SALIVARY
ESTRIOL / HS ESTRIOL
ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-1802, (Single) 96-Well Kit;
1-1802-5, (5-Pack) 480 Wells

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Intended Use

The Salimetrics® Estriol/High Sensitivity (HS) Estriol Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary Estriol. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. Salimetrics has not validated this kit for serum or plasma samples.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

This instruction booklet contains two assay protocols. Use "Method A: High Sensitivity Salivary Estriol Procedure" for expected values below 20 pg/mL, such as in normal adults. Use "Method B: Salivary Estriol Procedure" for expected values in the range of 20 pg/mL or higher, such as in pregnancy.

Introduction

Estriol (1,3,5(10)-estratriene-3,16α,17β-triol; E3) is a female sex steroid hormone largely associated with pregnancy and fetal development. Fetal adrenal DHEA-S is metabolized in the fetal liver to 16-OH-DHEA-S, which is then converted to Estriol in the placenta. Near term, the fetus is the source of 90% of the 16-OH-DHEA-S in the normal human pregnancy. Maternal circulating Estriol levels rise progressively during pregnancy, reaching a peak in the third trimester (1).

The physiological roles of Estriol in non-pregnant women are not well understood and are under investigation, particularly in connection with aging and post-menopausal health. With respect to estrogenic activity, Estriol is generally thought to be less potent than Estradiol or Estrone. However, with regard to nongenomic signaling pathways and functional responses in the pituitary, it has been pointed out that Estriol is a strong estrogen (2).

In blood, the majority of Estriol is bound by serum proteins, with about 14-16% remaining unbound (3). Unbound Estriol enters saliva from blood via intracellular mechanisms, and correlation between serum and saliva samples is highly significant (4).

Test Principle

This is a competitive immunoassay kit. Estriol in standards and samples compete with Estriol conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Estriol Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Estriol Enzyme Conjugate detected is inversely proportional to the amount of Estriol present in the sample (5).

Safety Precautions

Read Safety Data Sheets before handling reagents.

Hazardous Ingredients

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

Safety Data Sheets are available by contacting Salimetrics at support@salimetrics.com (See www.salimetrics.com for alternative contact options).

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date.

Specimen Collection

Avoid sample collection within 60 minutes 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 sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at www.salimetrics.com or upon request.

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination (6,7) using our Blood Contamination EIA Kit (Item Nos. 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

It is important to record the time and date of specimen collection.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20ºC within 4 hours of collection. (Samples may be stored at -20ºC for up to 6 months.) For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate or making dilutions. Pipette clear sample into appropriate wells, or dilution tubes. Re-freeze saliva samples as soon as possible after running assay. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Saliva samples must be pre-diluted for the Method A: HS Salivary Estriol Procedure ONLY (No pre-dilution needed for Method B.) See Procedures for details.

Materials Supplied with Single Kit

	Item	Quantity/Size
1	**Microtitre Plate** Coated with rabbit anti-Estriol antibodies.	1/96 well
2	**Estriol Standard** 4860 pg/mL, in a saliva-like matrix. Further dilution of standard is necessary only for Method A: HS Salivary Estriol Procedure (see Procedure for details). Contains: Estriol, buffer, preservative.	1 vial / 500 µL
3	**Estriol Controls** High, Low, in a saliva-like matrix. Further dilution of controls is necessary only for Method A: HS Salivary Estriol Procedure (see Procedure for details). Contains: Estriol, buffer, preservative.	2 vials / 500 µL each
4	**Estriol Enzyme Conjugate** Concentrate. Dilute before use with Estriol Assay Diluent. (See Procedure, methods A & B.) Contains: Estriol conjugated to HRP, preservative.	1 vial / 50 µL
5	**Estriol Assay Diluent** Contains: phosphate buffer, preservative.	1 bottle / 60 mL
6	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
7	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle / 25 mL
8	**Stop Solution**	1 bottle / 12.5 mL
9	**Non-Specific Binding (NSB) Wells** Do not contain anti-Estriol antibody. Break off and insert as blanks (optional) where needed.	1 strip
10	**Adhesive Plate Covers**	2

Materials Needed But Not Supplied

- Precision pipette to deliver 7 µL to 500 µL
- Precision multichannel pipette to deliver 50 µL, 100 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of operating at 500 rpm & 2-8°C.
- Plate reader with 450 nm and 490 to 492 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 15 mL
- Small disposable polypropylene tubes for dilution of standard (both Methods A & B), controls and samples (Method A only)
- Pipette tips
- Serological pipette to deliver up to 14 mL
- Refrigerator
- Centrifuge capable of 1500 x g

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 14 mL (Method A) or 12 mL (Method B) of Estriol Assay Diluent used in the conjugate dilutions to come to room temperature.
- Bring Microtitre Plate to room temperature before use. ***It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.***
- Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). ***Dilute only enough for current day's use and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
- See Procedures below (Methods A and B) for specific instructions on diluting the standard. (Instructions for diluting controls and saliva samples are included in the Procedure for Method A only.)

Method A: HS Salivary Estriol Procedure

(expected values < 20 pg/mL)

(Proceed to Method B if expected values are ≥ 20 pg/mL.)

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1215 Std	1215 Std	Ctrl-H	Ctrl-H								
B	405 Std	405 Std	Ctrl-L	Ctrl-L								
C	135 Std	135 Std	SMP-1	SMP-1								
D	45 Std	45 Std	SMP-2	SMP-2								
E	15 Std	15 Std	SMP-3	SMP-3								
F	5 Std	5 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSB wells included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

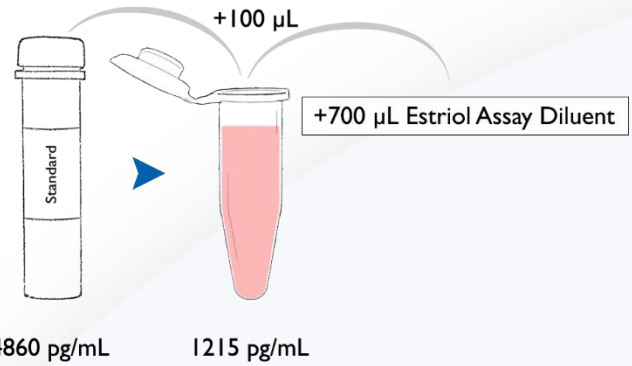
Cautions: 1. Extra NSB wells should not be used for determination of standards, controls, or unknowns.

2. Do not insert wells from one plate into a different plate.

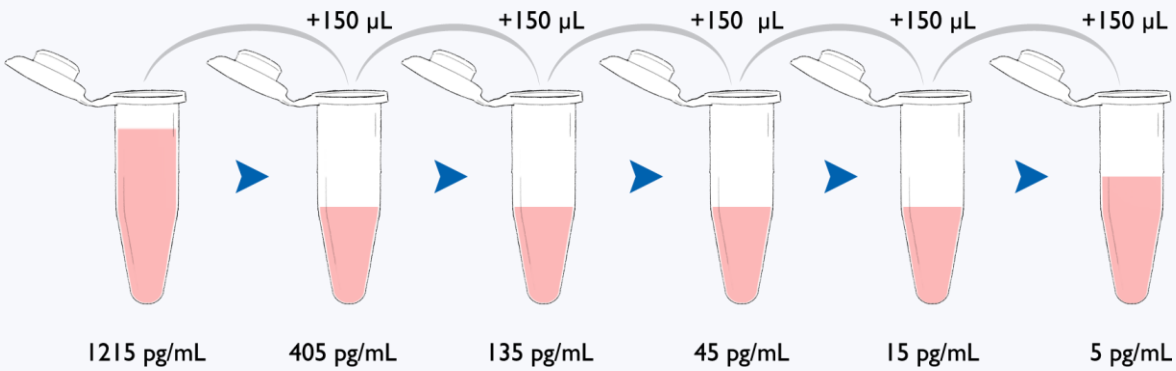
Step 3: Standard Dilution

- Label six polypropylene microcentrifuge tubes or other small tubes 1 through 6.
- Pipette 700 µL of Estriol Assay Diluent into tube 1.
- Dilute the 4860 pg/mL Estriol Standard 1:8 by pipetting 100 µL of the standard into tube 1. Label this tube 1215 pg/mL.

Note: The actual concentration of Estriol in the standard is 607.5 pg/mL. Given that samples are run at a 2X dilution, the concentration of the standard curve has been adjusted for your convenience in order to eliminate the need to multiply all sample results by 2.



- Pipette 300 µL of Estriol Assay Diluent into tubes 2 through 6.
- Serially dilute the standard 3X by adding 150 µL of the 1215 pg/mL standard (tube 1) to tube 2. Mix well.
- After changing pipette tips, remove 150 µL from tube 2 to tube 3. Mix well.
- Continue for tubes 4, 5, and 6.
- The final concentrations of standards for tubes 1 through 6 are, respectively, 1215 pg/mL, 405 pg/mL, 135 pg/mL, 45 pg/mL, 15 pg/mL, and 5 pg/mL. Standard concentrations in pmol/L are 4213.34, 1404.45, 468.15, 156.05, 52.02, and 17.34, respectively.



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Step 4: Pipette 14 mL of Estriol Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 7.

Step 5: Control and Sample Dilutions

- Label one polypropylene microcentrifuge tube 500 pg/mL and one tube 15 pg/mL.
    - Pre-dilute the 3000 pg/mL High Control 1:6 by adding 100 µL of the High Control to 500 µL of Estriol Assay Diluent in the tube labeled 500 pg/mL. Mix well.
    - Pre-dilute the 50 pg/mL Low Control 1:3.333 by adding 150 µL of the Low Control to 350 µL of Estriol Assay Diluent in the tube 15 pg/mL. Mix well.
- Label one polypropylene microcentrifuge tube with the identity of each control and saliva sample.
    - Further dilute both the 500 and 15 pg/mL High & Low Controls 2X: 150 µL control to 150 µL Estriol Assay Diluent. Mix well.
    - Dilute saliva samples 2X: 150 µL saliva sample to 150 µL Estriol Assay Diluent. Mix well.

Step 6:

- Pipette 100 µL of standards, diluted controls, and diluted saliva samples into appropriate wells.
- Pipette 100 µL of Estriol Assay Diluent into 2 wells to serve as the zero.
- Pipette 100 µL of Estriol Assay Diluent into each NSB well.

Step 7: Dilute the Enzyme Conjugate 1:2000 by adding 7 µL of the conjugate to the 14 mL tube of Estriol Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 8: Place adhesive cover provided over plate. Mix plate on a plate rotator continuously at 500 rpm for 20-24 hours at 2-8ºC.

Step 9: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 10: Add 200 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 11: Incubate the plate in the dark (covered) at room temperature for 45 minutes mixing constantly on a plate rotator at 500 rpm.

Step 12: Add 50 µL of Stop Solution with a multichannel pipette.

Step 13:

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow.

  Caution: Spillage may occur if mixing speed exceeds 600 rpm.
- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 490 to 492 nm is recommended.)

Quality Control

For both Methods A & B: The Salimetrics' High and Low Estriol Controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

The following Calculations, Results and Performance Characteristics apply to Method A only unless otherwise stated.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. The standard curve has been adjusted in order to automatically compensate for the 2X dilution of the saliva sample, therefore no further multiplication of the assay results is needed.
6. Samples (diluted 2X) with Estriol values greater than 1215 pg/mL should be diluted further with Estriol Assay Diluent and rerun for accurate results. If a further dilution of the 2X diluted sample is used, multiply the results by the additional dilution factor only.

   Example:
   1. Sample (diluted 2X) assay result = >1215 pg/mL.
   2. Dilute the 2X diluted sample further X4. Assay result = 1000 pg/mL.
   3. Final concentration of saliva sample = 1000 pg/mL x 4 = 4000 pg/mL.

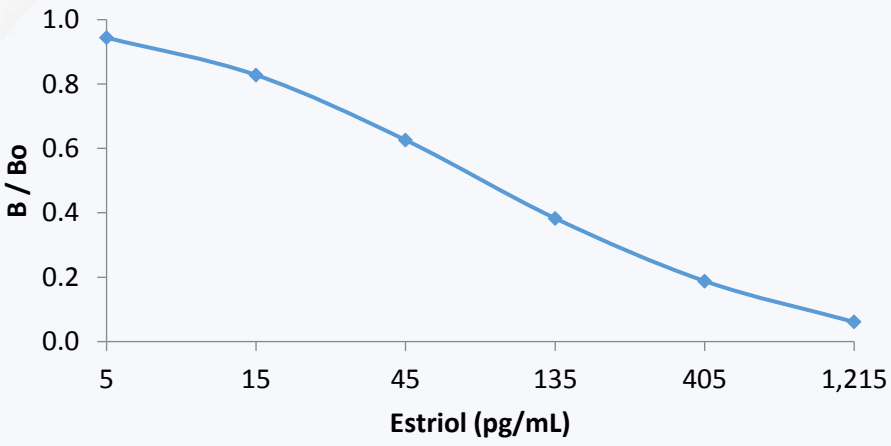
A new Standard Curve must be run with each full or partial plate.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	HS Estriol (pg/mL)
A1,A2	S1	0.119	0.096	0.061	1215
B1,B2	S2	0.318	0.295	0.188	405
C1,C2	S3	0.624	0.601	0.383	135
D1,D2	S4	1.004	0.981	0.626	45
E1,E2	S5	1.321	1.298	0.828	15
F1,F2	S6	1.503	1.480	0.944	5
G1,G2	Bo	1.591	1.568	NA	NA
H1,H2	NSB	0.023	NA	NA	NA

Example: HS Salivary Estriol 4-Parameter Curve Fit



Limitations

- For Method A (HS Salivary Estriol Procedure) only:
  - Samples (diluted 2X) with Estriol values greater than 1215 pg/mL should be diluted further with Estriol Assay Diluent and rerun for accurate results. If a further dilution of the 2X diluted sample is used, multiply the results by the additional dilution factor only. See also Calculations.
- For both Methods A & B:
  - See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
  - Samples collected with sodium azide are unsuitable for this assay.
  - Any quantitative results indicating abnormal Estriol levels should be followed by additional testing and evaluation.

Salivary Estriol Example Ranges*

(For both Methods A & B)

Group	Time	N	Range +/- 2SDs (pg/mL)	Absolute Range (pg/mL)
Premenopausal Adult Females	AM	17	0 - 16.4	0 - 28.12
Premenopausal Adult Females	PM	16	0 - 4.8	0 - 6.88

*To be used as a guide only. Each laboratory should establish its own range.

High Sensitivity Salivary Estriol EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from the mean of 12 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	534.5	10.84	2.0
L	12	15.04	1.25	8.3

The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	488.30	20.32	4.2
L	12	18.19	2.63	14.5

Recovery

Three saliva samples containing different levels of an endogenous Estriol were spiked with known quantities of Estriol and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	361.7	13.5	375.2	369.53	98.5
2	6.42	972	978.42	880.78	90.0
3	0	121.5	121.5	125.33	103.2

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 pg/mL level. The minimal concentration of Estriol that can be distinguished from 0 is 1 pg/mL.

Correlation with Serum

(For both Methods A & B)

The correlation between serum and saliva Estriol in pregnant and nonpregnant females was determined by assaying 35 matched samples. The serum-saliva correlation was highly significant, $r(33) = 0.87$, $p < 0.001$.

Sample Dilution Recovery

Two samples were serially diluted with Estriol Assay Diluent and assayed.

Saliva Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			156.71	
	1:2	78.36	68.60	87.5
	1:4	39.18	34.76	88.7
	1:8	19.59	18.44	94.1
	1:16	9.79	9.69	99.0
2			789.84	
	1:2	394.92	409.60	103.7
	1:4	197.46	214.78	108.8
	1:8	98.73	113.3	114.8
	1:16	49.37	53.53	108.4

Method B: Salivary Estriol Procedure

(expected values ≥ 20 pg/mL)

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	4860 Std	4860 Std	Ctrl-H	Ctrl-H								
B	1620 Std	1620 Std	Ctrl-L	Ctrl-L								
C	540 Std	540 Std	SMP-1	SMP-1								
D	180 Std	180 Std	SMP-2	SMP-2								
E	60 Std	60 Std	SMP-3	SMP-3								
F	20 Std	20 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

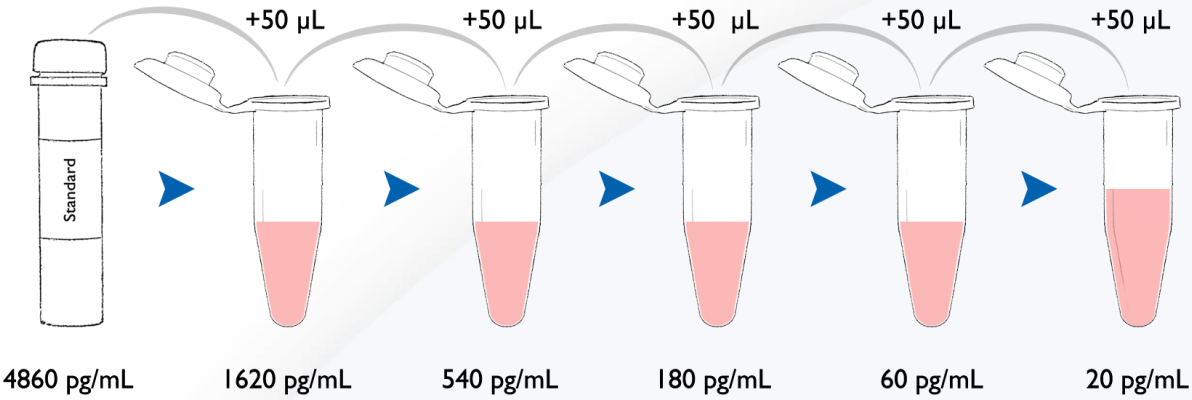
Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. If you choose to place non-specific binding wells in H-1, 2, remove strips 1 and 2 from the strip holder and break off the bottom wells. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSB wells included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Cautions: 1. Extra NSB wells should not be used for determination of standards, controls, or unknowns.

2. Do not insert wells from one plate into a different plate.

Step 3: Standard Dilution

- Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 100 µL of Estriol Assay Diluent into tubes 2 through 6.
- Serially dilute the standard 3X by adding 50 µL of the 4860 pg/mL standard (tube1) to tube 2. Mix well.
- After changing pipette tips, remove 50 µL from tube 2 to tube 3. Mix well.
- Continue for tubes 4, 5, and 6.
- The final concentrations of standards for tubes 1 through 6 are, respectively, 4860 pg/mL, 1620 pg/mL, 540 pg/mL, 180 pg/mL, 60 pg/mL, and 20 pg/mL. Standard concentrations in nmol/L are 16.85, 5.62, 1.87, 0.62, 0.21, and 0.07, respectively.



Step 4: Pipette 12 mL of Estriol Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 6.

Step 5:

- Pipette 25 µL of standards, controls, and saliva samples into appropriate wells.
- Pipette 25 µL of Estriol Assay Diluent into 2 wells to serve as the zero.
- Pipette 25 µL of Estriol Assay Diluent into each NSB well.

Step 6: Dilute the Enzyme Conjugate 1:800 by adding 15 µL of the conjugate to the 12 mL tube of Estriol Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 7: Place adhesive cover provided over plate. Mix plate on a plate rotator continuously at 500 rpm for 2 hours at room temperature.

Step 8: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 9: Add 100 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 10: Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark (covered) at room temperature for an additional 25 minutes.

Step 11: Add 100 µL of Stop Solution with a multichannel pipette.

Step 12:

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow.

  Caution: Spillage may occur if mixing speed exceeds 600 rpm.
- Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 490 to 492 nm is recommended.)

Quality Control

See page 12.

The following Calculations, Results and Performance Characteristics apply to Method B only unless otherwise stated.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with Estriol values greater than 4860 pg/mL should be diluted with Estriol Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

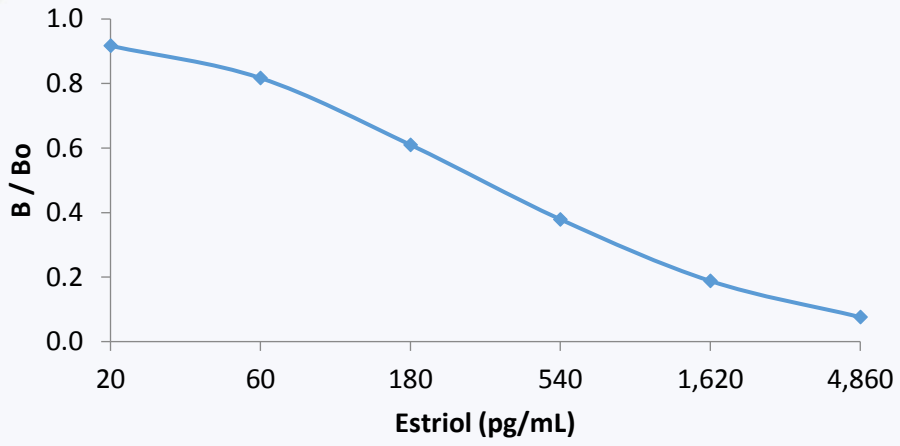
A new Standard Curve must be run with each full or partial plate.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Estriol (pg/mL)
A1,A2	S1	0.113	0.097	0.076	4860
B1,B2	S2	0.257	0.241	0.188	1620
C1,C2	S3	0.502	0.486	0.379	540
D1,D2	S4	0.798	0.782	0.610	180
E1,E2	S5	1.063	1.047	0.817	60
F1,F2	S6	1.192	1.176	0.917	20
G1,G2	Bo	1.298	1.282	NA	NA
H1,H2	NSB	0.016	NA	NA	NA

Example: Estriol 4-Parameter Curve Fit



Limitations

- For Method B (Salivary Estriol Procedure) only:
    - Samples with Estriol values greater than 4860 pg/mL should be diluted with Estriol Assay Diluent and rerun for accurate results. To obtain the final Estriol concentration, multiply the concentration of the diluted sample by the dilution factor.
- See page 14 for other Limitations.

Salivary Estriol Example Ranges*

See page 14.

Salivary Estriol EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from the mean of 12 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	3049.47	94.53	3.1
L	12	67.05	6.02	9.0

The inter-assay precision was determined from the mean of average duplicates for 12 separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	3173.1	182.79	5.8
L	12	60.47	5.06	8.4

Recovery

Three saliva samples containing different levels of an endogenous Estriol were spiked with known quantities of Estriol and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	0	2000.00	2000.00	2099.10	105.0
	0	486.00	486.00	510.80	105.3
2	381.65	2000.00	2381.65	2678.39	112.5
	381.65	486.00	867.65	967.46	111.5
	381.65	18.00	399.65	400.45	100.2
3	921.00	2000.00	2921.00	3225.35	110.4
	921.00	486.00	1407.00	1532.18	108.9
	921.00	18.00	939.00	989.53	105.4

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 pg/mL level. The minimal concentration of Estriol that can be distinguished from 0 is 16 pg/mL.

Correlation with Serum

See page 16.

Sample Dilution Recovery

Two samples were serially diluted with Estriol Assay Diluent and assayed.

Saliva Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			539.52	
	1:2	269.76	244.36	90.6
	1:4	134.88	112.89	83.7
	1:8	67.44	56.47	83.7
	1:16	33.72	31.09	92.2
2			1912.24	
	1:2	956.12	941.92	98.5
	1:4	478.06	472.93	98.9
	1:8	239.03	248.18	103.8
	1:16	119.52	125.71	105.2

Antibody Specificity

(For both Methods A & B.)

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Estriol / HS Estriol EIA
Estradiol	50	1.4
Estrone	10	ND
Progesterone	100	ND
17 α-Hydroxyprogesterone	1000	ND
Testosterone	100	0.0145
Cortisol	1000	ND
DHEA	1000	ND
Aldosterone	1000	ND
Cortisone	1000	ND
11-Deoxycortisol	1000	ND
21-Deoxycortisol	1000	ND
Dexamethasone	1000	ND
Triamincinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	1000	ND
Transferrin	1000	ND

ND = None detected (<0.004)

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