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DCM018-13
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URINARY CORTISOL ELISA

Direct immunoenzymatic determination of free Cortisol in urine

for routine analysis
for research use only

IVD | **LOT** See external label | 2°C 8°C | Σ = 96 tests | **REF DKO018**

INTENDED USE

The Eagle Biosciences Urinary Cortisol ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of free Cortisol concentration in Urine. Urinary Cortisol ELISA kit is for research use only and is not intended for diagnostic procedures.

1. CLINICAL SIGNIFICANCE

Cortisol is a steroid hormone released from the adrenal cortex in response to an hormone called ACTH (produced by the pituitary gland), it is involved in the response to stress; it increases blood pressure, blood sugar levels, may cause infertility in women, and suppresses the immune system.
Cortisol acts through specific intracellular receptors and has effects in numerous physiologic systems, including immune function, glucose-counter regulation, vascular tone, substrate utilization and bone metabolism. Cortisol is excreted primarily in urine in an unbound (free) form.
Cortisol is bound, in plasma, from corticosteroid-binding globulin (CBG, transcotin), with high affinity, and from albumin. Only free cortisol is available to most receptors.
These normal endogenous functions are the basis for the physiological consequences of chronic stress - prolonged cortisol secretion causes muscle wastage, hyperglycaemia, and suppresses immune / inflammatory responses. The same consequences arise from long-term use of glucocorticoid drugs.
The free cortisol fraction represents the metabolically active cortisol. In normal conditions, less then 1% it comes excrete in urines. In pathological conditions (syndrome of Cushing) the levels of free urinary cortisolo are elevate, because the CBG don't bound the plasmatic cortisol in excess and it was remove with urines.
During pregnancy or estro-progestogen treatment an increase of plasmatic cortisol caused by an increment of the production of the transport protein, but the levels of free urinary cortisol results normal to indicate a correct surrenic functionality.
This test is very useful to estimate the real surrenic function, because is dose the free cortisol, it is the metabolically active form. Moreover the measurement of free urinary cortisolo is the better parameter for the diagnosis of the Cushing's syndrome.

2. PRINCIPLE

The Cortisol (antigen) in the sample competes with the antigenic Cortisol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Cortisol coated on the microplate (solid phase).
After incubation, the bound/free separation is performed by a simple solid-phase washing.
Then, the enzyme HRP in the bound-fraction reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H_2SO_4) is added.
The colour intensity is inversely proportional to the Cortisol concentration of in the sample.
Cortisol concentration in the sample is calculated through a calibration curve.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

1. Calibrators (5 vials)

CAL0	(4 mL)	**REF DCE002/1806-0**
CAL1	(1 mL)	**REF DCE002/1807-0**
CAL2	(1 mL)	**REF DCE002/1808-0**
CAL3	(1 mL)	**REF DCE002/1809-0**
CAL4	(1 mL)	**REF DCE002/1810-0**

2. Controls (2 vial, 1 mL each, ready to use)

Low Control	**REF DCE045/1801-0**
High Control	**REF DCE045/1802-0**

3. Conjugate (1 vial, 33 mL)
Cortisol conjugated with horseradish peroxidase (HRP)
REF DCE002/1802-0

4. Coated Microplate (1 breakable microplate)
Anti Cortisol antibody adsorbed on the microplate
REF DCE002/1803-0

5. TMB Substrate (1 vial, 15 mL)
H_2O_2-TMB (0.26 g/L) *(avoid any skin contact)*
REF DCE004-0

6. Stop Solution (1 vial, 15 mL)
Sulphuric acid 0.15 mol/L *(avoid any skin contact)*
REF DCE005-0

7. 10X Conc. Wash Solution (1 vial, 50 mL)
Phosphate buffer 0.2M, Proclin < 0,0015%
REF DCE054-0

3.2. Reagents necessary not supplied

Distilled water

3.3. Auxiliary materials and instrumentation

Automatic dispenser
Microplates reader (450 nm, 620-630 nm)

Note

Store all reagents at 2÷8°C in the dark.
Open the bag of reagent 4 (Coated Microplate) only when it is at room temperature and close it immediately after use; once opened, the microplate is stable until expiry date of the kit.

4. WARNINGS

- This Urinary Cortisol ELISA Assay Kit is intended for research use only by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents of the Urinary Cortisol ELISA Assay Kit contain small amounts of Proclin 300^{R} as preservative. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.
- This method allows the determination of Cortisol from 0,47 ng/mL (LOD) to 200 ng/mL.
- The clinical significance of the Cortisol determination can be invalidated if the patient was treated with corticosteroids or natural or syntetic steroids.

5. PRECAUTIONS

- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. To improve the performance of the kit on automatic systems is recommended to increase the number of washes.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying control samples.
- Maximum precision is required for reconstitution and dispensation of reagents.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. STORAGE AND STABILITY

Store the kit at 2-8°C; the kit is stable until the expiry date claimed on the kit label and in the Certificate of Analysis.
Do not use the kit or its components after the expiry date.

7. PROCEDURE

7.1. Preparation of Calibrators and Controls

Before use, leave 5 minutes on a rotating mixer.
The Calibrators are ready to use and have the following concentration of Cortisol:

	C_0	C_1	C_2	C_3	C_4
ng/mL	0	1	5	30	200

The Controls are ready to use.
Once opened, Calibrators and Controls are stable 6 months at 2-8°C.

7.2. Preparation of the Conjugate

The Conjugate is ready to use.
Once opened, it stable 6 months at 2-8°C.

7.3. Preparation of the Sample

The determination of Cortisol with this kit should be performed in urine samples.
Important note: the kit has been designed to be used on untreated urine samples; acidification treatments of the urine that lead the pH to values below 5.0 could interfere with the assay and produce aberrant results.

It is not necessary to dilute the sample. The total volume of urine excreted during a 24 hours should be collected and mixed in a single container.
Urine samples which are not to be assayed immediately should be stored at 2-8°C or at -20°C for longer period (maximum 6 months).
Samples with concentration greater than 200 ng/mL has not to be diluted; such samples has to be reported as "> 200 ng/mL".

7.4. Preparation of Wash Solution

Dilute the content of each vial of the "10X Conc. Wash Solution" with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.
In concentrated wash solution is possible to observe the presence of crystals; in this case mix at room temperature until the complete dissolution of crystals; for greater accuracy, dilute the whole bottle of concentrated wash solution to 500 mL, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.

7.5. Procedure

- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay, store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_4), two for each Control, two for each sample, one for Blank.

Reagent	Calibrator	Samples/ Controls	Blank
Calibrator C_0-C_4	**10 µL**		
Samples/ Controls		**10 µL**	
Conjugate	**300 µL**	**300 µL**	
Incubate at 37°C for 1 hour. Remove the contents from each well. Wash the wells 3 times with 350 µL of diluted wash solution. **Important note**: during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel. **Automatic washer**: in case you use an automatic washer, it is advised to do 6 washing steps.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate at room temperature (22÷28°C) for 15 minutes in the dark.			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the microplate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

8. QUALITY CONTROL

Each laboratory should assay controls at normal, high and low levels range of urinary Cortisol for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

9. RESULTS

9.1. Mean Absorbance

Calculate the mean of the absorbance (Em) for each point of the calibration curve (C_0-C_4) and of each sample.

9.2. Calibration curve

Plot the values of absorbance (Em) of the Calibrators (C_0-C_4) against concentration. Draw the best-fit curve

through the plotted points (es: Four Parameter Logistic).

9.3. Calculation of Results

Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in ng/mL.

To calculate the cortisol concentration in urine, calculate as above and correct for total volume of volume of urine collected in 24 hours:

ng/mL x Vol(mL) urine 24 h/ 1000 = µg Cortisol/24h

10. REFERENCE VALUES

To determine the normal range for urine samples, 128 apparently healthy male and female adults were tested.

Result:

Normal range urine (24h)
1,5 - 63 µg/24h

11. PERFORMANCE AND CHARACTERISTICS

10.1. Analytical sensitivity

Analytical sensitivity was investigated through the LOB (white limit), the LOD (detection limit), the LOQ (quantification limit) and the anal sensitivity (A.S.).

The following table shows the criteria of the study and the results obtained.

	Criteria	***Results (ng/mL)***
LOB	60 replicates of Cal 0, used as "Blank", have been investigated in 5 different sessions over 3 days	0,28
LOD	6 urine samples with low cortisol concentration have been investigate over 10 assays in duplicate, performed in 5 days	0,47
LOQ	6 urine samples with low cortisol concentration have be en investigate over 10 assays in duplicate, performed in 5 days	0,56
A.S.	20 replicates of Cal 0 and 5 replicates of Cal 1 have been assayed. A.S. has been calculated by linear regression	0,22

10.2. Precision and reproducibility (complex precision)

Precision and reproducibility have been assessed through 6 different urine samples with different concentration of Cortisol.

The table below shows the Within Run and Total CV%.

Sample	n°	Mean (ng/mL)	Within Run CV%	Total CV%
PS2	20	112,141	6,6%	12%
PS4	20	64,563	8,1%	12%
CT High	20	50,577	7,3%	11%
PS5	20	25,878	7,6%	10%
PS6	20	9,269	7,6%	11%
CT Low	20	3,438	7,0%	9%

10.3. Analytical specificity

10.3.1. Interfering substances

Interference for Albumin, Acetylsalicylic Acid, Ibuprofen and Ascorbic Acid were studied by adding the interfering substance to the urine sample with a low and high Cortisol concentration, and by comparing its concentration to the unspiked sample.

The interference has been evaluated as "significant" if it causes a concentration bias >10% between spiked and unspiked sample.

The following table shows the results obtained:

Substance	**Concentration**	**Interference**
Albumin	5 mg/dL	No
Acetylsalicylic acid	3,62 mmol/L	No
Ibuprofen	2,42 mmol/L	No
Ascorbic Acid	5 mg/L	No

Conclusion: no interference has been found for Albumin, Acetylsalicylic Acid, Ibuprofen and Ascorbic Acid.

10.3.2. Cross-reactivity

The cross reactions of the antibody calculated at 50% according to Abraham are shown in the table:

Reagent	**Cross-reactivity**
Cortisol	100 %
Prednisolone	46.2 %
11-Deoxycortisol	4 %
Cortisone	3.69 %
Prednisone	3.10 %
11αOH Progesterone	1 %
Progesterone	< 0.1 %
Aldosterone	< 0.1 %
Pregnenolone	< 0.1 %
17b Estradiol	< 0.1 %
Estrone 3-solfato	< 0.1 %
Estriol	< 0.1 %
Testosterone	< 0.1 %
Spironolactone	< 0.1 %
DHEA	< 0.1 %
DHEA-S	< 0.1 %
Androstenedione	< 0.1 %

Androsterone	< 0.1 %
DHT	< 0.1 %
Danazol	< 0.1 %
Cholesterol	< 0.1 %
Dexamethasone	< 0.1 %

10.4. Correlation

137 urine samples were tested with the Urinary Cortisol Elisa kit and with a LC-MS method (reference)
The linear regression curve is:
Y = 1,008X - 0,5019
r^2 = 0,83

11. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

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	DIA.METRA SRL	
Mod. PIS	**PACKAGING INFORMATION SHEET**	

IT *Spiegazione dei simboli* **GB** *Explanation of symbols* **FR** *Explication des symboles* **ES** *Significado de los simbolos*

DE *Verwendete Symbole* **PT** *Explicaçao dos simbolos*

IVD	DE ES FR GB IT PT	In vitro Diagnostikum Producto sanitario para diagnóstico In vitro Dispositif medical de diagnostic in vitro In vitro Diagnostic Medical Device Dispositivo medico-diagnostico in vitro Dispositivos medicos de diagnostico in vitro		DE ES FR GB IT PT	Hergestellt von Elaborado por Fabriqué par Manufacturer Produttore Produzido por
REF	DE ES FR GB IT PT	Bestellnummer Nûmero de catálogo Réferéncès du catalogue Catalogue number Numero di Catalogo Número do catálogo	yyyy-mm	DE ES FR GB IT PT	Herstellungs datum Fecha de fabricacion Date de fabrication Date of manufacture Data di produzione Data de produção
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	DE ES FR GB IT PT	Gebrauchsanweisung beachten Consultar las instrucciones Consulter le mode d'emploi Consult instructions for use Consultare le istruzioni per l'uso Consultar instruções para uso	LOT	DE ES FR GB IT PT	Chargenbezeichnung Codigo de lote Numero de lot Batch code Codice del lotto Codigo do lote
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Max Min	DE ES FR GB IT PT	Temperaturbereich Límitaciôn de temperatura Limites de température de conservation Temperature limitation Limiti di temperatura Temperaturas limites de conservação			

SUGGERIMENTI PER LA RISOLUZIONE DEI PROBLEMI/TROUBLESHOOTING

ERRORE CAUSE POSSIBILI/ SUGGERIMENTI

Nessuna reazione colorimetrica del saggio

- mancata dispensazione del coniugato
- contaminazione del coniugato e/o del Substrato
- errori nell'esecuzione del saggio (es. Dispensazione accidentale dei reagenti in sequenza errata o provenienti da flaconi sbagliati, etc.)

Reazione troppo blanda (OD troppo basse)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo breve, temperatura di incubazione troppa bassa

Reazione troppo intensa (OD troppo alte)

- coniugato non idoneo (es. non proveniente dal kit originale)
- tempo di incubazione troppo lungo, temperatura di incubazione troppa alta
- qualità scadente dell'acqua usata per la soluzione di lavaggio (basso grado di deionizzazione,)
- lavaggi insufficienti (coniugato non completamente rimosso)

Valori inspiegabilmente fuori scala

- contaminazione di pipette, puntali o contenitori- lavaggi insufficienti (coniugato non completamente rimosso)

CV% intra-assy elevato

- reagenti e/o strip non portate a temperature ambiente prima dell'uso
- il lavatore per micropiastre non lava correttamente (suggerimento: pulire la testa del lavatore)

CV% intersaggio elevato

- condizioni di incubazione non costanti (tempo o temperatura)
- controlli e campioni non dispensati allo stesso tempo (con gli stessi intervalli) (controllare la sequenza di dispensazione)
- variabilità intrinseca degli operatori

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction

- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers

- contamination of pipettes, tips or containers

insufficient washing (conjugates not properly removed) too high within-run

- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)

too high between-run - incubation conditions not constant (time, CV % temperature)

- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation