Free Estriol Saliva ELISA Assay Kit

Catalog Number:

FES32-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

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INTENDED USE

The Eagle Biosciences Free Estriol Saliva ELISA Assay Kit is intended for the quantitative determination of Free Estriol (E3) in human serum and saliva by an enzyme immunoassay. The Eagle Biosciences Free Estriol Saiva ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

INTRODUCTION

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 moniroting of the estriol levels in women undergoing horomone replacement therapy (Falah et al., 2015).

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

PRINCIPLE OF THE ASSAY

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 samples and an enzyme-labeled 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 late 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 samples and controls.

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

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is for professional use only and for research use only.
2. Practice good laboratory practices when handling kit reagents and specimens. This includes:
    a. Do not pipette by mouth.
    b. Do not smoke, drinks, or eat in areas where specimens or kit reagents are handled.
    c. Wear protective clothing and disposable gloves,
    d. Wash hands thourougly after performing the test.
    e. 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 obtained 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 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 with affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
11. The substrate solution (TMB) is senstivie to light and should remain colorless is properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
12. When dispensing the substrate and stopping solutions, do not use pipettes in which these liquied 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 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 samoples simultaneously in the same run. The volume requred for the calibrators, controls, and samples is different depending on if serum or salliva samples will be run.
3. Do not used grossly hemolysed, 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 are for research use only

SAFETY CAUTIONS AND WARNINGS

BIOHAZARDS
The reagents shall be considered a potential biohazard and hand-led 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 labeled 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, PRETREATMENT AND STORAGE - SALIVA

Avoid sample collection within 1 hour after eating a major meal or within 12 hours after sonsuming 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 proproylene 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 labeled tube. The supernatant will be used in the assay procedure of the test.

Specimen Storage

Store pretreated saliva samples at 4C for up to 24 hours or freeze at or below -20C for up to 6 monts. Samples that have been stored should be inspected to ensure they are free from precipitates before veing 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 bottle 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 contorls 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: Dilute 1:10 in distilled or deionized water before use. If one 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 specimien 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 Was Buffer Concentration 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 10uL of each calibrator, control and specimen sample into planned microplate wells. Or, if running Saliva samples: Pipette 20uL 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 (350uL/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry. The use of a mircoplate 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 uL 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.

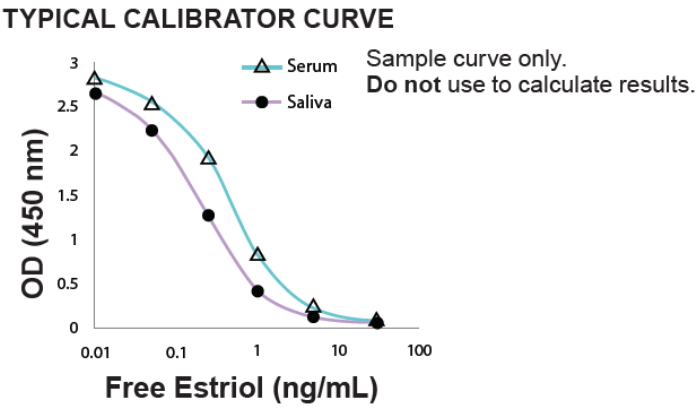
CALCULATIONS

1. Calculate the mean optical density of each calibrator 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 30ng/mL dilute it with calibrator A not more than 10-fold. The result obtained must be multiplied by the dilution factor.

TYPICAL TABULATED DATA
Sample data only. Do not use to calculate results.

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



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 0.058ng/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%.

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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 ug/mL, Daidzein, Genistein and Resveratol each up 200 ng/mL, HAMAS up to 1.2 ug/mL, and Rheumatoid Factor up to 1.2 IU/mL did not interfere with the assay.

PRECISION
The experimental protocol used a nested componets-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 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 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 Free Estriol Saliva ELISA kit (y) was compared to a commercial High Sensitivity Estriol ELISA kit (x). The comparison of 40 saliva samples yielded the follwoing linear regression results:
y + 1.32x + 0.06, r = 0.97

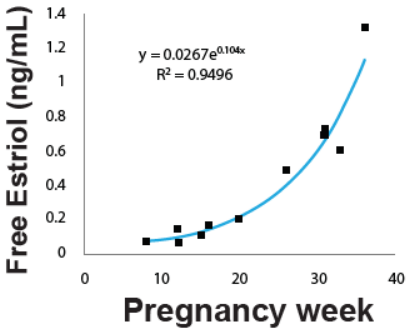
REFERENCE RANGES

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:



LITERATURE

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3. Kos-Kudla B, et al. Comparative studies of serum and salivary estriol concentrations in third trimester of normal pregnancy. *Med Sci Monit.* 1999;5(2):285-288.
4. Falah N, et al. Estriol review: Clinical applications and potential biomedical importance. *Clin Res Trials.* 2015;1(2):29-33.
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6. Montazeri S, et al. Investigating Relationship between Saliva Estriol to Progesterone Ratio and Preterm Delivery. *International Journal of Pharmaceutical Research & Allied Sciences.* 2016;5(3):264-9.
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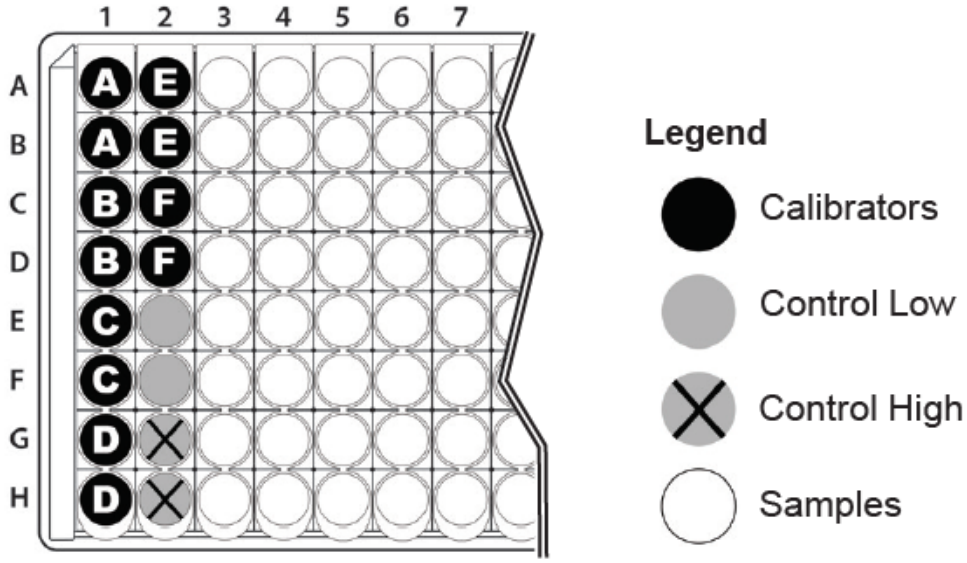
RECOMMENDED MICROPLATE LAYOUT



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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.