PROGESTERONE ELISA

Direct immunoenzymatic determination of Progesterone in human serum or plasma

INTENDED USE
The Eagle Biosciences Progesterone ELISA Assay Kit is a competitive immunoenzymatic colorimetric method for quantitative determination of Progesterone concentration in human serum or plasma. This Progesterone ELISA Assay Kit is intended for research use only and not to be used in diagnostic procedures.

1. CLINICAL SIGNIFICANCE
Progesterone is a C-21 steroid hormone involved in the female menstrual cycle, pregnancy (supports gestation) and embryogenesis of humans and other species. Progesterone is the major naturally occurring human progestagen.

Progesterone is important for aldosterone (mineralocorticoid) synthesis, as 17-hydroxyprogesterone is for cortisol (glucocorticoid). Progesterone levels are relatively low in children and postmenopausal women. Adult males have levels similar to those in women during the follicular phase of the menstrual cycle.

In women, progesterone levels are relatively low during the preovulatory phase of the menstrual cycle, rise after ovulation, and are elevated during the luteal phase. If pregnancy occurs, progesterone levels are maintained at luteal levels initially. After delivery of the placenta and during lactation, progesterone levels are very low. The fall in progesterone levels following delivery is one of the triggers for milk production.

Progesterone is produced in the adrenal glands, the gonads (specifically after ovulation in the corpus luteum), the brain, and, during pregnancy, in the placenta.

Progesterone converts the endometrium to its secretory stage to prepare the uterus for implantation. If pregnancy does not occur, progesterone levels will decrease, leading, in the human, to menstruation.

Progesterone belongs to the group of neurosteroids that are found in high concentrations in certain areas in the brain and are synthesized there. Neurosteroids affect synaptic functioning, are neuroprotective, and affect myelination.

Progesterone has multiple effects outside of the reproductive system. Progesterone is thermogenic, it reduces spasm and relaxes smooth muscle. Bronchi are widened and mucus regulated. Progesterone acts as an anti-inflammatory agent and regulates the immune response. Progesterone also assists in thyroid function, in bone building by osteoblasts. Measurement of serum progesterone concentrations have been used in evaluating ovarian function.

2. PRINCIPLE
In the Progesterone ELISA Assay Kit, the (antigen) in the sample competes with the antigenic Progesterone conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Progesterone 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 (H₂O₂) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H₂SO₄) is added. The color intensity is inversely proportional to the Progesterone concentration of in the sample. Progesterone 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. Progesterone Calibrators (5 vials, 1 mL each)
   - CAL0: REF DCE002/0606-0
   - CAL1: REF DCE002/0607-0
   - CAL2: REF DCE002/0608-0
   - CAL3: REF DCE002/0609-0
   - CAL4: REF DCE002/0610-0

2. Progesterone Control (1 vial, 1 mL)
   Concentration of Control is Lot specific and is indicated on Quality Control Report
   REF DCE045/0603-0

3. Conjugate (1 vial, 22 mL)
   Progesterone conjugated with horseradish peroxidase (HRP)
   REF DCE002/0602-0

4. Coated Microplate (1 microplate breakable coated with anti-Progesterone IgG)
   REF DCE002/0603-0

5. 10X Conc. Wash Solution (1 vial, 50 mL)
   Phosphate buffer 0.2M, proclin < 0.0015%
   REF DCE054-0

6. TMB Substrate (1 vial, 15 mL)
7. **Stop Solution** (1 vial, 15 mL)
   Sulphuric acid 0.15 mol/L (avoid any skin contact)  
   **REF** DCE005-0

3.2. **Reagents necessary not supplied**
Distilled water.

3.3. **Auxiliary materials and instrumentation**
Automatic dispenser.
Microplates reader (450 nm).

**Note**
Store all reagents between 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 Progesterone 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 Progesterone ELISA Assay Kit contain small amounts of Proclin 300® as preservatives. 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.
- This Progesterone ELISA Assay Kit allows the determination of Progesterone from 0.2 ng/mL to 40.0 ng/mL.
- For higher values, for example in pregnancy, dilute the sample; consider the diluting factor when calculating the final result.
- The clinical significance of the Progesterone determination can be invalidated if the sample was treated with cortisone or natural or synthetic steroids.

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 Instruction For Use.
- All reagents 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 kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.

- Do not interchange Progesterone 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 controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. **PROCEDURE**

6.1. **Preparation of the Calibrator** (C₀...C₄)
Before using leave 5 minutes on a rotary shaker.
The Calibrators are ready to use and have the following concentrations of Progesterone:

<table>
<thead>
<tr>
<th></th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/mL</td>
<td>0</td>
<td>0.2</td>
<td>1.0</td>
<td>8.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

The Calibrators are stable until the expiry date printed on the label. Once opened, the Calibrators are stable six months at 2-8°C.

6.2. **Preparation of the Sample**
The determination of Progesterone can be performed in human plasma as well as in serum.
Store reagent at -20°C if the determination is not performed on the same day of the sample connection. Avoid repetitive freezing and thawing of samples.
The Control is ready for use.

6.3. **Preparation of the Wash Solution**
Dilute the contents 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 it is possible to observe the presence of crystals. In this
case mix at room temperature until complete dissolution of crystals is observed. For greater accuracy dilute the whole bottle of concentrated wash solution to 500 mL, taking care also to transfer the crystals completely, then mix until the crystals are completely dissolved.

6.4. Procedure

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

7. QUALITY CONTROL

Each laboratory should assay controls at normal, high and low levels range of Progesterone for monitoring assay performance. 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 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 ($E_m$) for each point of the calibration curve ($C_0-C_4$) and of each sample.

8.2. Calibration curve

Plot the mean value of absorbance ($E_m$) 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.

9. REFERENCE VALUES

The serum or plasma Progesterone reference values are:

<table>
<thead>
<tr>
<th>Sex</th>
<th>Time</th>
<th>Reference Value (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEN</td>
<td>follicular phase</td>
<td>0.1 - 1.4</td>
</tr>
<tr>
<td></td>
<td>Midluteinic phase</td>
<td>4.0 - 25.0</td>
</tr>
<tr>
<td></td>
<td>menopause</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>WOMEN</td>
<td>pregnancy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18-21</td>
<td>53-76</td>
</tr>
<tr>
<td></td>
<td>22-25</td>
<td>60-86</td>
</tr>
<tr>
<td></td>
<td>26-29</td>
<td>71-133</td>
</tr>
<tr>
<td></td>
<td>30-33</td>
<td>86-142</td>
</tr>
<tr>
<td></td>
<td>34-37</td>
<td>104-175</td>
</tr>
<tr>
<td></td>
<td>38-41</td>
<td>117-187</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 measurements (20x) of two different control sera in one assay. The within assay variability is ≤ 4%.

10.1.2. Inter Assay Variation

Between run variation was determined by replicate measurements (10x) of three different control sera in different lots of kit. The between assay variability is ≤ 9.3%.
10.2. Accuracy
The recovery of 1.0 - 2.0 - 4.0 - 8.0 ng/mL of Progesterone added to sample gave an average value (±SD) of 100.88% ± 8.29% with reference to the original concentrations.

10.3. Sensitivity
The lowest detectable concentration of Progesterone that can be distinguished from the zero Calibrator is 0.05 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:

| Compound               | Specificity (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.37</td>
</tr>
<tr>
<td>17 αOH-progesterone</td>
<td>0.29</td>
</tr>
<tr>
<td>17ß Estradiol</td>
<td>0.0013</td>
</tr>
<tr>
<td>Estrone</td>
<td>&lt; 0.00053</td>
</tr>
<tr>
<td>Estriolo</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cortisolo</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

10.5. Correlation with RIA
Diametra Progesterone ELISA kit was compared to Adaltis Progesterone EIAGen kit commercially available. 31 serum samples were analysed according in both test systems. The linear regression curve was calculated:

\[(\text{Progest. Diametra}) = 0.97 \*(\text{Progest. Adaltis}) + 0.04\]

\[r^2 = 0.887\]

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