ESTRIOL SALIVA ELISA
Direct immunoenzymatic determination of Estriol in saliva.

INTENDED USE
Eagle Biosciences Estriol Saliva ELISA Assay Kit is a Competitive immunoenzymatic colorimetric method for quantitative determination of Estriol concentration in saliva. Estriol Saliva Assay Kit is intended for research use only and not to be used in diagnostic procedures.

1. CLINICAL SIGNIFICANCE
Estriol (also Oestriol) is one of the three main estrogens produced by the human body. It is only produced in significant amounts during pregnancy as it is made by the fetus. During pregnancy the production of estriol depends on an intact maternal-fetal unit. Fetal-placental production of estriol leads to a progressive rise in maternal circulating levels reaching a late-gestational peak several orders of magnitude greater than non-pregnant levels. In the maternal circulation, estriol undergoes a rapid conjugation in the liver followed by urinary excretion with a half-life of about 20 minutes. Since normal estriol production depends on an intact maternal-fetal circulation and functional fetal metabolism, maternal estriol levels have been used to monitor fetal status during pregnancy, particularly during the third trimester. DHEA-S is produced by the adrenal cortex of the fetus, this is converted to estriol by the placenta. If levels are abnormally low in a pregnant woman, this may indicate a problem with the development in the child. Levels of estriol in non-pregnant women do not change much after menopause, and levels are not significantly different from levels in men.

2. PRINCIPLE
In the Estriol Saliva ELISA Assay Kit, the (antigen) in the sample competes with the antigenic Estriol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Estriol 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 Estriol concentration in the sample.

Estriol 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. Estriol Calibrators (6 vials, 1 mL each)
   CAL0      REF DCE002/2606-0
   CAL1      REF DCE002/2607-0
   CAL2      REF DCE002/2608-0
   CAL3      REF DCE002/2609-0
   CAL4      REF DCE002/2610-0
   CAL5      REF DCE002/2611-0
2. Controls (2 vial, 1 mL each)
   Control L  REF DCE045/2601-0
   Control M  REF DCE045/2602-0
3. Incubation Buffer (1 vial, 30 mL)
   Phosphate buffer pH 7.5; BSA 1 g/L
   REF DCE010-0
4. Conjugate (1 vial, 1 mL)
   Estriol conjugated with horseradish peroxidase (HRP)
   REF DCE002/2602-0
5. Coated Microplate (1 microplate breakable)
   Antibody anti-estriol adsorbed on microplate
   REF DCE002/2603-0
6. 50X Conc. Wash solution (1 vial, 20 mL)
   NaCl 45 g/L; Tween-20 55 g/L
   REF DCE006-0
7. TMB Substrate (1 vial, 15 mL)
   H₂O₂-TMB 0.26 g/L (avoid any skin contact)
   REF DCE004-0
8. Stop solution (1 vial, 15 mL)
   Sulphuric acid 0.15 M (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)
Saliva Collection Device
Salivette Sarstedt
   REF DKO063
   REF 51.1534.500
4. WARNINGS
- This Estriol Saliva 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 Estriol 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 direct sunlight, metals or oxidants. Do not freeze the solution.
- This Estriol Saliva ELISA Assay Kit allows the determination of Estriol from 2.5 pg/mL to 4000 pg/mL.
- The clinical significance of Estriol determination can be invalidated if the patient was treated with 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 of Estriol 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 Estriol Saliva ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Estriol 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 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...C₅)
Before using, mix for 5 min with a rotating mixer.
The Calibrators are ready to use and have the following concentration of Estriol:

<table>
<thead>
<tr>
<th>pg/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
<th>C₃</th>
<th>C₄</th>
<th>C₅</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>15</td>
<td>100</td>
<td>600</td>
<td>4000</td>
</tr>
</tbody>
</table>

Once opened, the Calibrators are stable at 2-8°C for 6 months.
For SI UNITS: pg/mL x 3.5 = pmol/mL

6.2. Preparation of the Wash solution
Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. For smaller volumes respect the 1:50 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

6.3. Preparation of Diluted Conjugate
Prepare immediately before use.
Add 10 µL Conjugate (reagent 4) to 1.0 mL of Incubation Buffer (reagent 3). Mix gently. Stable 3 hours at room temperature (22-28°C).

6.4. Preparation of the Sample
The determination of Estriol with this kit should be performed in saliva samples.
It is recommended to collect saliva samples with a centrifuge glass tube and a plastic straw, with the Diametra Saliva Collection Device or with the “Salivette” (Sarstedt, Ref. 511534500). Other commercially available sample collector devices have not been tested.

6.4.1. Method and Limitations
Collect saliva samples at the times indicated. In order to have high reproducibility and accuracy, it is advisable to collect at least 3 samples in a period of
not less than 2 hours and pooling the samples before testing. 
If no specific instructions have been given, saliva samples may be collected at any time; for saliva collection, the following should be noted:

a) If saliva collection is carried out 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 extraneous materials.

6.4.2. Saliva Processing Instructions with Saliva Collection Device Diametra

1) Let the saliva flow down through the straw into 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 Sardstedt

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).
- 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-C₅), two for each Control, two for each sample, one for Blank.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Calibrator</th>
<th>Sample/Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₀-C₅</td>
<td>50 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample/Control L-M</td>
<td>50 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted Conjugate</td>
<td>100 μL</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
</tbody>
</table>

Incubate 1 h at room temperature (22±28°C). Remove the contents from each well; wash the wells 3 times with 300 μL of diluted wash solution.

| TMB Substrate            | 100 μL     | 100 μL          | 100 μL|

Incubate at room temperature (22±28°C) for 15 minutes in the dark.

| Stop Solution            | 100 μL     | 100 μL          | 100 μL|

Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 minutes.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of Estriol 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 (Em) for each point of the Calibration curve (C₀-C₅) and of each sample.

8.2. Calibration curve
Plot the mean value of absorbance (Em) of the Calibrators (C₀-C₅) 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 pg/mL.
9. REFERENCE VALUES
As the Estriol Saliva values follow a circadian pattern we suggest to collect the samples at the same time (8 A.M.). The following values should be used as preliminary guide until each laboratory has got his own reference range.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>N</th>
<th>Range +/- SD’s (pg/mL)</th>
<th>Absolute Range (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy weeks</td>
<td>Saliva (pg/mL)</td>
<td>22°</td>
<td>(700 ± 500)</td>
<td>0 - 210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24°</td>
<td>(900 ± 600)</td>
<td>0 - 220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26°</td>
<td>(1200 ± 700)</td>
<td>0 - 260</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28°</td>
<td>(1500 ± 800)</td>
<td>0 - 320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°</td>
<td>(1800 ± 800)</td>
<td>0 - 320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32°</td>
<td>(2200 ± 1100)</td>
<td>0 - 420</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34°</td>
<td>(3200 ± 1300)</td>
<td>0 - 500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36°</td>
<td>(4100 ± 1600)</td>
<td>0 - 600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°</td>
<td>(4500 ± 1700)</td>
<td>0 - 600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38°</td>
<td>(5000 ± 2000)</td>
<td>0 - 700</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39°</td>
<td>(5300 ± 2000)</td>
<td>0 - 700</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40°</td>
<td>(5700 ± 2000)</td>
<td>0 - 700</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 (16x) the measurement of two different saliva control in one assay. The within assay variability is ≤ 9.7%.

10.1.2. Inter Assay Variation
Between run variation was determined by replicate (10x) the measurement of two different saliva control with different lots of kit. The between assay variability is ≤ 13.7%.

10.2. Accuracy
The recovery of 50 – 300 – 2000 pg/mL of Estril added to “saliva-free” sample gave an average value (+SD) of 100.6% ± 14.6% with reference to the original concentrations.

10.3. Sensitivity
The lowest detectable concentration of Estril that can be distinguished from the Calibrator 0 is 1.0 pg/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>Estriol saliva</th>
<th>100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 epi-estriol</td>
<td>10.5 %</td>
</tr>
<tr>
<td>15 α-OH-estriol</td>
<td>7.0 %</td>
</tr>
<tr>
<td>Estril 3-Sulfate</td>
<td>2.0 %</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.1 %</td>
</tr>
<tr>
<td>17 epi-estriol</td>
<td>&lt; 1x10^-2 %</td>
</tr>
<tr>
<td>Estril 3α-Glucoronide</td>
<td>&lt; 1x10^-2 %</td>
</tr>
<tr>
<td>Estril 16α-Glucoronide</td>
<td>&lt; 1x10^-2 %</td>
</tr>
<tr>
<td>Prednisone</td>
<td>&lt; 1x10^-%</td>
</tr>
<tr>
<td>Estrone</td>
<td>&lt; 1x10^- %</td>
</tr>
</tbody>
</table>

10.5. Correlation
Diametra Estriol saliva ELISA kit was compared to another commercially available Estril saliva assay. 30 saliva samples were analysed according to both test systems. The linear regression curve was calculated:

\[ y = 1.03x + 0.68 \]
\[ r^2 = 0.988 \]
\[ y = \text{Estriol saliva Diametra Elisa kit} \]
\[ x = \text{Estriol saliva Salimetrics Elisa kit} \]

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

BIBLIOGRAPHY

Ed. 03/2012

DiaMetra S.r.l. Headquarter: Via Garibaldi, 18 20090 SEGRATE (MI) Italy
Tel. 0039-02-2139184 – 02-26921595
Fax 0039-02–2133354.

Distributed in the US/Canada by:

EAGLE BIOSCIENCES, INC.
20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 FAX: 617-419-1110
www.EagleBio.com • info@eaglebio.com
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