

## 1. NAME AND INTENDED USE

CORT-CT2 is a radioimmunoassay kit for the quantitative determination of cortisol in human serum, plasma, urine and saliva.

## 2. INTRODUCTION

Cortisol is a steroid hormone of M.W. 362.5 having the following formula:

4-pregnen-11  $\beta$ , 17  $\alpha$ , 21-triol, 3,20-dione

Cortisol is secreted by the adrenal cortex cells directly into the blood: under most physiological conditions about 90 % of plasma cortisol is strongly but reversibly bound to a carrier protein, Corticosteroid Binding Globulin (CBG, transcortin). A small amount is bound to albumin and about 10% is free.

The most important biological effects of cortisol are:

- deposit of glycogen in the liver
- production of glucose from amino acids
- delay of glucose oxidation

Cortisol is the only adrenocortical hormone capable of slowing down both production and release of ACTH from the pituitary, through the hypothalamic centres which produce CRF (Corticotrophin Releasing Factor). Cortisol is therefore directly involved in the mechanism of autoregulation of the hypothalamic-pituitary-adrenal cortex axis.

There are wide variations in cortisol blood levels throughout the day. The typical circadian rhythm of cortisol secretion can be correlated to the activity of the cerebral centres and the pituitary. Cortisolaemia reaches a maximum in the early morning hours, then it shows a sharp decrease during the afternoon and the evening; the lowest level is reached about midnight.

It is obvious that cortisol assay on a single blood sample taken at random during the day does not give useful indications about the functional behaviour of the adrenal cortex.

Moreover, it must be remembered that the blood level of a hormone does not depend only on the amount secreted by the gland cells, but also on the circulating carrier protein, the distribution of the hormone and the clearance rate from the blood due to both hepatic catabolism and renal excretion.

In spite of this, cortisol determination in plasma samples taken at a fixed time (e.g. 8-9 a.m) gives very useful information about gross impairment of adrenals, thus constituting an essential screening tool.

Determination of cortisol also plays an important role in several stimulation and suppression tests, which are used mainly to study the hypothalamus pituitary adrenal cortex axis.

## 3. PRINCIPLE

The principle of the assay is based on the competition between the labelled cortisol and cortisol contained in standards or specimens to be assayed for a fixed and limited number of antibody binding sites bound to the solid phase (coated tubes).

After incubation, the unbound tracer is easily removed by a washing step.

The amount of labelled cortisol bound to the antibody is inversely related to the amount of unlabelled cortisol initially present in the sample.

## 4. REAGENTS

Each kit contains enough reagents for 100 tubes. The expiry date is marked on the external label.

REAGENTS	QUANTITY	STORAGE
<b>COATED TUBES:</b> ready to use. Polyclonal rabbit cortisol antibodies coated onto the bottom of the tube.	100 tubes	2-8°C until the expiry date (in their original package).
<b><sup>125</sup>I-CORTISOL:</b> ready to use. <sup>125</sup> I labelled cortisol in tris buffer < 0.1%Thimerosal, NaN <sub>3</sub> , red colour additive. ≤ 250 KBq (≤ 6.75 $\mu$ Ci).	1 55 mL vial	2-8°C until the expiry date.
<b>STANDARDS:</b> lyophilized. Human serum and 0.1% sodium azide and Kathon. 0 - 20 - 75 - 500 et 2000 nmol/L (*).	5 vials reconstitute with 0.5 mL distilled water	2-8°C until the expiry date. 2-8°C 8 weeks after reconstitution.

(\*) The standards are calibrated relative to international standards measured by mass spectrometry coupled to gas chromatography.

## 5. PRECAUTIONS FOR USE

### 5.1. Safety measures

Raw materials of human origin contained in the reagents of this kit have been tested with licensed kits and found negative for the anti-HIV 1, anti-HIV 2, anti-HCV antibodies and the HBs antigen. However as it is impossible to strictly guarantee that such products will not transmit hepatitis, the HIV virus, or any other viral infection, all raw materials of human origin including the samples to be assayed must be treated as potentially infectious.

Do not pipette by mouth. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled.

Wear disposable gloves while handling kit reagents or specimens and wash hands thoroughly afterwards.

Avoid splashing.

Decontaminate and dispose of specimens and all potentially contaminated materials as if they contained infectious agents. The recommended method of doing this is autoclaving for a minimum of one hour at 121.5°C.

Sodium azide may react with lead or copper piping to form highly explosive metal azides. During waste disposal, flush the drains thoroughly to prevent a build-up of these products.

## 5.2. Basic radioprotection rules

This radioactive product may only be received, purchased, stored or used by persons so authorized, and by laboratories covered by such authorization. The solution should under no circumstances be administered to humans or to animals. The purchase, storage, use or exchange of radioactive products are subject to the laws in force in the user's country. The enforcement of the basic rules for handling radioactive products ensures adequate security.

A summary of these is given below:

Radioactive products must be stored in their original containers in a suitable area.

A record of the reception and storage of radioactive products must be kept up to date.

Handling of radioactive products should take place in a suitably-equipped area with restricted access (controlled zone).

Do not eat, drink, smoke or apply cosmetics in a controlled zone. Do not mouth-pipette radioactive solutions.

Avoid any direct contact with all radioactive products by using laboratory coats and protective gloves.

Contaminated laboratory equipment and glassware must be disposed of immediately after contamination to prevent cross-contamination of different isotopes.

Any contamination or radioactive substance loss should be dealt with in accordance with the established procedures.

All radioactive waste disposal must be carried out according to the regulations in force.

## 5.3. Handling precautions

Do not use kit components beyond their expiry date.

Do not mix reagents from different batches.

Avoid any microbial contamination of the reagents or of the water used for washing.

Fully respect the incubation times and the washing instructions.

Store coated tubes in their own package at 2-8°C. Take only the amount of coated tubes needed in one run.

Do not remove the unused tubes from the box cover.

## 6. SPECIMEN COLLECTION AND PREPARATION

### Serum and plasma

The assay is performed on sera or plasma (heparin or EDTA). Haemolyzed or hyperlipemic samples should not be used. If the test is to be carried out within 1 week, the samples must be refrigerated at 2-8°C. Otherwise, they should be divided into aliquots, deep frozen (-20°C) for up to 6 months and must be thawed only just before using. Do not refreeze samples for later use.

If elevated cortisol levels are suspected, standard "0" should be used for dilution. It is recommended that disposable plastic tubes be used when carrying out the dilutions.

### Urine

Diurnal urine samples should be collected (without the use of preservatives) which should have pH values between 4-11. Samples may be stored at 2-8°C for up to 48 h or deep-frozen for up to 6 months at -20°C. For dilution, saline solution (0.9 % NaCl) may be used.

### Saliva

Saliva samples should be collected using saliva collecting tubes (e.g. Salivette, Sarstedt), centrifuged and stored at -20°C for up to 6 months. For dilution, saline solution (0.9% NaCl) may be used.

## 7. ASSAY PROCEDURE

### 7.1. Material required

Precision micropipettes or similar, with disposable tips, capable of dispensing 20, 500 µL (sera), 100, 250, 300, 500 µL (urinary extraction procedure), 150 µL and 500 µL (saliva procedure). Their calibration should be checked regularly. Semiautomatic pipette for accurately dispensing 500 µL. Positive displacement pipette 1000 µL (for extraction). Reagent dispenser 1 mL (for washing) and 5 mL (for extraction). Distilled water. Dichloromethane, NaOH 0.1M and Tris-HCl buffer 0.1M pH 7.4 with 0.2% BSA. Nitrogen gas. Vortex type mixer. Absorbent paper. Water bath (37°C). Disposable plastic test-tubes and glass tubes for urinary extraction. Parafilm. Gamma scintillation counter calibrated for 125 Iodine.

### 7.2. Reconstitution of the standards

Reconstitute the standards with 0.5 mL of distilled water. Recap the vial. Mix gently to ensure complete dissolution of the lyophilized material.

N.B.:The reconstituted standards should stand at least 30 minutes after reconstitution before proceeding.

### 7.3. Protocol

All reagents should be brought to room temperature (18-25°C) at least 30 minutes before their use. Dispensing of reagents is also carried out at room temperature.

The assay requires the following groups of tubes:

T group, for the total activity determination. Standard groups, to establish the standard curve.

Reference group for the external controls.

Sx groups, for the test samples.

It is recommended to perform the assay in duplicate for the standard groups, controls and samples.

Strictly respect the order in which reagents should be added.

#### A- Procedure for direct measurement of serum and urine cortisol

**Dispense** 20 µL of standards, controls and samples to be assayed into the correspondingly-labelled coated tubes.

**Add** 500 µL of <sup>125</sup>I -cortisol to each tube (and T group).

**Mix** each tube gently with a Vortex-type mixer.

**Cover** tubes with plastic film.

**Incubate** 2 hours at 37°C

**Decant** liquid from each assay tube and **tap** the top of each tube firmly against absorbent paper (except T tubes).

**Wash** once with 1 mL of distilled water, shaking the rack by hand.

**Empty** the tubes and tap firmly onto absorbent paper. Leave the tubes standing upside down for at least 5 min. (except T tubes).

**Measure** the remaining radioactivity bound to the tubes with a gamma scintillation counter calibrated for 125 Iodine.

#### B- Extraction procedure for urinary cortisol measurement

As an alternative to the standard direct assay method, urinary cortisol may be assayed using the following extraction method:

Using an automatic reagent dispenser, **add** 5 mL dichloromethane to 250 µL aliquot of standards and 500 µL aliquot of samples in the corresponding glass tubes.

**Vortex** for 30 seconds in pulse of about 5 seconds. Allow the layers to separate.

After having discarded the aqueous layer, **wash** once with 1 mL, 0.1M NaOH and then twice with 1 mL distilled water, each time **vortexing** the samples for 10 seconds.

Use a positive displacement pipette to **transfer** precisely 1000 µL of eluates.

**Evaporate** to dryness under N<sub>2</sub>.

**Dissolve** the residue in 300 µL buffer (0.1M Tris-HCl, pH 7.4, 0.2% BSA) by mixing vigorously for 15 seconds.

**Dispense** 100 µL of extracted standards and patient samples to be assayed into the correspondingly-labeled coated tubes.

**Add** 500 µL of <sup>125</sup>I -cortisol to each tube (and T group).

**Mix** each tube gently with a Vortex-type mixer.

**Cover** tubes with plastic film.

**Incubate** 30 minutes at 37°C.

**Decant** liquid from each assay tube and **tap** the top of each tube firmly onto absorbent paper (except T tubes).

**Wash** once with 1mL of distilled water, shaking the rack by hand.

**Empty** the tubes and tap firmly onto absorbent paper. Leave the tubes standing upside down for at least 5 min. (except T tubes).

**Measure** the remaining radioactivity bound to the tubes with a gamma scintillation counter calibrated for 125 Iodine.

**Correct** for volume by dividing the cortisol concentration results by two.

#### C- Procedure for salivary cortisol measurement

Since the concentration of cortisol in saliva is very low, the method for serum and urine samples cannot be used directly. Instead the following procedure should be adopted:

Using the 2000 nmol/L standard, **prepare** the following dilution buffer (0.1 M Tris-HCl, pH 7.4, 0.2 % BSA):

0 - 1.0 - 4.0 - 20 and 100 nmol/L. Prepare the 0 standard with the buffer.

**Dispense** 150 µL of standards, controls and samples to be assayed into the correspondingly-labelled coated tubes.

**Add** 500 µL of <sup>125</sup>I -cortisol to each tube (and T group).

**Mix** each tube gently with a Vortex-type mixer.

**Cover** the tubes with plastic film (parafilm).

**Incubate** for 30 minutes at 37°C.

**Decant** liquid from each assay tube and **tap** the top of each tube firmly onto absorbent paper (except T tubes).

**Wash** once with 1 mL of distilled water, shaking the rack by hand.

**Empty** the tubes and tap firmly onto absorbent paper. Leave the tubes standing upside down for at least 5 min. (except T tubes).

**Measure** the remaining radioactivity bound to the tubes with a gamma scintillation counter calibrated for 125 Iodine.

#### 8. QUALITY CONTROL

Good laboratory practices require the use of quality control samples in each series of assays to check the quality of the results obtained. All specimens should be treated identically, and result analysis using the appropriate statistical methods is recommended.

#### 9. RESULTS

For each group of tubes compute the mean counts. Calculate B/Bo values. Draw up the standard curve by plotting the B/Bo of the standards against their concentration. Read sample values directly from the standard curve, and correct the read value for the dilution factor, if necessary.

This data must not be substituted for results obtained in the laboratory.

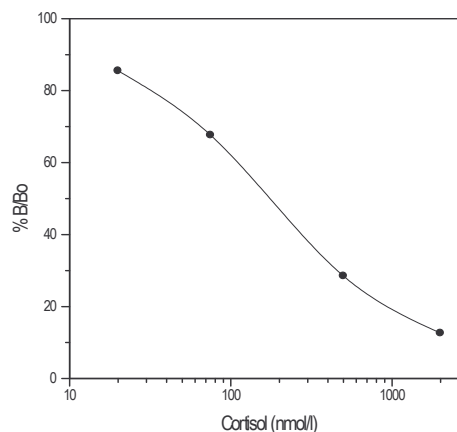
Conversion to ng/mL may be accomplished by using the following equation: Cortisol (ng/mL) = Cortisol (nmol/L) x 0.3625

If using the urine extraction method, the result must be corrected for volume by dividing the cortisol concentration by two.

The 24h urine cortisol excretion (nmol/L) is calculated by multiplying the urine cortisol concentration (nmol/L) with the volume of 24 h urine (L). Spline function curve is recommended.

**Typical standard curve:** example of result with 2h incubation procedure.

GROUPS OF TUBES	Mean CPM	B/Bo x 100	Concentration nmol/L
T	57157		
Standard 0 0 nmol/L	48389		
Standard 1 20 nmol/L	41352	85.5	
Standard 2 75 nmol/L	32700	67.6	
Standard 3 500 nmol/L	13804	28.5	
Standard 4 2000 nmol/L	6115	12.6	
Sample A	10596	21.9	796
Sample B	32459	67.1	77



## 10. PROCEDURAL LIMITATIONS

Strict adherence to the exact procedures described in this package insert and full technical care are necessary to obtain reliable results with the CORT-CT2 kit.

## 11. EXPECTED VALUES

Each laboratory must establish its own range of normal values.

Cortisol levels from healthy, fasting individuals obtained utilizing the CORT-CT2 kit are presented in the table below (indicative values at 0.025 fractile):

	Serum (nmol/L)		24 h urine (nmol/L)		Saliva (nmol/L)	
	8-10 am	3 - 5 pm	direct	Extr.	6-8 am	6-8 pm
n	220	60	45	43	28	28
Means (nmol/L)	308	182	147	123	22.1	2.7
Reference interval* (nmol/L)	131 - 642	61 - 429	54 - 319	26 - 354	6.2 - 38.1	0.6 - 4.9
Confidence intervals 0.90						
for lower reference limit	104 - 146					
for upper reference limit	576 - 709					

\* Reference interval = 0.025 and 0.975 fractiles

## 12. SPECIFIC CHARACTERISTICS OF THE ASSAY

### 12.1. Measurement range

The CORT-CT2 kit can be used to measure concentrations ranging from 20 to 2000 nmol/L (for serum and urine), and from 1 to 100 nmol/L (for saliva).

### 12.2. Precision

This was evaluated with 3 serum samples with different concentrations assayed 10 times in the same series according to the direct measurement procedure.

Samples	Within-run	
	Mean (nmol/L)	(%) CV
1	62	4.5
2	235	3.8
3	743	4.2

This was evaluated with 3 serum samples with different concentrations assayed 10 independent assays according to the direct measurement procedure.

Samples	Between-run	
	Mean (nmol/L)	CV (%)
4	73	5.5
5	436	4.4
6	768	4.3

### 12.3. Recovery test

Known quantities of cortisol were added to different serum pools. The recovery percentage of cortisol obtained were between 86 % and 107%.

	Mean R %	Range
Serum	99.1	85.9 - 107.1
Urine		
direct	104.4	89.3 - 114.8
extraction	123	111 - 137
Saliva	123	110 - 136

### 12.4. Specificity

Determined from equivalent displacement measurements at 50 % binding. The antiserum used in the test shows the following cross-reactions:

Compound	2 h at 37°C	Compound	2 h at 37°C
Cortisol	100 %	18-Hydroxycorticosterone	< 0.1
Prednisolone	42	17 $\alpha$ -Hydroxypregnenolone	< 0.1
Fludrocortisone	12.1	17 $\alpha$ -Hydroxyprogesterone	0.02
5 $\alpha$ -Dihydrocortisol	8.8	Beclomethasone	< 0.01
21-Deoxycortisol	8.6	Betamethasone	< 0.01
Corticosterone	2.8	Cortisol-21-sulphate, Na salt	< 0.01
6 $\alpha$ -Methylprednisolone	1.2	Dehydroepiandrosterone sulphate	< 0.01
Prednisone	1.2	Dexamethasone	< 0.01
Cortisone	0.9	20 $\alpha$ -Dihydrocortisol	< 0.01
6 $\beta$ -Hydroxycortisol	0.6	Oestradiol	< 0.01
20 $\beta$ -Dihydrocortisol	0.4	Progesterone	< 0.01
11-Deoxycortisol	0.3	Testosterone	< 0.01
11-Deoxycorticosterone	0.2		

Effect of the measured compounds

Compound	Therapeutical or physiological max concentration (nmol/L)	Cross reactivity (%)	Increase in cortisol concentration (nmol/L)
Aldosterone	10	-	< 4
Beclomethasone	100	< 0.01	< 4
Corticosterone	180	2.8	< 5
Cortisone	3000**	0.9	40
DHEA-S	8700	< 0.01	< 4
11-Deoxycorticosterone	0.303	0.2	< 4
11-Deoxycortisol	1000	0.3	5
Dexamethasone	100	< 0.01	< 4
20 $\alpha$ -Dihydrocortisol	3000**	< 0.01	< 4
20 $\beta$ -Dihydrocortisol	3000**	0.4	10
6 $\beta$ -Hydroxycortisol	3000**	0.6	20
Dihydrotestosterone	2.9	-	< 4
Estradiol	120	< 0.01	< 4
Estriol	104	-	< 4
Estrone	43	-	< 4
Fludrocortisone	100	12	< 40
18-Hydroxycortico- sterone	351	< 0.01	< 4
17 $\alpha$ -Hydroxypregnen olone	21	< 0.01	< 4
17 $\alpha$ -Hydroxyproge- sterone	1000	0.02	10
Prednisolone	1700	42	700
Progesterone	660	< 0.01	< 4
Testosterone	35	< 0.01	< 4

\* The maximum concentrations of the substances that may be encountered in patient samples according to Standardization of Hapten Immunoprocudures: Total Cortisol in Scandinavian Journal of Clin. Lab. Inv., Vol 53, Suppl. 216, 1993

\*\* In hyper cortisolism, the concentrations of these free cortisol metabolites may be this high or even higher in urine.

#### 12.5. Detection limit

The sensitivity of the method is defined as being the minimum detectable concentration equivalent to twice the standard deviation of the zero-binding value. It is approximately 4.6 nmol/L for direct measurement in serum and urine, 6 nmol/L with urinary extraction method and 0.8 nmol/L with saliva cortisol measurement.

#### 12.6. Dilution

High serum samples can be diluted up to 1:10 with zero serum, high urine and saliva samples can be diluted up to 1:10 with saline.

#### 12.7. Interfering substances

Serum bilirubin concentration ● 385  $\mu$ mol/L does not interfere. Serum hemoglobin concentration ● 6000 mg/L does not interfere.

The use of highly lipemic samples is not recommended.

**ASSAY FLOW-CHART**  
**(SERUM, PLASMA OR URINE)**

TUBES	Standards Controls Samples $\mu\text{L}$	$^{125}\text{I}$ - CORTISOL $\mu\text{L}$		Distilled water $\mu\text{L}$	
T	-	500	Mix ---- Cover the tubes. ---- Incubate 2 h at 37°C ---- Empty the tubes	-	Empty the tubes ---- Count
Standards	20	500		1000	
Controls and Samples	20	500		1000	

**(URINARY EXTRACTION)**

TUBES	Standards Controls Samples $\mu\text{L}$	Dichloro- methane $\mu\text{L}$		Solution 0.1M NaOH $\mu\text{L}$		Tampon Tris/HcL 0.1M + BSA $\mu\text{L}$	
Standards	250	5000	Vortex for 30 s in pulses of about 5 s Discard the aqueous Layer ----	Add 1 ml of 0.1 M NaOH solution. Mix. Discard the aqueous layer.	Transfer 1000 $\mu\text{L}$ of eluat.	<i>Redissolve in</i> 300	Mix vigorously
Controls and urinary samples	500	5000		Add 1 ml distilled water. Mix Discard the aqueous layer. Repeat once this operation with d.w.		Evaporate to dryness under N2 -----	

**(URINARY MEASUREMENT AFTER EXTRACTION)**

TUBES	Standards Controls Samples $\mu\text{L}$	$^{125}\text{I}$ - CORTISOL $\mu\text{L}$		Distilled water $\mu\text{L}$	
T	-	500	Mix. Cover the tubes. Incubate 30 min. at 37°C. Empty the tubes.	-	Empty the tubes. Count. Correct for volume.
Standards After extraction	100	500		1000	
Controls and Samples After extraction	100	500		1000	

**(SALIVA)**

TUBES	Standards Controls Samples $\mu\text{L}$	$^{125}\text{I}$ - CORTISOL $\mu\text{L}$		Distilled water $\mu\text{L}$	
T	-	500	Mix ---- Cover the tubes. ---- Incubate 30 min. at 37°C ---- Empty the tubes	-	Empty the tubes ---- Count --- Correct for volume
Standards after dilution of the 2000 nmol/L	150	500		1000	
Controls and samples	150	500		1000	