SALIVARY PROGESTERONE

ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-1502, (Single) 96-Well Kit;
1-1502-5, (5-Pack) 480 Wells

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Intended Use

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

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

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

Introduction

Progesterone (4-pregenene-3,20-dione) is a steroid hormone of primary importance in ovulation, fertility, pregnancy, and menopause. Synthesis of Progesterone takes place in the placenta, adrenal glands, and gonads (1-3). In normal, non-pregnant women during the mid-luteal phase of the menstrual cycle, Progesterone exhibits a prominent circadian rhythm with additional ultradian components. Peak production occurs in the evening around 6 PM (4). In pregnant women, Progesterone also exhibits a similar rhythm during the second and third trimester, with a nadir in the morning and a peak in the late evening (5). In males, Progesterone is thought to play some role in testicular physiology (6).

In addition to its role as a sex hormone, Progesterone also serves as a precursor compound for many of the other steroid hormones. Progesterone is also synthesized in the brain and nervous system, where it functions as a neurosteroid that can influence survival and growth of cells, (7,8) and it is involved in brain development and behavior (7-10).

In blood, only 1 to 10% of Progesterone is in its unbound or biologically active form. The remaining Progesterone is bound to serum proteins. Unbound Progesterone enters the saliva via intracellular mechanisms, and the majority of Progesterone in saliva is not protein-bound. Salivary Progesterone levels are unaffected by salivary flow rate or salivary enzymes (11). Correlations obtained between plasma and salivary levels measured in the same subjects have generally been quite high (12).

Test Principle

This is a competitive immunoassay kit. Progesterone in standards and samples compete with Progesterone conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Progesterone 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 Progesterone Enzyme Conjugate detected is inversely proportional to the amount of Progesterone present in the sample (13).

Safety Precautions

Read Safety Data Sheets before handling reagents.

Hazardous Ingredients

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

Handling

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

Emergency Exposure Measures

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

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

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

General Kit Use Advice

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

Storage

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

pH Indicator

Progesterone values from samples with a pH $\leq$ 4.0 or $\geq$ 9.0 may be inaccurate. A pH indicator in the Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the 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 $\leq$ 4.0 or $\geq$ 9.0 should be recollected (14).

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

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

Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination (15,16) using our Blood Contamination EIA Kit (Item Nos. 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

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

Sample Handling and Preparation

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

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

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. 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.

Materials Supplied with Single Kit

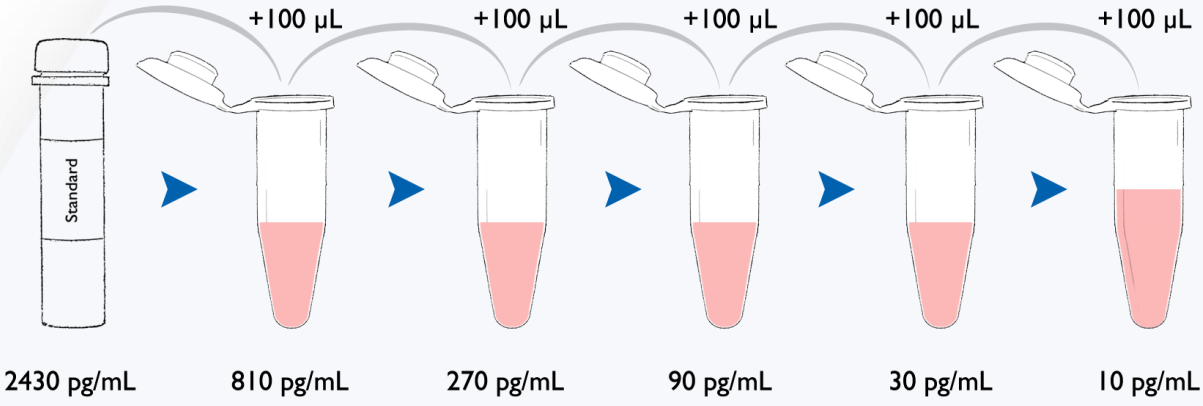
	Item	Quantity/Size
1	**Microtitre Plate** Coated with rabbit anti-Progesterone antibodies.	1/96 well
2	**Progesterone Standard** 2430 pg/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: Progesterone, buffer, preservative.	1 vial / 1 mL
3	**Progesterone Controls** High, Low, in a saliva-like matrix. Ready to use. Contain: Progesterone, buffer, preservative.	2 vials / 500 µL each
4	**Progesterone Enzyme Conjugate** Concentrate. Dilute before use with Assay Diluent. (See step 5 of Procedure.) Contains: Progesterone conjugated to HRP, preservative.	1 vial / 50 µL
5	**Assay Diluent** Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 60 mL
6	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
7	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle / 25 mL
8	**Stop Solution**	1 bottle / 12.5 mL
9	**Non-Specific Binding (NSB) Wells** Do not contain anti-Progesterone antibody. Break off and insert as blanks (optional) where needed.	1 strip
10	**Adhesive Plate Covers**	2

Materials Needed But Not Supplied

- Precision pipette to deliver 22.5 µL, 50 µL, 100 µL, and 200 µL
- Precision multichannel pipette to deliver 50 µL, 150 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 490 to 492 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 18 mL
- Five small disposable polypropylene tubes for dilution of standard
- Pipette tips
- Serological pipette to deliver up to 20 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 18 mL of 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 H_2O). ***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.)
- Prepare serial dilutions of the Progesterone Standard as follows:
    - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
    - Pipette 200 µL of Assay Diluent into 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 are, respectively, 2430 pg/mL, 810 pg/mL, 270 pg/mL, 90 pg/mL, 30 pg/mL, and 10 pg/mL. Standard concentrations in nmol/L are 7.733, 2.576, 0.859, 0.286, 0.095 and 0.032 nmol/L, respectively.



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	2430 Std	2430 Std	Ctrl-H	Ctrl-H								
B	810 Std	810 Std	Ctrl-L	Ctrl-L								
C	270 Std	270 Std	SMP-1	SMP-1								
D	90 Std	90 Std	SMP-2	SMP-2								
E	30 Std	30 Std	SMP-3	SMP-3								
F	10 Std	10 Std	SMP-4	SMP-4								
G	Zero	Zero	SMP-5	SMP-5								
H	NSB*	NSB*	SMP-6	SMP-6								

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

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

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

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

Step 3: Pipette 18 mL of Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 50 µL of standards, controls, and saliva samples into appropriate wells.
- Pipette 50 µL of Assay Diluent into 2 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 tube of 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 150 µL to each well using a multichannel pipette.

Step 6: Place adhesive cover provided over plate. Mix plate on a plate rotator ***continuously*** at 500 rpm for 1 hour 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 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 8: Add 200 µL 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 µL 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 490 to 492 nm is recommended.)

Quality Control

The Salimetrics' High and Low 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.

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 Progesterone values greater than 2430 pg/mL should be diluted with Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

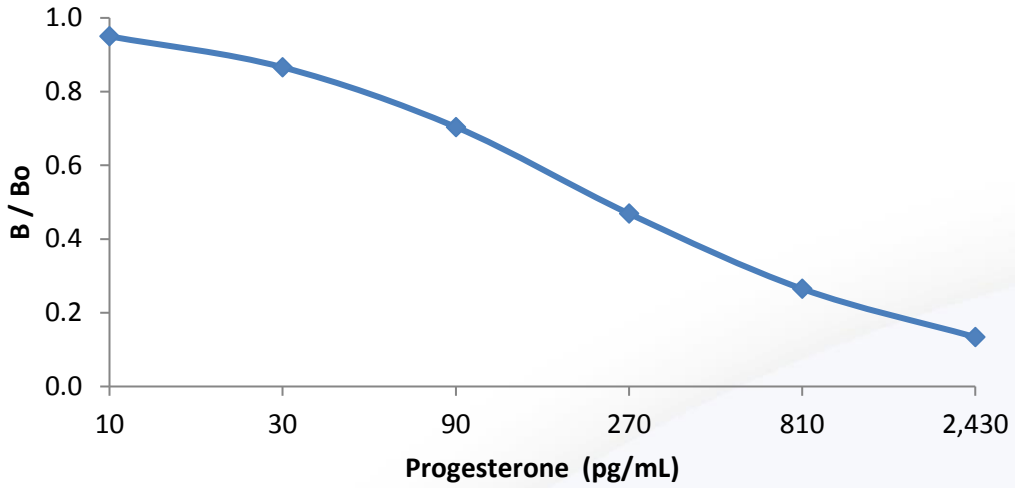
A new Standard Curve must be run with each full or partial plate.

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	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 Curve Fit



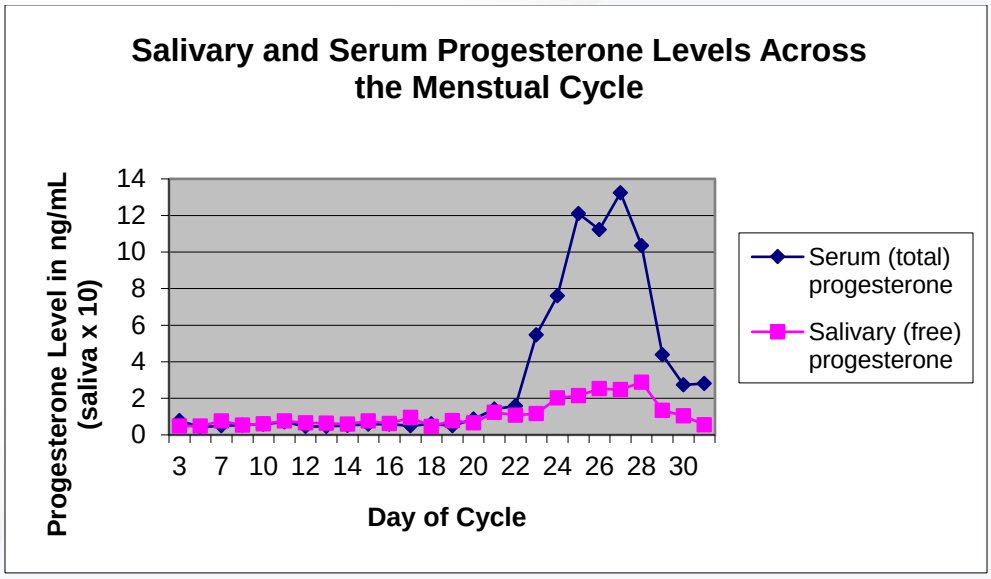
Limitations

- Samples with Progesterone values greater than 2430 pg/mL should be 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 the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values $\leq$ 4.0 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 Progesterone levels should be followed by additional testing and evaluation.

Salivary Progesterone Example Ranges*

Group	N	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.



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Salivary Progesterone EIA Kit Performance Characteristics

Precision

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

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	12	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.

Saliva 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

Recovery

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

Saliva 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	1943.90	93.0

Sensitivity

Analytical Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 20 replicates at the 0 pg/mL level. The minimal concentration of Progesterone that can be distinguished from 0 is 5 pg/mL.

Functional Sensitivity

The functional sensitivity was determined by assaying 60 saliva samples at a concentration level resulting in a CV of approximately 20%. The functional sensitivity of the salivary Progesterone ELISA is 12.37 pg/mL.

Correlation with Serum

The correlation between serum and saliva Progesterone was determined by assaying matched samples using the Diagnostic Systems Laboratories Serum Progesterone EIA and the Salimetrics Salivary Progesterone EIA.

The correlation between serum and saliva was highly significant, $\underline{r}$ (35) = 0.80 (females, $\underline{r}$ (25) = 0.87; males, $\underline{r}$ (8) = 0.67), $\underline{p} < 0.001$.

Sample Dilution Recovery

Three samples were serially diluted with Assay Diluent and assayed.

Saliva 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

Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Progesterone EIA
Prednisolone	1000	0.0021
Prednisone	1000	0.0038
Cortisone	1000	0.0106
11-Deoxycortisol	1000	0.0195
21-Deoxycortisol	1000	0.0082
17 α-Hydroxyprogesterone	1000	0.0723
Dexamethasone	1000	0.0014
Triamincinolone	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
Estriol	1000	ND

ND = None detected (<0.004)

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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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