



SALIVARY PROGESTERONE ENZYME IMMUNOASSAY KIT

Catalog No. 1-2502/1-2512, 96-Well Kit

Intended Use

Salimetrics' progesterone kit is a competitive enzyme immunoassay specifically designed for the quantitative *in vitro* diagnostic measurement of progesterone in saliva. This kit may be used to diagnose and treat disorders of the ovaries and as an aid to confirm that ovulation has taken place. Saliva progesterone accurately reflects the amount of serum progesterone in the circulation. This kit is not intended for use with serum/plasma. 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.

Summary and Explanation of the Test

Progesterone (4-pregnene-3,20-dione) is a steroid of primary importance in ovulation, fertility, menopause, and the regulation of pituitary gonadotropins. Synthesis of progesterone takes place in the placenta, adrenal glands, and gonads. During the luteal phase and late follicular phase, high progesterone levels are associated with endometriosis (1). Low levels have been associated with premenstrual syndrome (2) and strenuous physical exercise (3).

In the blood only 1 to 15% of progesterone is in its unbound or biologically active form. The remaining progesterone is bound to serum proteins. The majority of progesterone in saliva is not protein-bound and enters the saliva via intracellular mechanisms. Salivary progesterone levels are unaffected by salivary flow rate or salivary enzymes (4).

This kit is designed to measure progesterone levels in saliva. The standard is in a saliva-like matrix. In addition, a built-in pH indicator warns the user of acidic or basic samples.

Test Principle

A microtitre plate is coated with rabbit antibodies to progesterone. Progesterone in standards and unknowns competes with progesterone linked to horseradish peroxidase for the antibody binding sites. After incubation unbound components are washed away. Bound progesterone 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 progesterone peroxidase detected is inversely proportional to the amount of progesterone present (5).

pH Indicator

A pH indicator in the 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. Progesterone values from samples with a pH ≤ 4.0 or ≥ 9.0 may be artificially inflated or lowered (6).

Precautions

1. Stop Solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
2. This kit uses break-apart microtitre strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.
3. Do not mix components from different lots of kits.
4. 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.
5. See 'Material Safety Data' at the end of procedure.
6. We recommend that samples be screened for possible blood contamination (7,8) using a reliable screening tool such as the Salimetrics Blood Contamination EIA Kit (Cat. No.: 1-1302/1-1312). Do not use dipsticks, which result in false positive values due to salivary enzymes.
7. Routine calibration of pipettes is critical for the best possible assay performance.
8. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
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 - 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.
11. Use of progesterone enhanced cremes or supplements by the laboratory technician performing the analysis can adversely affect results.
12. Ranges for patients using cremes or supplements should be determined by the individual laboratory.

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

Reagents and Reagent Preparation

1. **Anti-Progesterone Coated Plate:** A ready-to-use microtitre plate pre-coated with rabbit anti-progesterone antibodies in a resealable foil pouch.
2. **Progesterone Standard:** One mL of progesterone in a saliva-like matrix with a non-mercury preservative, at a concentration of 2430 pg/mL.
3. **Progesterone Controls:** Two controls representing high and low levels of progesterone in a saliva-like matrix with a non-mercury preservative. Each vial contains 0.5 mL. See vials for ranges.
4. **Wash Buffer:** 100 mL of a 10X solution of phosphate buffer 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, it may be heated up to 60 °C for 15 minutes. Cool to room temperature before use in assay.*)
5. **Assay Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
6. **Enzyme Conjugate:** 50 μ L of a solution of progesterone labeled with horseradish peroxidase. Dilute prior to use with 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 (USA customers only). Stop solution is provided in powdered form to customers outside the USA. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before use.
9. **Non-specific Binding Wells:** These wells do not contain anti-progesterone 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 where needed.

Note: *The quantity of reagent provided with break-apart kits 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.*

Materials Needed But Not Supplied

- Precision pipette to deliver 22.5 μ L, 50 μ L, 100 μ L, and 200 μ L
- Precision multichannel pipette to deliver 50 μ L, 150 μ L, and 200 μ L
- Vortex
- Plate rotator
- Plate reader with a 450 nm filter
- Log-linear graph paper or computer software for data reduction
- Deionized water
- Reagent reservoirs
- One 18 mL disposable polypropylene tube
- Small disposable polypropylene tubes
- Pipette tips and a serological pipette

Specimen Collection

Due to the episodic secretion pattern of steroid hormones, we can only expect reproducible and reliable results in cases of multiple sampling. Therefore, we recommend taking 5 samples within at least a 2-hour period and pooling the samples before testing (9).

Collecting saliva by unstimulated passive drool is the preferred saliva collection method (6,10). **Do not use Salivettes, Sorbettes or cotton ropes to collect samples (10).** False high readings will result. 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. **Do not** add sodium azide to saliva samples as a preservative. Samples visibly contaminated with blood should be recollected. After collection it is important to keep samples cold. Refrigerate sample within 30 minutes, and freeze at or below -20°C within 4 hours of collection. Record the time and date of specimen collection. Keep saliva samples frozen until day of assay.

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-thaws cycles. Sample 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.

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

	1	2	3	4	5	6	7	8	9	10	11	12
A	2430 std	2430 std	Ctrl H	Ctrl H								
B	810 std	810 std	Ctrl L	Ctrl L								
C	270 std	270 std	Unk-1	Unk-1								
D	90 std	90 std	Unk-2	Unk-2								
E	30 std	30 std	Unk-3	Unk-3								
F	10 std	10 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 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 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 and refrigerate the pouch at 2 - 8°C.

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

Step 3:

- Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
- Pipette 200 µL of assay diluent in tubes 2 through 6.
- Serially dilute the standard 3X by adding 100 µL of the 2430 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 100 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 2430 pg/mL, 810 pg/mL, 270 pg/mL, 90 pg/mL, 30 pg/mL, and 10 pg/mL (values in nmol/L are 7.733, 2.576, 0.859, 0.286, 0.095 and 0.032 nmol/L respectively).
- Pipette 18 mL of assay diluent into the disposable tube. Set aside for Step 5.

Step 4:

- Pipette 50 µL of standards, controls and unknowns into appropriate wells. Standards, controls and unknowns should be assayed in duplicate.
- Pipette 50 µL of assay diluent into two wells to serve as the zero.
- Pipette 50 µL of assay diluent into each NSB well.

Step 5: Dilute the enzyme conjugate 1:800 by adding 22.5 µL of the conjugate to the 18 mL of assay diluent prepared in Step 3. If using 6 or fewer strips, 12.5 µL of conjugate to 10 mL of assay diluent may be used. Immediately mix the diluted conjugate solution and add 150 µL to each well using a multichannel pipette.

Step 6: Cover plate with adhesive cover provided. Rotate the plate *continuously* at 500 rpm for 1 hr at room temperature.

Step 7: Wash the plate four 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 flipping the liquid into 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 µL of TMB solution to each well with a multichannel pipette.

Step 9: Mix on a plate rotator for five minutes at 500 rpm 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 three minutes at 500 rpm. 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 500 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 492 to 620 is desirable).

Calculations

- Compute the average optical density (OD) for all duplicate wells.
- Subtract the average OD for the NSB wells 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.

Limitations

- Samples with progesterone values greater than 2430 pg/mL should be further diluted with assay diluent and rerun for accurate results. To obtain the final progesterone 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 assay diluent is added and the plate is mixed. Samples with pH values ≤ 4.0 or ≥ 9.0 should be recollected.
- See "Specimen Collection" recommendations to insure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.

Quality Control

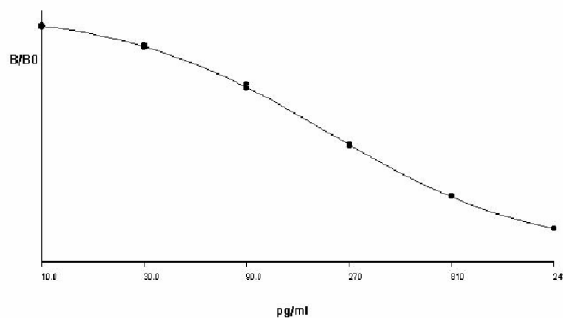
The Salimetrics' high and low salivary progesterone controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

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

Well	Sample	Average OD	B	B/Bo	Progesterone pg/mL
A1,A2	S1	0.155	0.152	0.134	2430
B1,B2	S2	0.303	0.300	0.265	810
C1,C2	S3	0.534	0.531	0.469	270
D1,D2	S4	0.800	0.797	0.704	90
E1,E2	S5	0.983	0.980	0.866	30
F1,F2	S6	1.075	1.072	0.950	10
G1,G2	Bo	1.135	1.132	NA	
H1,H2	NSB	0.003	NA	NA	

Example: Progesterone 4-Parameter Sigmoid Minus Curve Fit



Material Safety Data*

Hazardous Ingredients

Stop Solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.

Progesterone may cause cancer. Avoid inhalation, ingestion, and contact with skin. We recommend the procedures listed below for all kit reagents.

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 be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from contact with reagents.

Performance Characteristics

A. Recovery:

Six saliva samples containing different levels of endogenous progesterone were spiked with known quantities of progesterone and assayed.

Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	91.81	1000	1091.81	1188.34	108.8
2	31.14	1000	1031.14	1042.59	101.1
3	102.52	243	345.52	344.81	99.8
4	18.86	243	261.86	251.83	96.2
5	23.54	27	50.54	49.72	98.4
6	2063.56	27	2090.56	1943.9	93.0

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 (%)
H	12	884.61	35.1	4.0
L	12	39.23	3.3	8.4

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 (%)
H	12	884.15	48.7	5.5
L	12	28.04	2.7	9.6

C. Sensitivity:

The lower limit of sensitivity was determined by interpolating the mean minus 2 SD's for 20 zero standards. The minimal concentration of progesterone that can be distinguished from 0 is 5 pg/mL.

D. Linearity of Dilution:

Three saliva samples were diluted with assay diluent and assayed.

Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Sample 1			272.05	
	1:2	136.03	140.63	103.4
	1:4	68.01	61.09	89.8
	1:8	34.01	28.52	83.9
	1:16	17.00	14.97	88.1
Sample 2			282.90	
	1:2	141.45	131.74	93.1
	1:4	70.73	65.08	92.0
	1:8	35.36	36.72	103.8
	1:16	17.68	16.37	92.6
Sample 3			1188.34	
	1:2	594.17	581.16	97.8
	1:4	297.09	268.12	90.2
	1:8	148.54	134.85	90.8
	1:16	74.27	56.89	76.6

E. Specificity

The following compounds were tested at concentrations up to 1000 ng/mL for cross-reactivity:

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity
Prednisolone	1000	0.0021
Prednisone	1000	0.0038
Cortisone	1000	0.0106
11-Deoxycortisol	1000	0.0195
21-Deoxycortisol	1000	0.0082
17- α Hydroxy-progesterone	1000	0.0723
Dexamethasone	1000	0.0014
Triamcinolone	1000	ND
Corticosterone	500	0.1924
Testosterone	1000	ND
DHEA	1000	ND
Cortisol	1000	ND
Transferrin	1000	ND
Aldosterone	1000	ND
Estradiol	1000	ND
Estrone	1000	ND
Estril	1000	ND

ND = None detected (<0.004)

F. Correlation With Serum:

The correlation between serum and saliva progesterone was determined by assaying matched samples using the DSL serum progesterone EIA and the Salimetrics HS Salivary Progesterone EIA.

The correlation between serum and saliva was highly significant, $r(35) = 0.80$ (females, $r(25) = 0.87$, males, $r(8) = 0.67$).

Salivary Progesterone Expected Normal Ranges: *

Group	Number	Mean (pg/mL)	Standard Deviation (pg/mL)
Follicular phase	127	80.35	34.8
Luteal phase	202	131.00	54.5
Pre-menopausal, day 20	23	136.30	82.3
Post-menopausal, day 20	11	58.90	29.7

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

References

- Ayers, J., Birenbaum, D.L., Menon, K.M. (1987). Luteal phase dysfunction in endometriosis: elevated progesterone levels in peripheral and ovarian veins during the follicular phase. *Fertility and Sterility*, 47(6), 935-939.
- Redei, E., Freeman, E.W. (1995). Daily plasma estradiol and progesterone levels over the menstrual cycle and their relation to premenstrual symptoms. *Psychoneuroendocrinology*, 20(3), 259-267.
- De Cree, C., Lewin, R., Ostyn, M. (1990). The monitoring of the menstrual status of female athletes by salivary steroid determination and ultrasonography. *European Journal of Applied Physiology*, 60, 472-477.
- Vining, R.F., & McGinley, R.A. (1987). The measurement of hormones in saliva: Possibilities and pitfalls. *Journal of Steroid Biochemistry*, 27, 81-94.
- Chard, T. (1990). *An introduction to radioimmunoassay and related techniques*. Amsterdam: Elsevier.
- Schwartz, E.B., Granger, D.A., Susman, E.J., Gunnar, M.R., & Laird, B. (1998). Assessing salivary cortisol in studies of child development. *Child Development*, 69, 1503-1513.
- Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., & Curran, M. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behavior*, 46, 39-46.
- Schwartz, E., & Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry*, 50, 654-656.
- West, C.D., Mahajan, D.K., Chavre, V.J., Nabors, C.J. (1973). Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. *Journal of Clinical Endocrinology & Metabolism*, 36 No.6, 1230 - 1236.
- 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, 165-173.

For Additional Information

Lu, Y.C., Chatterton, R. T., Vogelsong, K. M., & May, L. K. (1997). Direct radioimmunoassay of progesterone in saliva. *Journal of Immunoassay*, 18, 149-163.

Kirschbaum, C., Read, G.F., & Hellhammer, D.H. (1992). *Assessment of hormones and drugs in saliva in biobehavioral research*. Kirkland, WA: Hogefe & Huber.

Riad-Fahmy, D., Read, G. F., Walker, R. F., & Griffiths, K. (1982). Steroids in saliva for assessing endocrine function. *Endocrine Reviews*, 3, 367-395.

Meulenber, P. M., & Hofman, J. A. (1989). Salivary progesterone excellently reflects free and total progesterone in plasma during pregnancy. *Clinical Chemistry*, 35, 168-172.

Tallon, D. F., Gosling, J. P., Buckley, P. M., Dooley, M. M., Cleere, W. F., O'Dwyer, E. M., & Fottrell, P. F. (1984). Direct solid-phase enzyme immunoassay of progesterone in saliva. *Clinical Chemistry*, 30, 1507-1511.

Ellison, P. T. (1994). Measurements of salivary progesterone. *Annals of the New York Academy of Sciences*, 709, 161-173.

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."

"European Authorized Representative"
Electra-Box Diagnostica AB, Tyresco-Stockholm
Solkraftsvagen 18B, 135 70 Stockholm, Sweden
(T) 46-40-457755

