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High Sensitivity
SALIVARY 17β-ESTRADIOL ENZYME IMMUNOASSAY KIT

Item No. 1-3702, (Single) 96-Well Kit;
1-3702-5, (5-Pack) 480 Wells

For Research Use Only

Intended Use

The Salimetrics™ estradiol kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary estradiol. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. Please read the complete kit insert before performing this assay. 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, E_2, 1,3,5(10)-estratriene-3, 17β-diol), a steroid hormone, is produced primarily by the ovarian follicles from testosterone (1,2). Estradiol is the most active naturally secreted estrogen (1). In men, estradiol originates in the testes and from extraglandular conversion of androgens (1).

Circulating estradiol levels are relatively high at birth in both males and females, but decrease postnatally (2). 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.

Research concerning estradiol has focused predominantly on reproductive issues such as conception, ovulation, infertility, and menopause (3,4,5). Yet, estradiol affects a diversity of biological processes involved with pubertal and reproductive capacity, establishment and maintenance of pregnancy, infant care, coronary artery disease, immunocompetence, and cancer susceptibility (6,7,8). Estradiol is also believed to affect individual differences in cognitive and socioemotional processes as well as psychopathology (9,10).

Estrogens have been measured by many immunoassay methods. Studies suggest that estradiol can be accurately measured in saliva (3,4,11,12).

Test Principle

A microtitre plate is coated with rabbit antibodies to estradiol. Estradiol in standards and unknowns competes with estradiol linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound estradiol peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of estradiol peroxidase detected is inversely proportional to the amount of estradiol present (13).

pH Indicator

A pH indicator in the estradiol assay diluent alerts the user to samples with high or low pH values. Acidic samples will turn the diluent yellow. Alkaline samples will turn the diluent purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Estradiol values from samples with a pH ≤ 5 or ≥ 9 may be artificially inflated or lowered. Samples with a pH ≤ 5 or ≥ 9 should be recollected.

Precautions

1. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in false values.
2. Liquid stop solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
3. This kit uses break-apart microtitre strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch with desiccant and used in the frame provided.
4. Do not mix components from different lots of kits.
5. When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
6. See 'Material Safety Data'at the end of procedure.
7. We recommend that samples be screened for possible blood contamination (14,15) using a reliable screening tool such as the Salimetrics Blood Contamination EIA Kit (Item No.: 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.
8. Routine calibration of pipettes is critical for the best possible assay performance.
9. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.
10. The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68 - 74ºF (20 - 23.3ºC). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
11. The quantity of reagent provided with this kit is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
12. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.

Storage

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

Reagents and Reagent Preparation

1. **Anti-Estradiol Coated Plate:** A ready-to-use, 96-well microtitre plate pre-coated with rabbit anti-estradiol antibodies in a resealable foil pouch.
2. **Estradiol Standard:** 1.6 mL of estradiol in a saliva-like matrix with a non-mercury preservative, at a concentration of 32 pg/mL.
3. **Estradiol Controls:** Two controls representing high and low levels of estradiol in a saliva-like matrix with a non-mercury preservative. Each vial contains 1 mL.
4. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute only the amount needed for current day's use. Discard any leftover reagent. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H_20). ***(Note**: If precipitate has formed in the concentrated wash buffer, heat to 40°C for 15 minutes to dissolve crystals. Cool to room temperature before use in assay.)*
5. **Estradiol Assay Diluent:** 60 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
6. **Enzyme Conjugate:** 50 μL of a solution of estradiol labeled with horseradish peroxidase. Dilute prior to use with estradiol assay diluent.
7. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic, ready-to-use solution.
8. **Stop Solution:** 12.5 mL of a solution of sulfuric acid.
9. **Non-specific Binding Wells (NSB):** One strip of wells that do not contain anti-estradiol antibody. They are located in the foil pouch. Wells may be broken off and inserted as blanks (optional) where needed.

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Materials Needed But Not Supplied

- Precision pipette to deliver 15 µL, 100 µL, and 300 µL
- Precision multichannel pipette to deliver 50 µL, 100 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17" orbit
- Plate reader with a 450 nm filter
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One 15 mL disposable tube
- Small disposable tubes for dilution of standard, controls, and samples
- Pipette tips
- Serological pipette to deliver 12 mL

Specimen Collection

Due to the episodic secretion pattern of steroid hormones, we can expect reproducible and reliable results only in cases of multiple sampling. Therefore, we recommend taking a minimum of 3 samples within at least a 2-hour period and pooling the samples before testing . Equal volumes from each of the samples should be pooled to create one sample that physically averages the fluctuations over that time period (16,17).

The preferred method of collecting whole saliva is by unstimulated passive drool. Collection protocols are available on request. **Do not use Salivettes, the Salimetrics Oral Swab (SOS), Sorbettes, cotton, or polyester materials to collect samples**. False readings will result (11). Do **not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected. 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. Record the time and date of specimen collection. 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 or lower for long term storage.)

Freezing saliva samples will precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. It is important to avoid additional freeze-thaw cycles. However, if samples have been refrozen, centrifuge again prior to assaying. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Particulate matter may interfere with antibody binding, leading to falsely elevated results.

Procedure

Bring all reagents to room temperature. A minimum of 1.5 hours is necessary for the 12 mL of estradiol assay diluent used in Step 5 (conjugate dilution) to come to room temperature. **Note**: *It is important to keep the zip-lock pouch with the plate strips closed until warmed to room temperature as humidity may have an effect on the coated wells. Mix all reagents before use.*

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	32 Std	32 Std	C-H	C-H								
B	16 Std	16 Std	C-L	C-L								
C	8 Std	8 Std	Unk 1	Unk 1								
D	4 Std	4 Std	Unk 2	Unk 2								
E	2 Std	2 Std	Unk 3	Unk 3								
F	1 Std	1 Std	Unk 4	Unk 4								
G	Zero	Zero	Unk 5	Unk 5								
H	NSB	NSB	Unk 6	Unk 6								

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. Break off the bottom wells in each strip. Place the strips back into the strip holder leaving H-1, 2 blank. Break off 2 NSB wells from the strip of NSBs included in the foil pouch. Place in H-1, 2. Alternatively, NSBs may be placed wherever you choose on the plate. Reseal the zip-lock foil pouch with unused wells and desiccant. Store at 2 - 8°C.

<u>Cautions</u>: *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:

- Label five microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 300 µL of estradiol assay diluent into tubes 2 through 6.

  Serially dilute the standard 2X by adding 300 µL of the 32 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 300 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 32 pg/mL, 16 pg/mL, 8 pg/mL, 4 pg/mL, 2 pg/mL, and 1 pg/mL, respectively. Standard concentrations in pmol/L are 117, 58.5, 29, 14.6, 7.3 and 3.65, respectively.
- 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.
- Pipette 100 µL of estradiol assay diluent into each NSB well.

  Note: *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.*

Step 5: Dilute the enzyme conjugate 1: 800 by adding 15 µL of the conjugate to the 12 mL of estradiol assay diluent prepared in Step 3. (Scale down proportionally if not using the entire plate.) Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 6: Cover plate with adhesive cover provided. Mix plate on rotator for 5 minutes at 500 rpm (or tap to mix) and incubate at room temperature for an additional 115 minutes.

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 µL 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 µL of TMB solution to each well with a multichannel pipette.

Step 9: Mix on a plate rotator for 5 minutes at 500 rpm (or tap to mix) and incubate the plate in the dark at room temperature for an additional 25 minutes.

Step 10: Add 50 µL 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:** *<u>Do not</u> mix at speeds over 600 rpm. Spillage may occur.*
- 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 is desirable.)

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 average OD of the zero, standards, controls, and unknowns.
3. Calculate the percent bound (B/Bo) for each standard, control, and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the controls and unknowns by interpolation using software capable of logistics. We recommend using a 4-parameter sigmoid minus curve fit.

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5. If a dilution of the sample is used, multiply the results by the dilution factor.

Quality Control

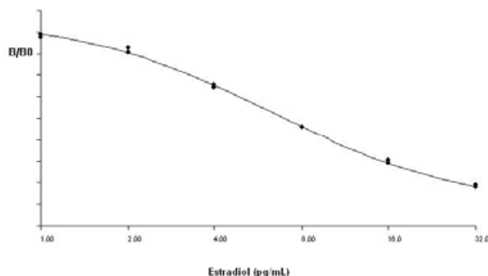
The Salimetrics' high and low salivary estradiol controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

The following results are shown 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

Example: HS Estradiol 4-Parameter Sigmoid Minus Curve Fit



Material Safety Data*

Hazardous Ingredients
Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. Specific kit component MSDS sheets are available from Salimetrics upon request.

Handling
Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard 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, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

Performance Characteristics

A. 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.

B. Precision:

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

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

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

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
High	10	24.62	1.47	6.0
Low	10	4.76	0.42	8.9

C. Linearity of Dilution:

Four saliva samples were diluted with estradiol assay diluent and assayed.

Sample	Dilution	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
I			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
II			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
III			6.78	
	1:2	3.39	3.07	90.6
	1:4	1.70	1.70	100.0
IV			8.54	
	1:2	4.27	4.55	106.6
	1:4	2.14	1.93	90.2

D. Specificity of Antiserum

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in HS Salivary Estradiol EIA
Estriol	10	0.234
Estrone	1	1.276
Progesterone	100	ND
17 α-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
Triamincinolone	1000	ND
Corticosterone	1000	ND
Prednisolone	1000	ND
Prednisone	100	0.016
Transferrin	1000	ND
Ethynodiol diacetate	1000	ND
Ethynylestradiol	10	0.189

ND = None detected (<0.004)

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E. Recovery:

Five saliva samples was spiked with different levels of estradiol and assayed.

Sample	Endogenous (pg/ml)	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
I	2.92	20.48	23.40	23.84	101.9
II	4.68	13.65	18.33	17.91	97.7
III	3.80	3.20	7.00	6.78	96.9
IV	5.41	20.48	25.89	28.2	108.9
V	3.69	3.20	7.16	8.26	115.4

F. Correlation With Serum:

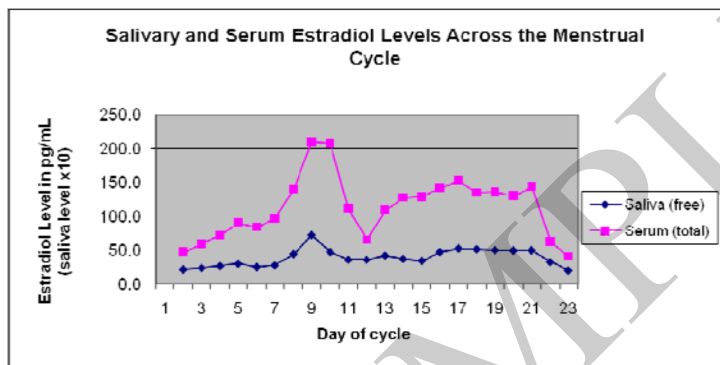
The correlation between saliva and serum estradiol in females was determined by assaying 11 matched samples. Samples were screened for pH and blood contamination. The magnitude of the saliva-serum correlation, $r(9)= 0.80$, $p = <0.001$, is consistent with the literature (4, 12, 18).

***Salivary Estradiol Expected 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:



Citations

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Seller's Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."

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