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PROGESTERONE SALIVA ELISA

USA: For Research Use Only. Not for Use in Diagnostic Procedures

REF: IB59401

Version: 7.0
Effective: September 13, 2018

INTENDED USE
For the quantitative determination of Progesterone by an enzyme immunoassay in human saliva.

PRINCIPLE OF THE TEST
The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabelled antigen (present in standards, controls and samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of progesterone in the sample. A set of standards is used to plot a standard curve from which the amount of progesterone in samples and controls can be directly read.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
11. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS
1. All the reagents within the kit are calibrated for the direct determination of progesterone in human saliva. The kit is not calibrated for the determination of progesterone in serum, plasma or other specimens of human or animal origin.
2. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
3. Only calibrator A may be used to dilute any high saliva samples. The use of any other reagent may lead to false results.
4. The occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the results obtained should include all aspects of a samples background including the frequency of exposure to animals/products if false results are suspected.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL
Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. No test method however, can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood sample.

CHEMICAL HAZARDS
Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SAMPLE COLLECTION AND STORAGE
Approximately 1 mL of saliva is required per duplicate determination. Collect 4–5 mL of saliva into a clean glass tube* between 7–10 am without force or inducement and before eating, drinking or brushing the teeth. Simply rinse the mouth with water before collection. Do not use blood-contaminated specimens. Store samples at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.
**Do not use cotton or polyester rolls or plastic collection tubes for collecting saliva samples in this assay, since it has been well established that false elevated results will occur.*

SAMPLE PRETREATMENT
Sample tubes are to be placed into a freezer and allowed to freeze. When ready to use, the specimens are to be thawed, heated at 60°C for 1 hour, and then centrifuged. The supernatants are to be collected and poured into freshly labelled tubes. Do not use blood-contaminated specimens. If samples are to be used at a later date store frozen.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 50, 100, 150 and 300 µL
2. Disposable pipette tips
3. Deionized water
4. Plate shaker
5. Bench top centrifuge
6. Water bath set to 60°C
7. Microplate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater* (see assay procedure step 10)

REAGENTS PROVIDED

1. Rabbit Anti-Progesterone Antibody-Coated Break-Apart Well Microplate — Ready To Use
- Contents: One 96-well (12x8) polyclonal antibody-coated microplate in a resealable pouch with desiccant.
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.

2. Progesterone-Horseradish Peroxidase (HRP) Conjugate Concentrate — Requires Preparation X100
- Contents: Progesterone-HRP conjugate in a protein-based buffer with a non-mercury preservative.
- Volume: 300 µL/vial
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.
- Preparation: Dilute 1:100 in assay buffer before use (eg. 20 µL of HRP in 2 mL of assay buffer). If the whole plate is to be used dilute 120 µL of HRP in 12 mL of assay buffer. Discard any that is left over.

3. Progesterone Saliva Calibrators— Ready To Use
- Contents: Six vials containing progesterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of progesterone.

* Listed below are approximate concentrations, please refer to bottle labels for exact concentrations.

Calibrator	Concentration	Volume/Vial
Calibrator A	0 pg/mL	2.0 mL
Calibrator B	20 pg/mL	0.5 mL
Calibrator C	100 pg/mL	0.5 mL
Calibrator D	500 pg/mL	0.5 mL
Calibrator E	2000 pg/mL	0.5 mL
Calibrator F	5000 pg/mL	0.5 mL

- Storage: Refrigerate at 2–8°C
- Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Controls — Ready To Use
- Contents: Two vials containing progesterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with defined quantities of progesterone. Refer to vial labels for the acceptable range.
- Volume: 0.5 mL/vial
- Storage: Refrigerate at 2–8°C
- Stability: 12 months in unopened vial or as indicated on label. Once opened, the controls should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate — Requires Preparation X10
- Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
- Volume: 50 mL/bottle
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.
- Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 mL of the wash buffer concentrate in 450 mL of water.

6. Assay Buffer — Ready To Use*
- Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
- Volume: 15 mL/bottle
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.

** Warm to completely dissolve before use.*

7. TMB Substrate — Ready To Use
- Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
- Volume: 16 mL/bottle
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.

8. Stopping Solution — Ready To Use
- Contents: One bottle containing 1M sulfuric acid.
- Volume: 6 mL/bottle
- Storage: Refrigerate at 2–8°C
- Stability: 12 months or as indicated on label.

ASSAY PROCEDURE

Sample Pretreatment: **Freezing, Heating at 60°C for 1 Hour & Centrifugation.**

All reagents must reach room temperature before use. Calibrators, controls and samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the progesterone-HRP conjugate and wash buffer.
2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 µL of each calibrator, control and sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the conjugate working solution into each well. (We recommend using a multichannel pipette.)
5. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.
6. Wash the wells 3 times with 300 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)
7. Pipette 150 µL of TMB substrate into each well at timed intervals.
8. Incubate on a plate shaker for 10–20 minutes at room temperature (or until calibrator A attains dark blue colour for desired OD).
9. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 7.
10. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.

* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of samples or controls.

CALCULATIONS

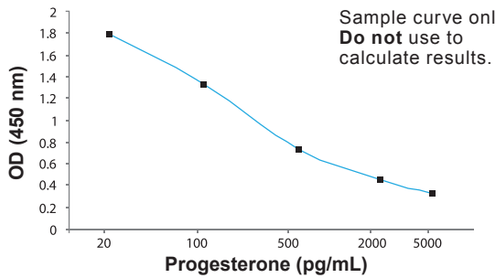
1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 5000 pg/mL, then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA

Sample data only. **Do not** use to calculate results.

Calibrator	OD 1	OD 2	Mean OD	Value (pg/mL)
A	2.252	2.320	2.286	0
B	1.795	1.768	1.782	20
C	1.352	1.322	1.337	100
D	0.730	0.736	0.733	500
E	0.453	0.451	0.452	2000
F	0.341	0.307	0.324	5000
Unknown	0.915	0.919	0.917	300

TYPICAL CALIBRATOR CURVE



Sample curve only. **Do not** use to calculate results.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Progesterone Saliva ELISA kit is **20 pg/mL**.

SPECIFICITY (CROSS-REACTIVITY)

The following compounds were tested for cross-reactivity with the Progesterone Saliva ELISA kit with progesterone cross-reacting at 100%.

Steroid	% Cross Reactivity
Progesterone	100
11α-OH-Progesterone	100
Deoxycorticosterone	1.7
17-OH-Progesterone	0.4
5α-Androstane-3β, 17β-diol	0.3
Corticosterone	0.3
Pregnenolone	0.2

The following steroids were tested but cross-reacted at less than 0.1%: Cortisol, Cortisone, Danazol, DHEAS, Estradiol, 5β-Pregnan-3α, 17α, 21α-triol-20-one, 5β-Pregnan-3α, 17-diol-20-one, Pregnan-3α, 20α-diol and Testosterone.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in pg/mL) are tabulated below:

Sample	Mean	SD	CV %
1	32.93	4.39	13.3
2	78.73	4.63	5.9
3	302.67	22.30	7.37

INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of four weeks. The results (in pg/mL) are tabulated below:

Sample	Mean	SD	CV %
1	30.83	3.90	12.7
2	75.03	7.73	7.7
3	241.06	26.23	10.9

RECOVERY

Spiked samples were prepared by adding defi ned amounts of progesterone to two saliva samples (1:1). The results (in pg/mL) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1 Unspiked	4.38	-	-
+ 100	58.93	52.19	112.9
+ 500	240.57	252.19	95.4
+ 2000	851.70	1002.19	85.0
2 Unspiked	7.49	-	-
+ 100	46.27	53.75	86.1
+ 2000	894.58	1003.75	89.1
+ 5000	2694.49	2503.75	107.6

LINEARITY

Three saliva samples were diluted with calibrator A. The results (in pg/mL) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1	1005.66	-	-
1:2	473.10	502.83	94.1
1:4	218.29	251.41	86.8
1:8	115.63	125.71	92.0
2	1462.5	-	-
1:2	700.48	731.25	95.8
1:4	327.69	365.62	89.6
1:8	172.12	182.81	94.1
3	2279.9	-	-
1:2	1061.0	1139.95	93.1
1:4	497.67	569.98	87.3
1:8	239.59	284.99	84.1

EXTRACTION VS. NON-EXTRACTION COMPARITIVE STUDY

The Progesterone Saliva ELISA method was validated by the following comparative study between:

1. Prior extraction of saliva samples with diethyl acetate.
2. Prior heating of saliva samples for 1 hour at 60–70°C.

The data from these 22 random saliva samples show a strong correlation of r = 0.91. As a result, the heating method was chosen due to its easier and less time consuming technique.

REFERENCES

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SYMBOLS

CE European Comformity
IVD In vitro diagnostic device
Consult instructions for use
Contains sufficient for <n> tests
Storage Temperature
Legal Manufacturer
Use by
REF Catalogue Number
EC REP Authorized representative
LOT Lot number
X # Dilute 1: # Before use