2. Control materials should be included in every run at a high level.

1. Users should have a thorough understanding of this protocol.

PROCEDURAL CAUTIONS AND WARNINGS

- 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.
- When reading the microplate, the presence of bubbles in the wells will affect the optical density (ODs). A subjective removal may not remove all bubbles before performing the reading step.
- The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
- When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
- To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
- 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.
- Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

- All the reagents within the kit are calibrated for the direct determination of cortisol in human serum. The kit is not calibrated for the determination of cortisol in saliva, plasma or other specimen matrices.
- Do not use grossly hemolysed, grossly lipemic, icteric or improperly stored serum.
- Any samples or controls containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
- Only calibrator A may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
- The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of hirsutism of human or animal origin. Most circulating cortisol is bound to cortisol binding globulin or transcortin. Therefore, the free cortisol concentration excreted in the urine is very small, and the 24-hour collection of the urine is a must in order to attain an accurate measurement of urinary cortisol. Cortisol in blood shows a diurnal rhythm with the highest levels in the morning and the lowest levels at night.

PROCEDURAL CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

- 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.
- Control materials should be included in every run at a high and low level for assays of the reagents.
- When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
- In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.

SAFETY CAUTIONS AND WARNINGS

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.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.1 ml of serum is required per duplicate determination. Collect 4–5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store 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 specimens as potentially infectious materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

REAGENTs AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 20, 50, 150 and 300 μL.
2. Disposable pipette tips
3. Distilled deionized water
4. Plate shaker
5. Microplate reader with a filter set at 450 nm and an upper wavelength of 900 nm
6. Assay Buffer
7. TMB Substrate

REAGENTS PROVIDED

5. Calibrator A 0.5 μg/dL 0.3 mL—Ready To Use
6. Calibrator B 0.5 µg/dL 0.3 mL—Ready To Use
7. Calibrator C 2 µg/dL 0.3 mL—Ready To Use
8. Calibrator D 5 µg/dL 0.3 mL—Ready To Use
9. Calibrator E 10 µg/dL 0.3 mL—Ready To Use
10. Calibrator F 30 µg/dL 0.3 mL—Ready To Use
11. Calibrator G 60 µg/dL 0.3 mL—Ready To Use

Storage: Refrigerate at 2–8°C
Stability: 12 months in unopened vial as indicated on label.

4. Controls — Ready To Use
Contents: One vial containing cortisol in a human serum-based buffer with a non-mercury preservative.
Prepared by spiking serum with defined quantities of cortisol. Refer to vial labels for the acceptable range.
Volume: 50 μL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

5. Wash Buffer Concentrate — Requires Preparation
Contents: One bottle containing a 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.

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: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

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

2. Cortisol-Horseradish Peroxidase (HRP) Conjugate
Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 100 μL
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:100 in assay buffer before use (eg. 20 μL of cocktail in 1980 μL of assay buffer). If the whole plate is to be used dilute 120 μL of conjugate in 12 mL of assay buffer. Discard any that is left over.

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

4. Controls — Ready To Use
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 50 μL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

1. Rabbit Anti-Cortisol Antibody-Coated Break-Apart Well Microwell — Ready To Use
Contents: One 96-well (12x8) polyclonal antibody-coated microwell in a resealable pouch with desiccant.
Volume: 200 μL
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

2. Rabbit Anti-Cortisol Antibody-Coated Break-Apart Well Microwell — Ready To Use
Contents: One 96-well (12x8) polyclonal antibody-coated microwell in a resealable pouch with desiccant.
Volume: 200 μL
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

3. Cortisol-Horseradish Peroxidase (HRP) Conjugate
Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 100 μL
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:100 in assay buffer before use (e.g. 20 μL of cocktail in 1980 μL of assay buffer). If the whole plate is to be used dilute 120 μL of conjugate in 12 mL of assay buffer. Discard any that is left over.

4. Controls — Ready To Use
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 50 μL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

5. Wash Buffer Concentrate — Requires Preparation
Contents: One bottle containing a 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.

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: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

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

2. Cortisol-Horseradish Peroxidase (HRP) Conjugate
Contents: Cortisol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 100 μL
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:100 in assay buffer before use (e.g. 20 μL of cocktail in 1980 μL of assay buffer). If the whole plate is to be used dilute 120 μL of conjugate in 12 mL of assay buffer. Discard any that is left over.

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

4. Controls — Ready To Use
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 50 μL/bottle
Storage: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.

5. Wash Buffer Concentrate — Requires Preparation
Contents: One bottle containing a 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.

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: Refrigerate at 2–8°C
Stability: 12 months or as indicated on label.
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 20 μL of each calibrator, control and specimen sample into corresponding labelled wells in duplicate.
4. Pipette 100 μL of the conjugate working solution into each well. (We recommend using a multi-channel pipette.)
5. Incubate on a plate shaker for 15–20 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
6. Pipette 150 μL of TMB substrate into each well at the same timed intervals as in step 7.
7. Incubate for 5 minutes at room temperature (or until TMB turn blue colour for desired OD).
8. Pipette 50 μL of stopping solution into each well at the same timed intervals as in step 7.
9. Read the plates at a microplate reader at 450 nm within 20 minutes after addition of the stopping 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 patient/control samples. No cross reaction was detected with DHEAS and Tetrahydrocortisone.

**SPECIFICITY (CROSS-REACTIVITY)**

The following compounds were tested for cross-reactivity with the Direct Cortisol 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>Pseudocortisone</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>Deoxycorticisol</td>
<td>5.6</td>
</tr>
<tr>
<td>Prednisone</td>
<td>5.6</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.8</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 pseudocortisone. Since pseudocortisone is converted to pseudocortisone in vivo, caution must be exercised when assaying the cortisol levels of patients undergoing either therapy.

**TYPICAL CURVE**

Sample curve only. Do not use to calculate results.

**TYPICAL TABULATED DATA**

Sample data only. Do not use to calculate results.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean OD</th>
<th>Value (μg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.263</td>
<td>2.183</td>
<td>2.223</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>2.071</td>
<td>2.001</td>
<td>2.036</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>1.717</td>
<td>1.719</td>
<td>1.716</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>1.382</td>
<td>1.402</td>
<td>1.395</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>0.985</td>
<td>0.953</td>
<td>0.959</td>
<td>10</td>
</tr>
<tr>
<td>F</td>
<td>0.385</td>
<td>0.373</td>
<td>0.379</td>
<td>30</td>
</tr>
<tr>
<td>G</td>
<td>0.174</td>
<td>0.176</td>
<td>0.175</td>
<td>60</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.697</td>
<td>0.722</td>
<td>0.710</td>
<td>15.3</td>
</tr>
</tbody>
</table>

**CALCULATIONS**

1. Calculate the mean optical density of each step.
2. Calculate the mean optical density of each calibrator.
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 60 μg/dL, 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.

**EXTRA-ASSAY PRECISION**

Three samples were assayed ten times each on the same calibrator curve. The results (in μg/dL) 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>1.44</td>
<td>0.14</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>14.06</td>
<td>0.41</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>37.55</td>
<td>1.87</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**INTRA-ASSAY PRECISION**

Three samples were assayed ten times over a period of four weeks. The results (in μg/dL) 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>1.60</td>
<td>0.13</td>
<td>8.1</td>
</tr>
<tr>
<td>2</td>
<td>15.01</td>
<td>0.74</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>38.18</td>
<td>1.43</td>
<td>3.8</td>
</tr>
</tbody>
</table>

**LINEARITY**

Three patient serum samples were diluted with calibrator A. The results (in μg/dL) are tabulated below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Observation</th>
<th>Result</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.86</td>
<td>9.06</td>
<td>123.2</td>
</tr>
<tr>
<td>2</td>
<td>3.61</td>
<td>9.33</td>
<td>116.1</td>
</tr>
<tr>
<td>3</td>
<td>6.12</td>
<td>9.33</td>
<td>89.4</td>
</tr>
</tbody>
</table>

**REFERENCES**