Diagnostics Biochem Canada Inc.
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Free Estriol ELISA

EU: CE IVD CAN: IVD USA: For Research Use Only. Not for Use in Diagnostic Procedures

REF: CAN-E-620

Version 3.1 (COMB)
Effective: October 17, 2023

INTENDED USE

For the quantitative determination of Free Estriol (E3) in human serum and saliva by an enzyme immunoassay. For *in vitro* use only.

PRINCIPLE OF THE TEST

The Free Estriol (also referred to as unconjugated estriol or uE3 in the literature) ELISA is a competitive immunoassay. Competition occurs between Estriol present in calibrators, controls and patient samples and an enzyme-labelled antigen (conjugate) for a limiting number of anti-Estriol antibody binding sites on the microplate wells. After a washing step that removes unbound materials, the enzyme substrate is added, and approximately 15–20 minutes later the enzymatic reaction is terminated by addition of stopping solution. The resulting optical density (OD), measured with a microplate reader, is inversely proportional to the concentration of Estriol in the sample. A calibrator curve is plotted with a provided set of calibrators to calculate directly the concentration of estriol in patient samples and controls.

Serum and salivary assays follow the same procedure except that the volume of the calibrators, controls and samples dispensed into the microplate wells is 10 µL for serum assays and 20 µL for salivary assays.

CLINICAL APPLICATIONS

Since the production of estriol in pregnant women depends on a healthy maternal-placental-fetal system, the estriol concentration is a marker of both placental and fetal normal development and metabolism; hence the determination of serum or saliva estriol concentration is instrumental for the assessment of fetus health in advanced pregnancy (Berkane et al., 2017).

In non-pregnant women and men, estriol levels are low. Notwithstanding, one common application of salivary tests is the monitoring of the estriol levels in women undergoing hormone replacement therapy (Falah et al, 2015).

Due to the significant temporal fluctuations in the concentrations of this hormone, multiple tests are recommended to obtain reliable results (Fleck et al, 2018).

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is for professional use only and for in vitro use only.
2. Practice good laboratory practices when handling kit reagents and specimens. This includes:
   - Do not pipette by mouth.
   - Do not smoke, drink, or eat in areas where specimens or kit reagents are handled.
   - Wear protective clothing and disposable gloves.
   - Wash hands thoroughly after performing the test.
   - Avoid contact with eyes; use safety glasses; in case of contact with eyes, flush eyes with water immediately and contact a doctor.
3. 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.
4. Avoid microbial contamination of reagents.
5. A calibrator curve must be established for every run.
6. It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
7. The controls (included in kit) must be included in every run and their results must fall within the ranges stated in the quality control certificate; a failed control result might indicate improper procedural techniques or pipetting, incomplete washing or improper reagent storage.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
9. All kit reagents and specimens must be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of specimens.
10. 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.
11. 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.
12. When dispensing the substrate and stopping solutions, do not use pipettes in which these liquids will come into contact with any metal parts.
13. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, calibrator and control.
14. Do not use kit components from different kit lots within a test and do not use any component beyond the expiration date printed on the label.
15. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

LIMITATIONS

1. This kit is calibrated for the determination of Estriol in either human serum or human saliva. The kit is not calibrated for the determination of Estriol in other specimens of human or animal origin.
2. This kit shall not be used to test serum and saliva samples simultaneously in the same run. The volume required for the calibrators, controls and samples is different depending on if serum or saliva samples will be run.
3. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
4. Do not use blood contaminated saliva samples.
5. Samples or control sera containing azide or thimerosal are not compatible with this kit, they may lead to false results.
6. Only calibrator A may be used to dilute high serum and saliva samples. The use of any other reagent will lead to false results.
7. The results obtained with this kit shall never be used as the sole basis for a clinical diagnosis. For example, some drugs and the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products have the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should comprise all aspects of a patient's background including the frequency of exposure to animals/products.

SAFETY CAUTIONS AND WARNINGS

BIOHAZARDS

The reagents shall be considered a potential biohazard and handled with the same precautions applied to any blood specimen.

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 - SERUM

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 later. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN COLLECTION, PRE-TREATMENT AND STORAGE - SALIVA

Avoid sample collection within 1 hour after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before the sample is collected. Do not use blood-contaminated specimens.

Specimen Collection

Approximately 0.1 mL of saliva is required per duplicate determination. Rinse mouth thoroughly with water 10 minutes before the sample is collected. Collect 1–2 mL of saliva into a clean polypropylene tube without force or inducement.

Specimen Pre-Treatment

Following collection, the sample must be pretreated according to the following procedure:

1. Freeze the sample for a minimum of 2 hours.
2. Thaw the sample.
3. Vortex to mix and centrifuge the sample at 2000x g for 10 minutes.
4. Carefully remove the supernatant and transfer to a new labelled tube. The supernatant will be used in the assay procedure of the test.

Specimen Storage

Store pretreated saliva samples at 4°C for up to 24 hours or freeze at or below -20°C for up to 6 months. Samples that have been stored should be inspected to ensure they are free from precipitates before being used in the assay. If there are precipitates present, follow steps 3–4 in the specimen pretreatment section. Consider all human specimens as possible biohazardous materials.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 10, 20, 50, 150 and 350 µL
2. Disposable pipette tips
3. Distilled or deionized water
4. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater
5. Microplate washer (recommended)

REAGENTS PROVIDED

1. Anti-Estriol Antibody-Coated Break-Apart Well Microplate — Ready To Use

Contents: One monoclonal antibody-coated 96-wells (12x8) microplate in a resealable pouch with desiccant.
Storage: Refrigerate at 2–8°C

2. Estriol-Horseradish Peroxidase (HRP) Conjugate — Ready to Use

Contents: One bottle containing Estriol-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 20 mL/bottle
Storage: Refrigerate at 2–8°C

3. Free Estriol Calibrators — Ready to Use

Contents: Six vials containing estriol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of estriol.

* Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
Calibrator A	0 ng/mL	2.0 mL
Calibrator B	0.05 ng/mL	1.0 mL
Calibrator C	0.25 ng/mL	1.0 mL
Calibrator D	1 ng/mL	1.0 mL
Calibrator E	5 ng/mL	1.0 mL
Calibrator F	30 ng/mL	1.0 mL

Storage: Refrigerate at 2–8°C
Stability: Once opened, the calibrators should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Free Estriol Controls — Ready To Use

Contents: Two vials containing estriol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of estriol. Refer to vial labels for the acceptable ranges.
Volume: 1.0 mL/vial
Storage: Refrigerate at 2–8°C
Stability: Once opened, the controls 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
Preparation of working wash buffer: 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. 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

7. Stopping Solution — Ready To Use

Contents: One bottle containing 1M sulfuric acid.
Volume: 6 mL/bottle
Storage: Refrigerate at 2–8°C

ASSAY PROCEDURE

Specimen Pretreatment

Serum: None **Saliva:** See Specimen Collection, Pre-Treatment and Storage Section - Saliva

All kit components, controls and specimen samples must reach room temperature prior to use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. After all kit components have reached room temperature, mix gently by inversion. Prepare the working wash buffer (see Wash Buffer Concentrate in the REAGENTS PROVIDED section).
2. Plan the microplate wells to be used for calibrators, controls and samples. See Recommended Microplate Layout section. Remove the strips from the microplate frame that will not be used and place them in the bag with desiccant. Reseal the bag with the unused strips and return it to the refrigerator.
3. If running Serum samples: Pipette 10 µL of each calibrator, control and specimen sample into planned microplate wells.
   or
   If running Saliva samples: Pipette 20 µL of each calibrator, control and specimen sample into planned microplate wells.
4. Pipette 150 µL of the Estriol-HRP conjugate into each microplate well (the use of a multi-channel pipette is recommended).
5. Gently tap the microplate frame for 10 seconds to mix the contents of the wells and incubate the microplate at room temperature (no shaking) for 60 minutes.
6. Wash the microplate wells 3 times with working wash buffer (350 µL/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry. The use of a microplate washer is highly recommended. If a microplate washer is not available, ensure that the wash buffer reaches the top edge of the wells and that no liquid remains in the microplate after the final washing, avoid splashing.
7. Pipette 150 µL of TMB substrate into each microplate well at timed intervals (the use of a multi-channel pipette is recommended).
8. Incubate the microplate at room temperature (no shaking) for 15–20 minutes.
9. Pipette 50 µL of stopping solution into each microplate well at the same timed intervals as in step 7 and gently tap the microplate frame to mix the contents of the wells (the use of a multi-channel pipette is recommended).
10. Read the optical density (absorbance) in the microplate wells using a microplate reader set at 450 nm, within 20 minutes after addition of the stopping solution.

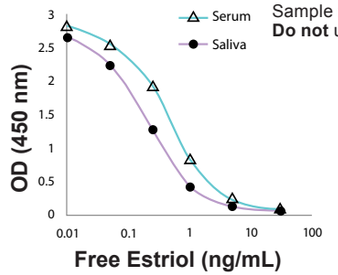
CALCULATIONS

1. Calculate the mean optical density of each calibrator, control and sample duplicate.
2. Use a 4-parameter or 5-parameter curve fit with immunoassay software to generate a calibrator curve.
3. Read the values of the unknowns directly off the calibrator curve.
4. If a sample reads more than 30 ng/mL dilute it with calibrator A not more than 10-fold. The result obtained must be multiplied by the dilution factor.

TYPICAL TABULATED DATA

Calibrator	Mean OD (450 nm)	% Binding	Value (ng/mL)
A	2.822	100	0
B	2.542	90	0.05
C	1.924	68	0.25
D	0.840	30	1
E	0.224	8	5
F	0.072	3	30
Unknown	1.929	-	0.24

TYPICAL CALIBRATOR CURVE



Sample curve only.
Do not use to calculate results.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit was calculated following EP17-A. Sixty replicates of the matrix and a low concentration sample were run in independent tests with two lots of the kit.

Serum: The Limit of Background was determined to be 0.027 ng/mL and the Limit of Detection was determined to be 0.058 ng/mL.

Saliva: The Limit of Background was determined to be 0.017 ng/mL and the Limit of Detection was determined to be 0.034 ng/mL

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with estriol reacting at 100%.

Compound	% Cross-Reactivity
Estriol	100
Estriol-3-Sulfate	0.6
Estriol-3-Glucuronide	1.3
Estradiol	< 0.1
17α-Estradiol	< 0.1
Estradiol Sulfate	< 0.01
Estrone	< 0.1
Estrone Sulfate	< 0.01
Cholesterol	< 0.0001
Corticosterone	< 0.01
DHEAS	< 0.1
Equilin	< 0.1
Prednisone	< 0.001

INTERFERENCES

Hemoglobin up to 2 g/L, Bilirubin conjugated and unconjugated up to 20 mg/dL, Triglycerides up to 5 mg/mL, Biotin up to 10 µg/mL, Daidzein, Genistein and Resveratrol each up to 200 ng/mL, HAMAS up to 1.2 µg/mL, and Rheumatoid Factor up to 1.2 IU/mL did not interfere with the assay.

PRECISION

The experimental protocol used a nested components-of- variance design with 10 testing days, two lots and two scientists per day. Each scientist ran two tests with two lots per day and two replicate measurements per run (a 10 x 2 x 2 x 2 design) for each sample. The results were analyzed with a two-way nested ANOVA and summarized in the table below.

Sample	Mean (ng/mL)	Within Run SD	Within Run CV	Total SD	Total CV
1	0.167	0.023	13.6%	0.026	15.6%
2	0.264	0.032	12.3%	0.036	13.8%
3	0.946	0.062	6.5%	0.066	7.0%
4	4.841	0.326	6.7%	0.366	7.6%
5	11.89	1.107	9.3%	1.148	9.7%
6	16.10	1.621	10.1%	1.639	10.2%
7	3.544	0.232	6.5%	0.256	7.2%
8	1.927	0.110	5.7%	0.119	6.2%
9	5.932	0.403	6.8%	0.448	7.5%
10	9.127	0.606	6.6%	0.619	6.8%

LINEARITY

The linearity study was performed with four human serum and four human saliva samples covering the range of the assay and following CLSI guideline EP06-A. The samples were diluted in calibrator A at several equidistant concentration levels and up to ten-fold (1:10), tested in duplicate, and the results (y) compared to the predicted concentration (x). The statistical analysis shows that the assay is sufficiently linear up to a 1:10 dilution throughout the dynamic range of the kit when using calibrator A as the diluent.

Serum: y = 1.04x – 0.71; r = 0.99

Saliva: y = 0.95x – 0.08; r = 0.99

COMPARATIVE STUDIES

The DBC Free Estriol ELISA kit (y) was compared to a commercial Estriol Immunofluorescence assay (x) used for IVD. The comparison of 61 serum samples yielded the following linear regression results:
y = 0.92x – 0.12, r = 0.99

The DBC Free Estriol ELISA kit (y) was compared to a commercial High Sensitivity Estriol ELISA kit (x). The comparison of 40 saliva samples yielded the following linear regression results:
y = 1.32x + 0.06, r = 0.97

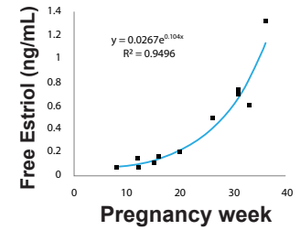
REFERENCE RANGES

Serum Cohort Group	n	Median (ng/mL)	95% Range (ng/mL)	Total Range (ng/mL)
Adult Males and Non-Pregnant Females	120	< 0.058	ND–0.11	ND–0.12
Pregnant Females First Trimester	30	0.15	—	ND–2.95
Pregnant Females Second Trimester	50	1.20	0.46–3.04	0.45–3.07
Pregnant Females Third Trimester	25	9.5	—	3.6–14.3

Saliva Cohort Group	n	Median (ng/mL)	95% Range (ng/mL)	Total Range (ng/mL)
Adult Males and Non-Pregnant Females	80	0.05	ND–0.08	ND–0.08
Pregnant Females Third Trimester	4	0.71	—	0.6–1.3

ND = Non-determined; value less than the limit of detection.

The concentration of estriol in the saliva of pregnant women increases with the gestation period as follows:



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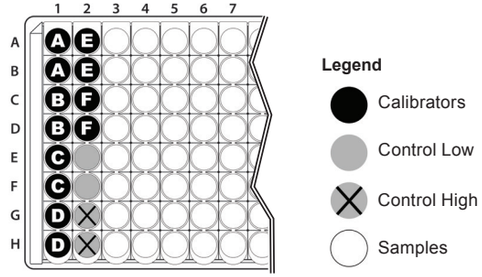
RECOMMENDED MICROPLATE LAYOUT



SYMBOLS

CE European Comformity
IVD In vitro diagnostic device
Consult instructions for use
Contains sufficient for <n> tests
Storage Temperature
Legal Manufacturer
Use by
REF Catalogue Number
EC REP Authorized representative
LOT Lot number
X # Dilute 1: # Before use