

SALIVARY ESTRONE ENZYME IMMUNOASSAY KIT

Catalog No. 1-3202, (Single) 96-Well Kit,
1-3202-5, (5-Pack) 480 Wells

For Research Use Only

Intended Use

The Salimetrics™ estrone kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary estrone. 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

Estrone [3-hydroxy-1,3,5(10)-estratrien-17-one: E1] is a naturally occurring steroidal hormone. A major portion of estrone is produced from androstenedione in prepubertal children, men, and postmenopausal women. (1,2) Circulating estrone levels are relatively high at birth in both males and females, decrease postnatally, and increase during puberty. (3) Of the three major estrogens, estrone is predominant after menopause in women. Estrone is primarily secreted by the ovaries in premenopausal women, peaking in the preovulatory phase with a smaller secondary increase during the luteal phase. (2,3)

Estrone is a primary component of many pharmaceutical preparations. Research concerning estrone is often focused on pregnancy, reproduction, and menopause. However, estrogens affect a diverse group of biological processes such as arterial vasodilation, bone density, cognitive function, and neuroprotection. (4-7) Estrogens are also studied in regard to coronary artery disease, immunocompetence, cancer susceptibility and polycystic ovarian syndrome. (8-12)

Test Principle

A microtitre plate is coated with sheep antibodies to estrone. Estrone in standards and unknowns competes with estrone linked to horseradish peroxidase for the antibody binding sites. After incubation, unbound components are washed away. Bound estrone 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 estrone peroxidase detected is inversely proportional to the amount of estrone present. (13)

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.: 1-1302/1-1312). 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. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
10. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.
11. 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 will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
12. 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.
13. 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-Estrone Coated Plate:** A ready-to-use, 96-well microtitre plate pre-coated with sheep anti-estrone antibodies in a resealable foil pouch.
2. **Estrone Standard:** 1.6 mL of estrone in a saliva-like matrix with a non-mercury preservative, at a concentration of 300 pg/mL.
3. **Estrone Controls:** Two controls representing high and low levels of estrone 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 H₂O). (*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. **Estrone Assay Diluent:** 60 mL of a phosphate buffered solution containing a non-mercury preservative.
6. **Enzyme Conjugate:** 100 µL of a solution of estrone labeled with horseradish peroxidase. Dilute prior to use with estrone assay diluent.
7. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic, ready-to-use solution.
8. **Stop Solution:** 12.5 mL of a 2-molar solution of sulfuric acid.
9. **Non-specific Binding Wells (NSB):** These wells do not contain anti-estrone antibody. In order to support multiple use, a strip of NSB wells is included. They are located in the foil pouch. Wells may be broken off and inserted as blanks (optional) where needed.

Materials Needed But Not Supplied

- Precision pipette to deliver 75 µL, 100 µL, 200 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. (16,17)

The preferred method for 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 (18). 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 -20°C within 4 hours of collection. (Samples may be stored at -20°C or lower for long term storage.)

Freezing saliva samples will precipitate mucins. On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding, leading to falsely elevated 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. Centrifuge/re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Procedure

Bring all reagents 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	300 Std	300 Std	C-H	C-H								
B	120 Std	120 Std	C-L	C-L								
C	48 Std	48 Std	Unk 1	Unk 1								
D	19.2 Std	19.2 Std	Unk 2	Unk 2								
E	7.7 Std	7.7 Std	Unk 3	Unk 3								
F	3.1 Std	3.1 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.

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:

- Label five microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 300 µL of estrone assay diluent into tubes 2 through 6. Serially dilute the standard 2.5X by adding 200 µL of the 300 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 200 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, respectively, are 300 pg/mL, 120 pg/mL, 48 pg/mL, 19.2 pg/mL, 7.7 pg/mL, and 3.1 pg/mL. Standard concentrations in pmol/L are 1109.47, 443.79, 177.51, 71.01, 28.48, and 11.46 pmol/L, respectively.
- Pipette 12 mL of estrone assay diluent into a disposable tube. (Scale down proportionally if not using the full plate.) Set aside for Step 5.

Step 4:

- Pipette 100 µL of standards, controls, and unknown samples into appropriate wells. Samples do not need to be diluted. Standards, controls, and unknown samples should be assayed in duplicate.
- Pipette 100 µL of estrone assay diluent into 2 wells to serve as the zero.
- Pipette 100 µL of estrone assay diluent into each NSB well.

Step 5: Dilute the enzyme conjugate 1:160 by adding 75 µL of the conjugate to the 12 mL of estrone assay diluent prepared in Step 3. (Scale down proportionally if not using the full 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. Incubate at room temperature for 3 hours mixing constantly at 500 rpm.

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 and incubate 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:** Do not mix at speeds over 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 is desirable.)

Calculations

- Compute the average optical density (OD) for all duplicate wells.
- Subtract the average OD for the NSB wells (if used) from the average OD of the zero, standards, controls, and unknowns.
- 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).
- 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.
- If a dilution of the sample is used, multiply the assay results by the dilution factor.

Quality Control

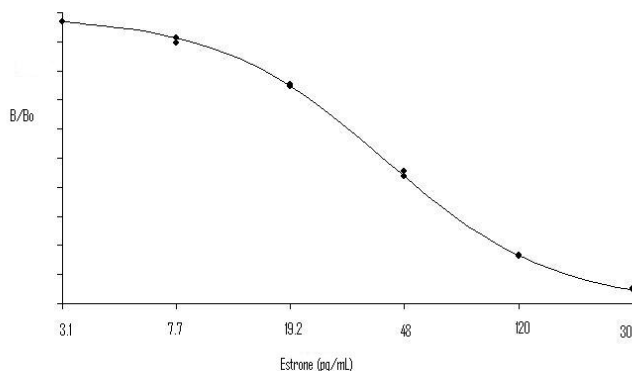
The Salimetrics' high and low salivary estrone controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

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	Estrone (pg/mL)
A1,A2	S1	0.057	0.047	0.025	300
B1,B2	S2	0.195	0.185	0.099	120
C1,C2	S3	0.560	0.550	0.295	48
D1,D2	S4	1.141	1.131	0.606	19.2
E1,E2	S5	1.499	1.489	0.798	7.7
F1,F2	S6	1.725	1.715	0.920	3.1
G1,G2	Bo	1.875	1.865	NA	NA
H1,H2	NSB	0.010	NA	NA	NA

Example: Estrone 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. Recovery:

Two saliva samples were spiked with 3 different levels of estrone and assayed.

Sample	Endogenous (pg/ml)	Added (pg/ml)	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
I	28.42	210	238.42	263.38	110.5
	27.50	30	57.5	58.37	101.5
	27.50	4.8	32.3	28.06	86.9
II	23.16	210	233.16	261.41	112.1
	22.41	30	52.41	56.53	107.9
	22.41	4.8	27.21	26.89	98.8

B. Precision:

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

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
High	12	172.76	3.77	2.2
Low	12	25.12	1.41	5.6

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

Sample	N	Mean (pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
High	12	178.38	3.46	1.9
Low	12	16.72	1.77	10.6

C. Linearity of Dilution:

Three saliva samples were diluted with estrone assay diluent and assayed.

Sample	Dilution Factor	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
I			260.04	
	1:2	130.02	123.03	94.6
	1:4	65.01	59.40	91.4
	1:8	32.51	28.91	88.9
	1:16	16.25	16.93	104.2
	1:32	8.13	6.63	81.5
II			299.98	
	1:2	150.00	140.65	93.8
	1:4	75.00	67.73	90.3
	1:8	37.5	35.21	93.9
	1:16	18.75	17.45	93.1
	1:32	9.38	9.59	102.2
III			258.24	
	1:2	129.12	115.66	89.6
	1:4	64.56	56.46	87.5
	1:8	32.28	29.20	90.5
	1:16	16.14	15.32	94.9
	1:32	8.07	7.50	92.9

D. 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 estrone that can be distinguished from 0 is < 1 pg/mL.

E. Specificity of Antiserum

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Estrone EIA
Estrone-sulfate	0.200	35.5
Estradiol	50	0.145
Estriol	10	ND
Progesterone	100	0.008
17 α -Hydroxyprogesterone	1000	ND
Testosterone	100	0.020
Cortisol	1000	ND
DHEA	1000	ND
Androstenedione	1000	0.0045
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	1000	ND
Transferrin	6600	ND

ND = None detected (<0.004)

*Salivary Estrone Example Ranges:

Pre-menopausal Adult Women	N	Mean (pg/mL)	Standard Deviation (pg/mL)
Follicular	21	14.14	11.28
Mid-cycle	23	11.92	7.36
Luteal	21	13.06	7.92

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

Citations

- Baird, D.T., Horton, R., Longcope, C., & Tait, J.F. (1969). Steroid dynamics under steady state conditions. *Recent Prog Horm Res*, 25, 611-64.
- Cañez, M.S., Lee, K.J., & Olive, D.L. (1992). Progestogens and estrogens. *Infert Reprod Med Clin North Amer*, 3, 59-78.
- Faiman, C., Winter, J.S.D., & Reyes, F.I. (1976). Patterns of gonadotrophins and gonadal steroids throughout life. *Clin Obst Gynecol*, 3(3), 467-83.
- Kim, H.P., Lee, J.Y., Jeong, J.K., Bae, S.W., Lee, H.K., & Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor α localized in caveolae. *Biochem Biophys Res Commun*, 263(1), 257-62.
- White, R.E., Darkow, D.J., & Lang, J.L.F. (1995). Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. *Circ Res*, 77(5), 936-42.
- Green, P.S., Bishop, J., and Simpkins, J.W. (1997). 17 α -estradiol exerts neuroprotective effects on SK-N-SH cells. *J Neurosci*, 17(2), 511-15.
- Garcia-Segura, L., Naftolin, F., Hutchison, J.B., Ascotia, I., & Chown, J.A. (1999). Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. *J Neurobiol*, 40(4), 574-84.
- Mendelsohn, M.E., & Karas, R.H. (1999). The protective effects of estrogen on the cardiovascular system. *N Eng J Med*, 340(23), 1801-11.
- Otsuki, M., Dahlman-Wright, K., & Gustafsson, J.-Å. (2003). Cardiovascular roles of estrogen receptors: Insights gained from knockout models. *Nucl Recept Signal*, 1, e003.
- Silva, I., Mor, G., & Naftolin, F. (2001). Estrogen and the aging brain. *Maturitas*, 38(1), 95-100.
- Lipworth, L., Adami, H.O., Trichopoulos, D., Carlström, K., & Mantzoros, C. (1996). Serum steroid hormone levels, sex hormone-binding globulin, and body mass index in etiology of postmenopausal breast cancer. *Epidemiology*, 7(1), 96-100.
- Deligdisch, L., & Holinka, C.F. (1987). Endometrial carcinoma: Two diseases? *Cancer Detect Prev*, 10(3-4), 237-46.
- Chard, T. (1990). *An introduction to radioimmunoassay and related techniques* (4th ed.). Amsterdam: Elsevier.

14. Kivlighan, K. T., Granger, D.A., Schwartz, E.B., Nelson, V., Curran, M. & Shirtcliff, E.A. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Horm Behav.* 46(1), 39-46.
15. Schwartz, E.B. & Granger, D.A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin Chem.* 50(3), 654-56.
16. West, C.D., Mahajan, D.K., Chavre, V.J., Nabors, C.J. (1973). Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. *J Clin Endocrinol Metab.* 36(6), 1230-36.
17. Brambilla, D.J., O'Donnell, A.B., Matsumoto, A.M., & McKinlay, J.B. (2007). Intraindividual variation in levels of serum testosterone and other reproductive and adrenal hormones in men. *Clin Endocrinol (Oxf)*, 67(6), 853-62.
18. Shirtcliff, E.A., Granger, D.A., Schwartz, E., & Curran, M.J. (2001). Use of salivary biomarkers in biobehavioral research: Cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology*, 26(2), 165-73.

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”