INTENDED USE
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 Assay 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 blue color that changes into yellow when the Stop Solution ($H_2SO_4$) is added.

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

2. Controls (2 vial, 1 mL each, ready to use) REF DCE045/1801-0
   Low Control
   High Control

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 microplate
5. TMB Substrate (1 vial, 15 mL)  
H₂O₂-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  
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.

4. WARNINGS  
- This Urinary Cortisol ELISA Assay Kit is intended for research use 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.  
- Some reagents of the Urinary Cortisol ELISA Assay Kit contain small amounts of Proclin 300® 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/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.  
- This Urinary Cortisol ELISA Assay Kit allows the determination of Cortisol from 10 ng/mL to 500 ng/mL.  
- The clinical significance of the Cortisol

5. PRECAUTIONS  
- Please adhere strictly to the sequence of pipetting steps provided in this protocol.  
- All reagents of the Urinary Cortisol ELISA Assay Kit 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 Urinary Cortisol ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.  
- Do not interchange Urinary Cortisol ELISA Assay 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.  
- 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. PROCEDURE  

6.1. Preparation of the Calibrators (C₀…C₄)  
Before use, leave 5 minutes on a rotating mixer. The Calibrators are to use and have the following concentration of Cortisol:

<table>
<thead>
<tr>
<th>ng/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>150</td>
<td>500</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable 6 months at 2-8°C.

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

6.3. Preparation of the Sample  
The determination of Cortisol with this kit should be performed in urine samples. 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. The Controls are ready to use. In case of samples with concentration greater than 500 ng/mL dilute with Calibrator 0 (consider this dilution in the calculation of final concentration).

6.4. 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.

6.5. Procedure

- Allow all reagents to reach room temperature (22-28°C). 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.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Samples/ Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator $C_0-C_4$</td>
<td>10 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted samples/ Controls</td>
<td></td>
<td>10 µL</td>
<td></td>
</tr>
<tr>
<td>Conjugate</td>
<td>300 µL</td>
<td>300 µL</td>
<td></td>
</tr>
</tbody>
</table>

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. RESULTS

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

8.2. 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).

8.3. 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:

$$\text{ng/mL x Vol(mL)} \text{ urine 24 h} \times 1000 = \mu g \text{ Cortisol/24h}$$

9. REFERENCE VALUES
The urinary Cortisol concentration during the 24 hours are included in the following range:

50 - 190 µg / 24 hours

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision
10.1.1. Intra Assay Variation
Within run variation was determined by replicate (20x) the measurement of three different urine samples in one assay. The within assay variability is ≤ 6.5%.

10.1.2. Inter Assay Variation
Between run variation was determined by replicate (10x) the measurement of three different urine samples in different lots of kit. The between assay variability is ≤ 7.2%.

10.2. Accuracy
The recovery of 12.5 - 25 - 50 - 100 ng/mL of Cortisol added to a sample gave an average value (±SD) of 107.48% ± 8.16% with reference to the original concentrations.

10.3. Sensitivity
The lowest detectable concentration of urinary Cortisol that can be distinguished from the Calibrator 0 is 2.95 ng/mL at the 95% confidence limit.

10.4. Specificity
The cross reaction of the antibody calculated at 50% according to Abraham are shown in the table:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100%</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>46.2%</td>
</tr>
<tr>
<td>11-Deoxycorticisol</td>
<td>4%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>3.69%</td>
</tr>
<tr>
<td>Prednisone</td>
<td>3.10%</td>
</tr>
<tr>
<td>11αOH Progesterone</td>
<td>1%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>17b Estradiol</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Estrone-3-sulfato</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Estriol</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>DHEA</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Androsterone</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>DHT</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Danazol</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&lt; 0.1%</td>
</tr>
</tbody>
</table>

10.5. Correlation
The new Diametra Urinary Cortisol ELISA kit was compared to the old Diametra Urinary Cortisol ELISA kit. 100 urine samples were analysed. The linear regression curve was calculated:

\[ Y = 0.90 \times X + 9.95 \]
\[ r^2 = 0.836 \]

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
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