frequency).

The use of repetitive pipettes is particularly convenient. The contamination of solutions should be avoided.

Incubation in the dark is not necessary. However, avoid a direct exposure to sunlight.

Since the enzymatic reaction is temperature-dependent, different absorbances can be obtained according to the laboratory temperature. If in a test run the absorbance of the zero calibrator is lower than 1.3 or higher than 2.0, because the lab temperature is lower than 21°C or higher than 28°C, the next runs should be carried out setting the incubation time for the colour development to 20 and 10 minutes respectively. However, in case of absorbances higher than 2.0 it is not necessary to shorten the incubation time for the enzymatic reaction when the microplate reader is linear up to 2.5-3.0 absorbance units.

9.0 CALCULATION OF RESULTS

Calculate the mean absorbance of calibrators and samples (A).

Subtract the absorbance of the chromogen blank (Ac) from all the means.

Divide the corrected mean absorbance obtained by the corrected mean absorbance of the zero calibrator (Ao) and multiply by 100.

$$B/B_0 \times 100 = \frac{A - A_c}{A_0 - A_c} \times 100$$

Plot $B/B_0 \times 100$ versus the respective cortisol concentrations on logit/log or semilog graph paper and determine the concentrations of cortisol in the samples by interpolation from calibration curve.

The results can also be calculated with normal programs for automatic data processing, i.e. 4 Parameters, Spline, Logit-Log.

Should cortisol value exceed the highest calibrator value, dilute the sample with the zero calibrator and re-run the assay. Multiply the result obtained by the sample dilution factor.

10.0 LIMITATION OF THE PROCEDURE

- 10.1 Reliable results are obtained by a thorough understanding of the package insert supported by the use of a skillful technique and strict adherence to the instructions.
- 10.2 A calibration curve must be accomplished in duplicate in each run.
- 10.3 Reagents from different kits and lots should not be mixed. It is advised not to exchange strips of different plates even if 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.
- 10.4 Do not use expired kits.
- 10.5 Grossly lipemic or haemolyzed samples should not be used.
- 10.6 The antibody shows some cross-reactions with 11-deoxycortisol and prednisolone. This interference should be considered in the case of patients receiving prednisolone therapy or undergoing metyrapone suppression test, because of the resulting accumulation of 11-deoxycortisol in serum.
The same caution should be taken in case of patients being treated with prednisone therapy because of its conversion to prednisolone in the body.

11.0 EXPECTED VALUES

Cortisol value in serum or plasma ranges from 50 to 230 ng/ml (138-635 nmol/l) between 8:00 - 10:00 a.m., and from 30 to 150 ng/ml (82.8-414 nmol/l) at 4:00 p.m.

These values are from Tietz's Textbook (1) and may be used as main guidelines until each laboratory establishes its own normal ranges.

12.0 PERFORMANCE FEATURES

12.1 Lower Limit of Detection

The lower limit of detection - defined as the cortisol concentration given by the mean absorbance of the zero calibrator minus 2 standard deviations - was assessed to be approx. 2.5 ng/ml (6.9 nmol/l).

12.2 Specificity

The specificity of the DRG Cortisol Kit was assessed according to Abraham's method:

Steroid	Cross-reactivity
Cortisol	100.0%
Prednisolone	60.0%
Corticosterone	29.0%
Cortisone	3.0%
11-Deoxycortisol	< 1.0%
17-OH Progesterone	< 0.5%
Prednisone	< 0.1%
Progesterone	< 0.1%
Dexamethazone	< 0.1%
Desoxycorticosterone	< 0.1%

Dehydroepiandrosterone sulfate	< 0.1%
Estradiol	< 0.1%
Estriol	< 0.1%
Estrone	< 0.1%
Testosterone	< 0.1%

12.3 Precision

Inter Assay

Serum	n	<X> ± SD ng/ml	CV %
1	24	26.14 ± 1.30	4.96
2	24	167.96 ± 9.59	9.59
3	26	266.92 ± 17.68	6.62

Intra Assay

Serum	n	<X> ± SD ng/ml	CV %
1	18	26.68 ± 11.8	4.41
2	20	172.86 ± 6.87	3.98
3	20	278.80 ± 13.11	4.70

12.4. Accuracy

The accuracy of the assay was evaluated by recovery and dilution tests.

Recovery test

Serum	Endog. Cortisol ng/ml	Added Cortisol ng/ml	Measured conc. ng/ml	Expected conc. ng/ml	Recovery %
1	84	0.0	84.00		100
		50	133.66	134.00	99.75
		100	170.15	184.00	92.47
		200	270.48	284.00	95.24
2	14.90	0.0	14.90		100
		50	66.73	64.90	102.82
		100	109.72	114.90	95.49
		200	202.91	214.90	94.42

Dilution test

Serum	Dilution factor	Measured conc. ng/ml	Expected conc. ng/ml	Recovery %
1	Undiluted	287.27		
	1:2	136.87	139.14	98.37
	1:4	69.21	69.57	99.49
	1:8	31.84	34.78	91.54
2	Undiluted	259.61		
	1:2	128.99	129.81	99.37
	1:4	65.77	64.90	101.34
	1:8	30.98	32.45	95.47
3	Undiluted	157.94		
	1:2	83.00	78.97	105.10
	1:4	40.89	39.49	103.56
	1:8	23.20	19.74	117.51

13.0 REFERENCES

- (1) Tietz, N.W. Testbook of Clinical Chemistry, Saunders, 1986.