1. PROCEDURAL CAUTIONS AND WARNINGS

- Consider all human specimens as possible biohazardous substances, gloves should be worn when handling kit contents.
- Kit reagents must be regarded as hazardous waste and disposed of appropriately.
- All reagents used in this test are to be stored at 2–8°C and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- 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.
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ASSAY PROCEDURE

Sample Pretreatment: None.

1. Prepare working solutions of the 3α-Diol G-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 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 10–15 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 10–15 minutes at room temperature.
9. Pipette 50 μl of TMB substrate into each well at timed intervals.
10. Read the plate on 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 sample reads more than 50 ng/mL then dilute it with wash buffer.

**Calculations**

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibration 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. The results (in ng/mL) are tabulated below:

**TYPICAL TABULATED DATA**

<table>
<thead>
<tr>
<th>Sample OD</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.480</td>
<td>2.474</td>
<td>2.477</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.102</td>
<td>2.106</td>
<td>2.104</td>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.428</td>
<td>1.413</td>
<td>1.421</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.977</td>
<td>0.883</td>
<td>0.880</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.360</td>
<td>0.368</td>
<td>0.364</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.147</td>
<td>0.143</td>
<td>0.145</td>
<td>50</td>
<td>0</td>
<td>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>0.98</td>
<td>0.10</td>
<td>10.4</td>
</tr>
<tr>
<td>2</td>
<td>7.05</td>
<td>0.46</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>20.92</td>
<td>2.26</td>
<td>10.8</td>
</tr>
</tbody>
</table>

**EXPECTED VALUES**

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

**REFERENCES**