



High Sensitivity

# SALIVARY 17 $\beta$ -ESTRADIOL

ENZYME IMMUNOASSAY KIT



For Diagnostic In-Vitro Use

Item No. 1-4702, (Single) 96-Well Kit;  
1-4702-5, (5-Pack) 480 Wells



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## Intended Use

The Salimetrics® 17β-Estradiol Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the *in vitro* quantitative measurement of salivary Estradiol. Measurements obtained by this device may be used in the diagnosis and treatment of various hormonal sexual disorders. This test is not intended for assessing placental function in complicated pregnancy. Salivary Estradiol accurately reflects the amount of serum Estradiol in the circulation (1). Salimetrics has not validated this kit for use with serum/plasma samples. This kit is validated for measuring salivary Estradiol in females.

**This instruction booklet contains two assay protocols.** Use “Method A: High Sensitivity Salivary 17β-Estradiol Procedure” for expected values between 1 and 32 pg/mL. Use “Method B: Lower Range Salivary 17β-Estradiol Procedure” to report values between 0.5 and 32 pg/mL.

**Warning:** The drug fulvestrant (FASLODEX®) has been shown to cross react with antibodies used in Estradiol immunoassays and may cause falsely elevated Estradiol results. Due to the risk of this cross reactivity, the Salimetrics® High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay Kit should not be used for patients being treated with the drug fulvestrant (FASLODEX®). Fulvestrant (FASLODEX®) is used to treat a certain type of estrogen receptor positive breast cancer in postmenopausal women. Falsely elevated Estradiol results could lead to misinterpretation of the menopausal status of these women, resulting in the fulvestrant (FASLODEX®) treatment being incorrectly altered or discontinued.

***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.

## Introduction

Estradiol (17β-Estradiol, E2, 1,3,5(10)-estratriene-3, 17β-diol), a steroid hormone, is produced primarily by the ovarian follicles from testosterone (2,3). Estradiol is the most active naturally secreted estrogen (2). In men, Estradiol originates in the testes and from extraglandular conversion of androgens (2).

Circulating Estradiol levels are relatively high at birth in both males and females, but decrease postnatally (3). In prepubertal children and men, levels are non-cyclic and low. During puberty, there are gradual increases in Estradiol levels in both males and females. Interactions between luteinizing hormone (LH) and follicle-stimulating hormone (FSH) cause the release of Estradiol from the ovaries in premenopausal women. Estradiol secretion is low in postmenopausal women.



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Research concerning Estradiol has focused predominantly on reproductive issues such as conception, ovulation, infertility, and menopause (4,5,6). Yet, Estradiol affects a diversity of biological processes involved with reproductive capacity, (7) establishment and maintenance of pregnancy, (8) parenting, (9) coronary artery disease, (10) immunocompetence, (11) cancer susceptibility, (12) and neuroprotection (13). Estradiol is also believed to affect individual differences in cognitive and socioemotional processes as well as psychopathology (14,15).

Estrogens have been measured by many immunoassay methods. Studies suggest that Estradiol can be accurately measured in saliva (4,5,16,17).

## Test Principle

This is a competitive immunoassay kit. Estradiol in standards and samples compete with Estradiol conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Estradiol 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 Estradiol Enzyme Conjugate detected is inversely proportional to the amount of Estradiol present in the sample (18).



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## 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](mailto:support@salimetrics.com) (See [www.salimetrics.com](http://www.salimetrics.com) for alternative contact options).



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## 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.
- We recommend saving all reagents until data analysis has confirmed a successful run to facilitate troubleshooting if necessary.
- Prior to sample addition, please label each strip to assure plate orientation and sample order when data is acquired on plate reader.

## Storage

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

## pH Indicator

Estradiol values from samples with a pH  $\leq 5.0$  or  $\geq 9.0$  may be inaccurate. A pH indicator in the HS Estradiol Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the HS Estradiol Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH  $\leq 5.0$  or  $\geq 9.0$  should be recollected.



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## 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](http://www.salimetrics.com) or upon request.

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination (19,20) 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.

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 -20°C within 4 hours of collection. (Samples may be stored at -20°C 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. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.



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## Materials Supplied with Single Kit

	Item	Quantity/Size
1	<b>Microtitre Plate</b> Coated with rabbit anti-Estradiol antibodies.	1/96 well
2	<b>HS Estradiol Standard</b> 32 pg/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Further dilution of standard is necessary for Method B: Lower Range Salivary 17 $\beta$ -Estradiol Procedure Contains: Estradiol, buffer, preservative.	1 vial / 1.6 mL
3	<b>HS Estradiol Controls</b> High, Low, in a saliva-like matrix. Ready to use. Contain: Estradiol, buffer, preservative.	2 vials / 1 mL each
4	<b>Estradiol Enzyme Conjugate</b> Concentrate. Dilute before use with HS Estradiol Assay Diluent. (See step 5 of Procedure.) Contains: Estradiol conjugated to HRP, preservative.	1 vial / 50 $\mu$ L
5	<b>HS Estradiol Assay Diluent</b> Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 60 mL
6	<b>Wash Buffer Concentrate (10X)</b> Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
7	<b>TMB Substrate Solution</b> Non-toxic, ready to use.	1 bottle / 25 mL
8	<b>Stop Solution</b>	1 bottle / 12.5 mL
9	<b>Non-Specific Binding (NSB) Wells</b> Do not contain anti-Estradiol antibody. Break off and insert as blanks (optional) where needed.	1 strip
10	<b>Adhesive Plate Covers</b>	2





## Materials Needed But Not Supplied

- Precision pipette to deliver 15  $\mu$ L, 100  $\mu$ L, and 300  $\mu$ L
- Precision multichannel pipette to deliver 50  $\mu$ L, 100  $\mu$ L, and 200  $\mu$ L
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 620 to 630 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 12 mL
- Small disposable polypropylene tubes for dilution of standard (Five for Method A, Six for Method B)
- Pipette tips
- Serological pipette to deliver up to 12 mL
- 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 12 mL of HS Estradiol Assay Diluent used in Step 5 (conjugate dilution) 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.)



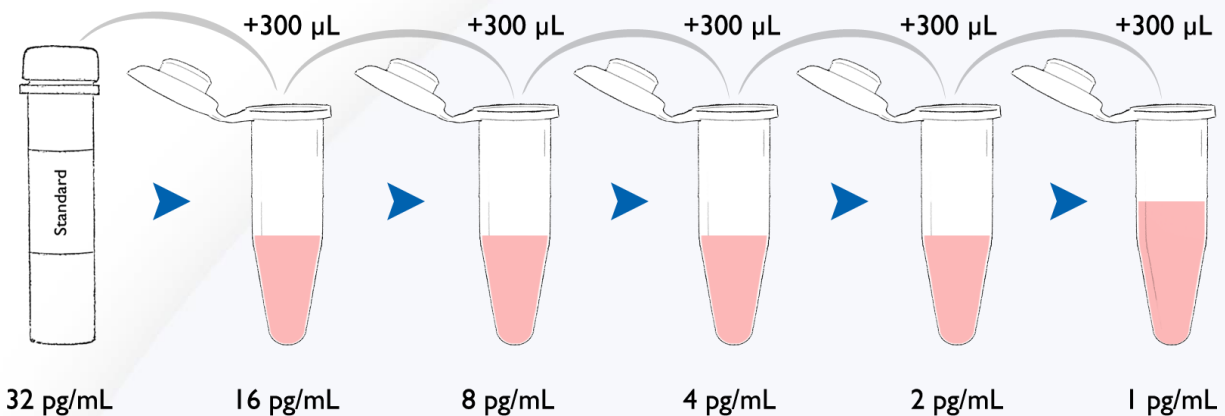
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## Method A: High Sensitivity Salivary 17 $\beta$ -Estradiol Procedure

(Expected values between 1 and 32 pg/mL)

(Proceed to Method B if expected values are between 0.5 and 32 pg/mL)

- Prepare serial dilutions of the HS Estradiol Standard as follows:
  - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
  - Pipette 300  $\mu$ L of HS Estradiol Assay Diluent into tubes 2 through 6.
  - Serially dilute the standard 2X by adding 300  $\mu$ L of the 32 pg/mL standard (tube 1) to tube 2. Mix well.
  - After changing pipette tips, remove 300  $\mu$ L 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, 32 pg/mL, 16 pg/mL, 8 pg/mL, 4 pg/mL, 2 pg/mL, and 1 pg/mL. Standard concentrations in pmol/L are 117, 58.5, 29, 14.6, 7.3, and 3.65 respectively.



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**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.)

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	32 Std	32 Std	Ctrl-H	Ctrl-H								
<b>B</b>	16 Std	16 Std	Ctrl-L	Ctrl-L								
<b>C</b>	8 Std	8 Std	SMP-1	SMP-1								
<b>D</b>	4 Std	4 Std	SMP-2	SMP-2								
<b>E</b>	2 Std	2 Std	SMP-3	SMP-3								
<b>F</b>	1 Std	1 Std	SMP-4	SMP-4								
<b>G</b>	Zero	Zero	SMP-5	SMP-5								
<b>H</b>	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:** Pipette 12 mL of HS Estradiol Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.

**Step 4:**

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



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**Step 5:** Dilute the Enzyme Conjugate 1:800 by adding 15  $\mu\text{L}$  of the conjugate to the 12 mL tube of HS Estradiol 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  $\mu\text{L}$  to each well using a multichannel pipette.

**Step 6:** Place adhesive cover provided over plate. Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 2 hours.

**Step 7:** 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  $\mu\text{L}$  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 8:** Add 200  $\mu\text{L}$  of TMB Substrate Solution to each well with a multichannel pipette.

**Step 9:** 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 10:** Add 50  $\mu\text{L}$  of Stop Solution with a multichannel pipette.

**Step 11:**

- 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 620 to 630 nm is recommended.)

## Quality Control

The Salimetrics' High and Low Estradiol 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.



## 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 Estradiol values greater than 32 pg/mL should be diluted with HS Estradiol 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

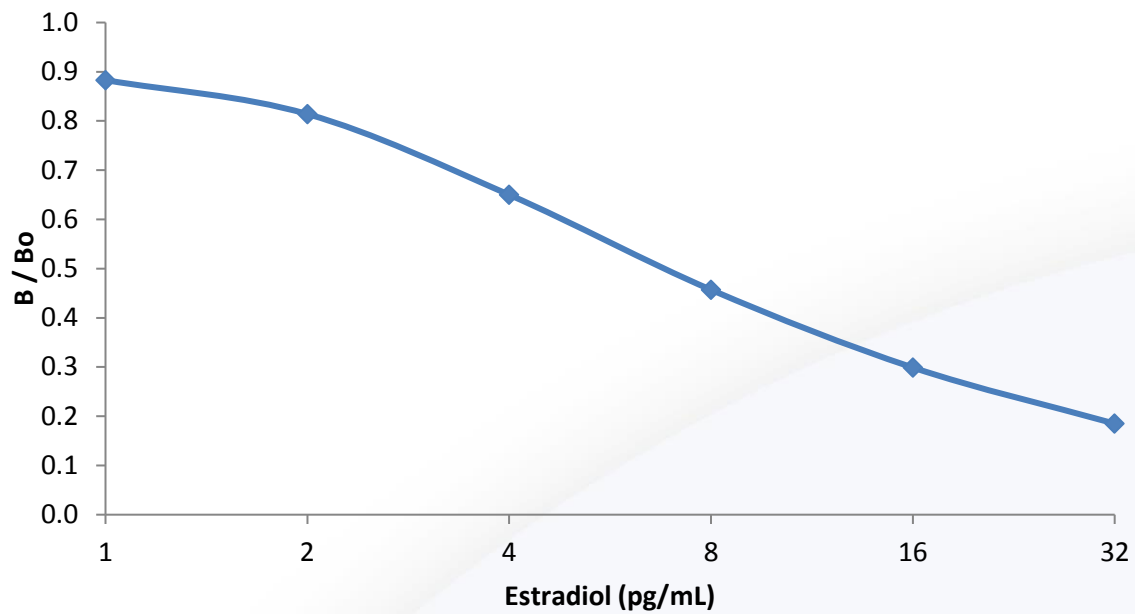
The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Estradiol (pg/mL)
A1,A2	S1	0.183	0.174	0.185	32
B1,B2	S2	0.290	0.280	0.299	16
C1,C2	S3	0.438	0.429	0.457	8
D1,D2	S4	0.619	0.609	0.650	4
E1,E2	S5	0.773	0.764	0.814	2
F1,F2	S6	0.837	0.828	0.883	1
G1,G2	Bo	0.947	0.937	NA	NA
H1,H2	NSB	0.009	NA	NA	NA



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## Example: HS 17 $\beta$ -Estradiol 4-Parameter Curve Fit

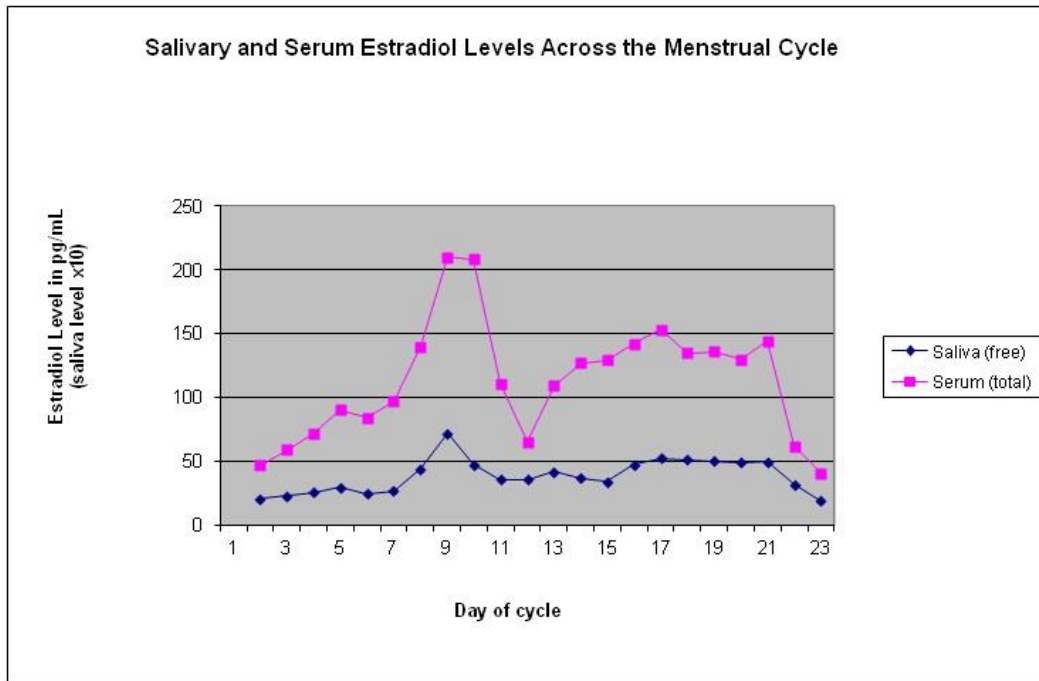


## Salivary Estradiol Example Ranges\*

Pre-menopausal Adult Women	N	Mean (pg/mL)	Standard Deviation (pg/mL)
Follicular	20	1.35	0.80
Mid-Cycle	20	2.97	1.58
Luteal	20	2.56	0.84

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

## Example of the Variation of Estradiol Levels during the Menstrual Cycle of One Woman



## HS Salivary 17 $\beta$ -Estradiol EIA Kit Performance Characteristics

### ***Precision***

The intra-assay precision was determined from the mean of 14 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
High	14	20.26	1.42	7.0
Mid	14	7.24	0.45	6.3
Low	14	3.81	0.31	8.1



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The inter-assay precision was determined from the mean of average duplicates for 10 separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	10	24.62	1.47	6.0
L	10	4.76	0.42	8.9

### ***Recovery***

Five saliva samples containing different levels of endogenous Estradiol were spiked with known quantities of Estradiol and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	2.92	20.48	23.40	23.84	101.9
2	4.68	13.65	18.33	17.91	97.7
3	3.80	3.20	7.00	6.78	96.9
4	5.41	20.48	25.89	28.2	108.9
5	3.69	3.20	6.89	8.26	120.0

### ***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 Estradiol that can be distinguished from 0 is 0.1 pg/mL.

### ***Correlation with Serum***

The correlation between serum and saliva Estradiol in females was determined by assaying 11 matched samples. Samples were screened for pH and blood contamination. The magnitude of the serum-saliva correlation,  $r(9) = 0.80$ ,  $p = <0.001$ , is consistent with the literature (5,17,21).





## ***Sample Dilution Recovery***

Four samples were serially diluted with HS Estradiol Assay Diluent and assayed.

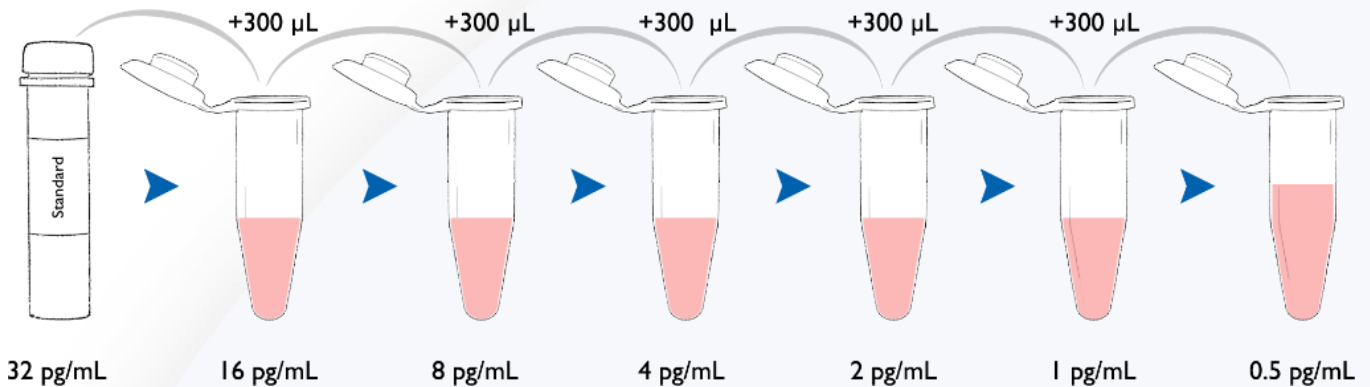
<b>Saliva Sample</b>	<b>Dilution Factor</b>	<b>Expected (pg/mL)</b>	<b>Observed (pg/mL)</b>	<b>Recovery (%)</b>
1			28.98	
	1:2	14.49	13.57	93.7
	1:4	7.25	7.24	99.9
	1:8	3.62	3.73	103.0
2			23.84	
	1:2	11.92	12.03	100.9
	1:4	5.96	5.56	93.3
	1:8	2.98	3.60	120.8
3			6.78	
	1:2	3.39	3.07	90.6
	1:4	1.70	1.70	100.0
4			8.54	
	1:2	4.27	4.55	106.6
	1:4	2.14	1.93	90.2



## Method B: Lower Range Salivary 17 $\beta$ -Estradiol Procedure

(Expected values between 0.5 and 32 pg/mL)

- Prepare serial dilutions of the HS Estradiol Standard as follows:
  - Label six polypropylene microcentrifuge tubes or other small tubes 2 through 7.
  - Pipette 300  $\mu$ L of HS Estradiol Assay Diluent into tubes 2 through 7.
  - Serially dilute the standard 2X by adding 300  $\mu$ L of the 32 pg/mL standard (tube 1) to tube 2. Mix well.
  - After changing pipette tips, remove 300  $\mu$ L from tube 2 to tube 3. Mix well.
  - Continue for tubes 4, 5, 6 and 7.
  - The final concentrations of standards for tubes 1 through 7 are, respectively, 32 pg/mL, 16 pg/mL, 8 pg/mL, 4 pg/mL, 2 pg/mL, 1 pg/mL and 0.5 pg/mL. Standard concentrations in pmol/L are 117, 58.5, 29, 14.6, 7.3, 3.65 and 1.83 respective.



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## Procedure

**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	32 Std	32 Std	Ctrl-H	Ctrl-H								
B	16 Std	16 Std	Ctrl-L	Ctrl-L								
C	8 Std	8 Std	SMP-1	SMP-1								
D	4 Std	4 Std	SMP-2	SMP-2								
E	2 Std	2 Std	SMP-3	SMP-3								
F	1 Std	1 Std	SMP-4	SMP-4								
G	0.5 Std	0.5 Std	SMP-5	SMP-5								
H	Zero	Zero	SMP-6	SMP-6								

Note: The lower range procedure does not incorporate NSB wells.

**Step 2:** Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. Reseal the zip-lock 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:** Pipette 12 mL of estradiol assay diluent into a disposable tube. (Scale down proportionally if not using the entire plate.) Set aside for Step 5.

### Step 4:

- Pipette 100 µL of standards, controls, and unknown samples into appropriate wells. Standards, controls, and unknown samples should be assayed in duplicate.
- Pipette 100 µL of Estradiol Assay Diluent into 2 wells to serve as the zero.



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**Step 5:** Dilute the Enzyme Conjugate 1:800 by adding 15  $\mu\text{L}$  of the conjugate to the 12 mL tube of HS Estradiol 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  $\mu\text{L}$  to each well using a multichannel pipette.

**Step 6:** Cover plate with adhesive cover provided. Mix plate on rotator continuously for 4 hours at 500 rpm at room temperature.

**Step 7:** 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  $\mu\text{L}$  of wash buffer into each well and then decanting the liquid into a sink. After each wash, blot plate on paper towels before turning upright. ***If using a plate washer, blotting is still recommended after the last wash.***

**Step 8:** Add 200  $\mu\text{L}$  of TMB solution to each well with a multichannel pipette

**Step 9:** Mix on a plate rotator for 5 minutes at 500 rpm and incubate the plate in the dark at room temperature for an additional 25 minutes.

**Step 10:** Add 50  $\mu\text{L}$  of Stop Solution with a multichannel pipette.

**Step 11:**

- Mix on a plate rotator for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to 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. (Correction at 630 nm is desirable.)

## Quality Control

The Salimetrics' High and Low Estradiol 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.



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## Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. 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.)
3. 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.
4. Samples with Estradiol values greater than 32 pg/mL should be diluted with HS Estradiol 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

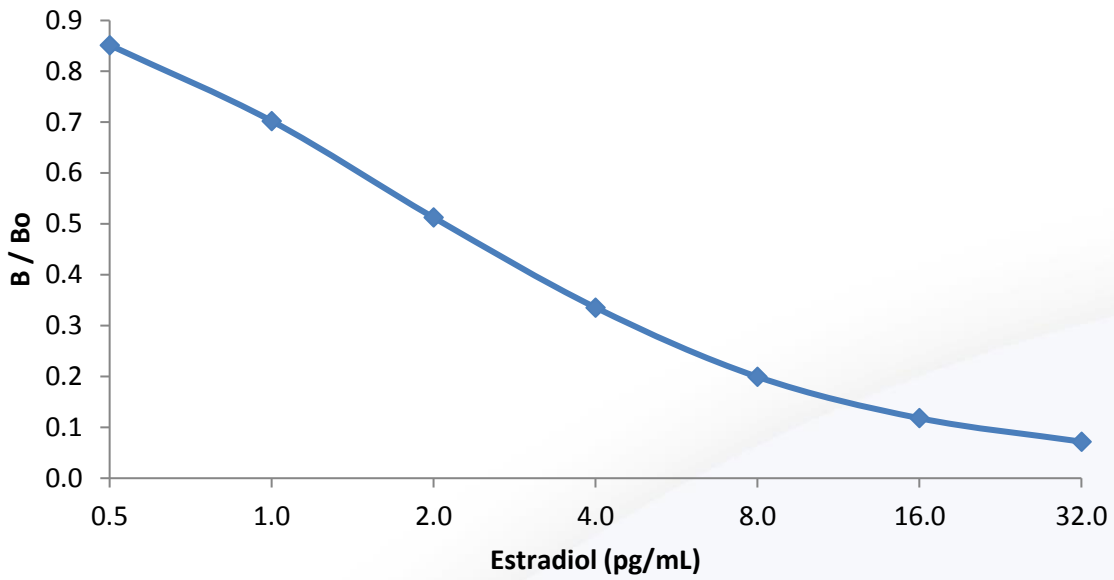
The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	B	B/Bo	Estradiol (pg/mL)
A1,A2	S1	0.110	0.160	0.072	32
B1,B2	S2	0.180	0.174	0.118	16
C1,C2	S3	0.309	0.294	0.199	8
D1,D2	S4	0.509	0.495	0.336	4
E1,E2	S5	0.785	0.756	0.513	2
F1,F2	S6	1.052	1.036	0.702	1
G1,G2	S7	1.261	1.256	0.852	0.5
H1,H2	Bo	1.489	1.475	N/A	NA



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## Example: HS 17β-Estradiol 4-Parameter Curve Fit



## HS Salivary 17β-Estradiol EIA Kit Performance Characteristics

### ***Precision***

The intra-assay precision was determined from the mean of 5 saliva samples and assayed.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
L1	20	1.71	0.07	4.3
L2	20	3.31	0.16	4.9
L3	20	5.01	0.24	4.8
L4	20	11.00	0.76	6.9
L5	20	12.27	0.95	7.8



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The plate to plate variability was determined from five saliva samples at various concentrations using the mean of four plates.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
L1	18	1.92	0.08	4.3
L2	18	3.64	0.20	5.4
L3	18	6.19	0.33	5.3
L4	18	10.50	0.81	7.8
L5	18	14.07	1.12	8.0

### ***Sensitivity***

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 16 sets of duplicates at the 0 pg/mL level, using the mean of two runs. The minimal concentration of Estradiol that can be distinguished from 0 is 0.086 pg/mL.

### **Limitations (Methods A & B)**

- Samples with Estradiol values greater than 32 pg/mL should be diluted with HS Estradiol Assay Diluent and rerun for accurate results. To obtain the final Estradiol concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values  $\leq 5.0$  or  $\geq 9.0$  should be recollected.
- 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 Estradiol levels should be followed by additional testing and evaluation.
- Ranges are established for females only.



## Antibody Specificity (Methods A & B)

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS 17 $\beta$ -Salivary Estradiol EIA
Estriol	10	0.234
Estrone	1	1.276
Progesterone	100	ND
17 $\alpha$ -Hydroxyprogesterone	1000	ND
Testosterone	1000	ND
Cortisol	1000	ND
DHEA	1000	ND
Androstenedione	1000	ND
Aldosterone	1000	ND
Cortisone	1000	ND
11-Deoxycortisol	1000	ND
21-Deoxycortisol	1000	ND
Dexamethasone	1000	ND
Triamcinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	100	0.016
Transferrin	1000	ND
Ethinodiol diacetate	1000	ND
Ethinylestradiol	10	0.189

ND = None detected (<0.004)



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**Updated: March 2, 2018**



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