Cortisol (Saliva) ELISA

For the quantitative determination of cortisol in human saliva.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 11-CORHU-E01-SLV
Size: 96 Wells
Version: 6.0 – ALPCO October 19, 2018
INTENDED USE
For the quantitative determination of Cortisol by an enzyme immunoassay in human saliva. Not for use in diagnostic procedures.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, controls and samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stop solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample. A set of standards is used to plot a standard curve from which the amount of cortisol in samples and controls can be directly read.

RESEARCH APPLICATIONS
Cortisol is the most abundant circulating steroid and the major glucocorticoid secreted by the adrenal cortex. Cortisol is physiologically effective in blood pressure maintenance and anti-inflammatory activity. It is also involved in calcium absorption, gluconeogenesis as well as the secretion of gastric acid and pepsin. It is increased under stress situations, physical exercise and external administration of ACTH.

Most circulating cortisol is bound to cortisol binding globulin or transcortin and albumin. The free cortisol, which is considered the active part of blood, is about 1–2%. In the absence of appreciable amounts of the cortisol binding proteins in saliva, salivary cortisol is considered to be free and shows a diurnal rhythm with the highest levels in the morning and the lowest levels at night.

PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human samples.
5. All kit reagents and samples should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and samples.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
11. When dispensing the substrate and stop solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the direct determination of cortisol in human saliva. The kit is not calibrated for the determination of cortisol in serum, plasma or other samples of human or animal origin.
2. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
3. Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
SAFETY CAUTIONS AND WARNINGS POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and controls has been tested and found to be nonreactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. No test method however, can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood sample.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SAMPLE COLLECTION AND STORAGE

Approximately 1 mL of saliva is required per duplicate determination. Collect 4–5 mL of saliva into a clean glass tube (Salivette by Sarstedt may be used) without force or inducement and before eating, drinking or brushing the teeth. Simply rinse the mouth with water before collection. Do not use blood-contaminated samples. Store samples at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human samples as possible biohazardous materials and take appropriate precautions when handling.

SAMPLE PRETREATMENT

Sample tubes are to be placed into a freezer and allowed to freeze. When ready to use, the samples are to be thawed and centrifuged. The supernatants are to be collected and poured into freshly labelled tubes.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 50, 100, 150 and 300 μL
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Benchtop centrifuge
6. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10)

REAGENTS PROVIDED

1. Rabbit Anti-Cortisol Antibody-Coated Break-Apart Well Microplate — Ready To Use
   Contents: One 96-well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.

2. Cortisol-Horseradish Peroxidase (HRP) Conjugate Concentrate — Requires Preparation X50
   Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   Volume: 300 μL/vial
   Storage: Refrigerate at 2–8°C
   Stability: 12 months or as indicated on label.
   Preparation: Dilute 1:50 in assay buffer before use (eg. 40 μL of concentrate in 2 mL of assay buffer). If the whole plate is to be used dilute 240 μL of concentrate in 12 mL of assay buffer. Discard any that is left over.

3. Cortisol Saliva Calibrators — Ready To Use
   Contents: Six vials containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of cortisol.
   * Listed below are approximate concentrations, please refer to bottle labels for exact concentrations.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume/Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator A</td>
<td>0 ng/mL</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>1 ng/mL</td>
<td>0.6 mL</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>3 ng/mL</td>
<td>0.6 mL</td>
</tr>
</tbody>
</table>

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com
<table>
<thead>
<tr>
<th>Calibrator D</th>
<th>10 ng/mL</th>
<th>0.6 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator E</td>
<td>30 ng/mL</td>
<td>0.6 mL</td>
</tr>
<tr>
<td>Calibrator F</td>
<td>100 ng/mL</td>
<td>0.6 mL</td>
</tr>
</tbody>
</table>

Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. **Controls** — Ready To Use
Contents: Two vials containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of cortisol. Refer to vial labels for the acceptable range.
Volume: 0.6 mL/vial
Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. **Wash Buffer Concentrate** — Requires Preparation [X10]
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. **Assay Buffer** — Ready To Use
Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 15 mL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
7. **TMB Substrate** — Ready To Use
   Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
   Volume: 16 mL/bottle
   Storage: Refrigarate at 2–8°C
   Stability: 12 months or as indicated on label.

8. **Stop Solution** — Ready To Use
   Contents: One bottle containing 1M sulfuric acid.
   Volume: 6 mL/bottle
   Storage: Refrigerate at 2–8°C Stability: 12 months or as indicated on label.

### ASSAY PROCEDURE

Sample Pretreatment: Freezing and Centrifugation.

All reagents must reach room temperature before use. Calibrators, controls and sample samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the cortisol-HRP conjugate and wash buffer.
2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 µL of each calibrator, control and sample sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the conjugate working solution into each well. (It is recommended to use a multichannel pipette.)
5. Incubate on a plate shaker (approximately 200 rpm) for 45 minutes at room temperature.
6. Wash the wells 3 times with 300 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)
7. Pipette 150 µL of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 15–20 minutes at room temperature (or until calibrator A attains dark blue color for desired OD).
9. Pipette 50 µL of stop solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stop solution.

* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of samples.

### CALCULATIONS

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 100 ng/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

### TYPICAL TABULATED DATA

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean OD</th>
<th>Value (ng/mL)</th>
</tr>
</thead>
</table>

Sample data only. **Do not** use to calculate results.
### Performance Characteristics

**Sensitivity**

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Cortisol Saliva ELISA kit is 1.0 ng/mL.

**Specificity (Cross-Reactivity)**

The following compounds were tested for cross-reactivity with the Direct Cortisol Saliva ELISA kit with cortisol cross-reacting at 100%.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross-Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>13.6</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>7.6</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>7.2</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7.2</td>
</tr>
<tr>
<td>Cortisone</td>
<td>6.2</td>
</tr>
<tr>
<td>Deoxycortisol</td>
<td>5.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>5.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.6</td>
</tr>
</tbody>
</table>

No cross-reaction was detected with DHEAS and Tetrahydrocortisone.

*Please note that there is an observed cross-reactivity of 13.6% with prednisolone. Since prednisone is converted to prednisolone in vivo, caution must be exercised when assaying the cortisol levels.*

### Intra-Assay Precision

Three samples were assayed ten times each on the same calibrator curve. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>0.68</td>
<td>10.3</td>
</tr>
<tr>
<td>2</td>
<td>24.8</td>
<td>1.98</td>
<td>8.0</td>
</tr>
</tbody>
</table>
### INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of four weeks. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3</td>
<td>0.63</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>23.7</td>
<td>2.06</td>
<td>8.7</td>
</tr>
<tr>
<td>3</td>
<td>51.8</td>
<td>3.37</td>
<td>6.5</td>
</tr>
</tbody>
</table>
RECOVERY

Spiked samples were prepared by adding defined amounts of cortisol to three saliva samples (1:1). The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs. Result</th>
<th>Exp. Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unspiked</td>
<td>6.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 1.0</td>
<td>4.14</td>
<td>3.64</td>
<td>113.7</td>
</tr>
<tr>
<td>+ 10</td>
<td>9.05</td>
<td>8.14</td>
<td>111.2</td>
</tr>
<tr>
<td>+ 100</td>
<td>61.85</td>
<td>53.14</td>
<td>116.4</td>
</tr>
<tr>
<td>2 Unspiked</td>
<td>8.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 3.0</td>
<td>6.05</td>
<td>5.52</td>
<td>109.6</td>
</tr>
<tr>
<td>+ 30</td>
<td>20.64</td>
<td>19.02</td>
<td>108.5</td>
</tr>
<tr>
<td>+ 100</td>
<td>52.20</td>
<td>54.02</td>
<td>96.6</td>
</tr>
<tr>
<td>3 Unspiked</td>
<td>6.98</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ 3.0</td>
<td>5.38</td>
<td>4.99</td>
<td>107.8</td>
</tr>
<tr>
<td>+ 10</td>
<td>8.76</td>
<td>8.49</td>
<td>103.2</td>
</tr>
<tr>
<td>+ 30</td>
<td>19.00</td>
<td>18.49</td>
<td>102.8</td>
</tr>
</tbody>
</table>

LINEARITY

Three saliva samples were diluted with calibrator A. The results (in ng/mL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Obs. Result</th>
<th>Exp. Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>10.32</td>
<td>9.09</td>
<td>113.5</td>
</tr>
<tr>
<td>1:4</td>
<td>5.09</td>
<td>4.55</td>
<td>112.1</td>
</tr>
<tr>
<td>1:8</td>
<td>2.20</td>
<td>2.27</td>
<td>96.7</td>
</tr>
<tr>
<td>2</td>
<td>49.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>28.03</td>
<td>24.95</td>
<td>112.3</td>
</tr>
<tr>
<td>1:4</td>
<td>13.29</td>
<td>12.47</td>
<td>106.6</td>
</tr>
<tr>
<td>1:8</td>
<td>7.97</td>
<td>7.24</td>
<td>110.1</td>
</tr>
<tr>
<td>3</td>
<td>68.53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:2</td>
<td>34.27</td>
<td>31.49</td>
<td>91.9</td>
</tr>
<tr>
<td>1:4</td>
<td>17.13</td>
<td>13.81</td>
<td>80.6</td>
</tr>
<tr>
<td>1:8</td>
<td>8.57</td>
<td>7.48</td>
<td>87.3</td>
</tr>
</tbody>
</table>

EXPECTED VALUES

As for all assays each laboratory should collect data and establish their own range of expected normal values.

REFERENCES

