The Eagle Biosciences Salivary Cortisol ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of Cortisol concentration in saliva. Cortisol Saliva kit is intended for research use only.

1. CLINICAL SIGNIFICANCE

Cortisol is a steroid hormone released from the adrenal cortex in response to a 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. The majority of cortisol in saliva is not-bound and enters the saliva via intracellular mechanisms. Salivary cortisol levels are unaffected by salivary flow rate or salivary enzymes. It is a high correlations between serum and saliva cortisol levels. 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.

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) of the Cortisol Saliva ELISA Assay Kit. 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 (H₂O₂) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H₂SO₄) 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 Cortisol Saliva ELISA Assay Kit

1. Cortisol Calibrators (7 vials, 1 mL each)
   - CAL0  REF DCE002/2006-0
   - CAL1  REF DCE002/2007-0
   - CAL2  REF DCE002/2008-0
   - CAL3  REF DCE002/2009-0
   - CAL4  REF DCE002/2010-0
   - CAL5  REF DCE002/2011-0
   - CAL6  REF DCE002/2012-0

2. Incubation Buffer (1 vial, 30 mL)
   - Phosphate buffer 50 mM pH 7.4, BSA 1 g/L  REF DCE001-0

3. Conjugate (1 vial, 1 mL)
   - Cortisol conjugated to horseradish peroxidase (HRP)  REF CE002/2002-0

4. Coated Microplate (1 breakable microplate)
   - Antibody anti Cortisol adsorbed on microplate  REF DCE002/2003-0

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 but not supplied
Distilled water.

### 3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplates reader (450 nm, 620-630 nm)
Saliva Collection Device **REF DKO063**
Salivette Sarstedt **REF 51.1534.500**

**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, it is stable up to expiry date of the kit.*

### 4. WARNINGS
- This Cortisol Saliva ELISA Assay Kit is intended for in vitro 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 Cortisol Saliva 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 Cortisol Saliva ELISA Assay Kit allows the determination of Cortisol from 0.5 ng/mL to 100 ng/mL.

### 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 Cortisol Saliva ELISA Instruction For Use.
- All reagents of the Cortisol Saliva 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 Cortisol Saliva ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Cortisol Saliva 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 Cortisol Saliva ELISA Assay 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…C₆)
Before using, leave the Calibrators on a rotating mixer for at least 5 minutes. The Calibrators are ready 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>
<th>C₅</th>
<th>C₆</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable six months at 2-8°C. For SI Units: ng/mL x 2.76 = nmol/L

6.2. Preparation of Diluted Conjugate
Prepare immediately before use. Add 10 μL of Conjugate (reagent 3) to 1 mL of Incubation Buffer (reagent 2). Mix gently. Stable for 3 hours at room temperature (22-28°C).

6.3. 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.

6.4. Preparation of the Sample
The determination of Cortisol should be performed in saliva.

b) During the day allow 1 hour after a meal, oral intake of pharmaceutical drugs or tooth cleaning.

c) It is very important that a good clear sample is received – i.e. no contamination with food, lipstick, blood (bleeding gums) or other such extraneous materials.

6.4.2. Saliva Processing Instructions with Saliva Collection Device
1. Let the saliva flow down through the centrifuge glass tube.
2. Centrifuge the sample for 15 minutes at 3000 rpm
3. Store at – 20°C for at least 1 hour
4. Centrifuge again for 15 minutes at 3000 rpm
5. The saliva sample is now ready to be tested.
6. Store the sample at 2-8°C for one week or at – 20°C for longer time.

6.4.3. Saliva Processing Instructions with Salivette Sarstedt
1. Remove the swab from the suspended insert of the Salivette
2. Gently chewing the swab for 1 minute produces a sufficient quantity of saliva.
3. Replace the swab into the Salivette and firmly close the tube using the stopper.
4. Centrifuge the Salivette for 2 minutes at 1000g (rcf) for saliva generation.
5. Remove the insert complete with the swab from the centrifuge vessel and discard. The clear saliva is now ready for analysis (at least 1 mL of saliva should be recovered with this method).

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 (C₀-C₆), two for each Control, two for each sample, one for Blank.

6.4.1. Method and Limitations
Collect saliva samples at the times indicated. If no specific instructions have been given, saliva samples may be collected at any time; however the following should be noted:

a) If saliva is collected in the morning ensure that this is carried out prior to brushing teeth

b) During the day allow 1 hour after a meal, oral intake of pharmaceutical drugs or tooth cleaning.

c) It is very important that a good clear sample is received – i.e. no contamination with food, lipstick, blood (bleeding gums) or other such extraneous materials.
7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of Cortisol for monitoring assay performance in the Cortisol Saliva ELISA Assay Kit. 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 Cortisol Saliva ELISA Assay Kit reagents. Fresh reagents should be used to determine the reason for the variations.

8. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the calibration curve (C<sub>0</sub>-C<sub>6</sub>) and of each sample.

8.2. Calibration curve
Plot the mean value (Em) of absorbance of the Calibrators (C<sub>0</sub>-C<sub>6</sub>) against concentration. Draw the best-fit curve through the plotted points. (ex: 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.

9. REFERENCE VALUES
The following values can be used as preliminary guideline until each laboratory established its own normal range.

<table>
<thead>
<tr>
<th></th>
<th>A.M.</th>
<th>P.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>range</td>
<td>3 – 10 ng/mL</td>
<td>0.6 – 2.5 ng/mL</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

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 saliva samples in one assay. The within assay variability is ≤ 10%.

10.1.2. Inter Assay Variation
Between run variation was determined by replicate (10x) the measurement of three different saliva samples in different lots of kits. The between assay variability is ≤ 8.3%.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Samples</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator C&lt;sub&gt;0&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;</td>
<td>25 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples</td>
<td>25 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted Conjugate</td>
<td>200 µL</td>
<td>200 µL</td>
<td></td>
</tr>
</tbody>
</table>
10.2. Accuracy
The recovery of 6.25 - 12.5 - 25 - 50 ng/mL of Cortisol added to a saliva sample gave an average value (±SD) of 95.42% ± 9.11% with reference to the original concentrations.

10.3. Sensitivity
The lowest detectable concentration of Cortisol that can be distinguished from the Calibrator 0 is 0.12 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>Compound</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-Deoxy cortisol</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>17β Estradiol</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Estrone 3-solfato</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Estradiol</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 Cortisol Saliva ELISA Assay Kit was compared to the old Diametra Cortisol saliva ELISA kit. 35 saliva samples were analysed. The linear regression curve was calculated:

\[ Y = 0.94X - 0.11 \]

\[ r^2 = 0.799 \]

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

BIBLIOGRAPHY
- Kirschbaum C. et al, Psychoneuroendocrinology, 19: 313-333
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