



17- α -OH Progesterone-ELISA

IN VITRO DIAGNOSTIC USE



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1 INTRODUCTION

The steroid 17- α -Hydroxyprogesterone (17- α -OHP) is produced by both the adrenal cortex and gonads. Even though 17- α -OHP has relatively little progestational activity, it is of intense clinical interest because it is the immediate precursor to 11-desoxycortisol (Cpd-S). Because Cpd-S is produced by 21-hydroxylation of 17- α -OHP, measurement of 17- α -OHP is a useful indirect indicator of 21-hydroxylase activity. In congenital 21-hydroxylase deficiency, the most common variety of congenital adrenal hyperplasia (CAH), 17- α -OHP is secreted in abundant excess. It is moderately elevated in the 11- β -hydroxylase deficiency as well. Measurement of 17- α -OHP is therefore valuable in the initial diagnosis of CAH.

2 CLINICAL PHYSIOLOGY

2.1 Adult non-pregnant women

In adult non-pregnant women in the childbearing age group, 17- α -OHP concentrations vary over the menstrual cycle with luteal phase concentrations being higher than follicular phase concentrations. This is because 17- α -OHP is secreted parallel with progesterone from maturing follicles or from the corpus luteum. There is also a diurnal variation of 17- α -OHP concentrations.

This rhythm is parallel with adrenal cortisol secretion such that maximum 17- α -OHP concentrations are measured in samples obtained between midnight and 8:00 am.

2.2 Adult males

There is little information available on the systematic variability of 17- α -OHP concentration in adult males.

2.3 Pregnant women and newborn children

The steroid 17- α -OHP is produced in large amounts by the fetus and the adrenals. It is secreted in abundance into both the fetal and maternal circulation. The maternal concentrations of 17- α -OHP increase very sharply after 32 weeks gestational age to about 4-fold above basal concentrations at term.

3 CLINICAL APPLICATIONS

3.1 Congenital adrenal hyperplasia

The principal application of the 17- α -OHP ELISA is in the diagnosis of CAH in newborns with ambiguous genitalia and in virilized adolescent girls. Since 17- α -OHP is the immediate precursor to 11-desoxycortisol, basal 17- α -OHP concentrations are sharply elevated in patients with 21-hydroxylase deficiency and to a lesser degree in patients with 11-hydroxylase deficiency.

Because 17- α -OHP concentrations are so markedly elevated in newborns and adolescent girls afflicted with CAH, a single basal measurement is all that is normally required to make the diagnosis.

3.2 Late onset adrenal hyperplasia

More recently, 17- α -OHP concentrations have been utilized in the evaluation of androgenized women where late onset 21-hydroxylase is suspected. This condition is clinically very subtle and since the presentation is the same as classical polycystic ovarian disease, basal plasma 17- α -OHP concentrations, unlike classical congenital adrenal hyperplasia, are normal. The diagnosis is made by administration of an ACTH stimulation test.

3.3 Other applications


Measurement of 17- α -OHP concentrations is also utilized in evaluation of both men and women with acne vulgaris, male pattern baldness and in some subtle forms of infertility. Experience with these applications are very limited.

4 PRINCIPLE

The **Bio-Line 17- α -OH Progesterone ELISA KIT** is based on the competition principle and the microtiterplate separation. An unknown amount of 17- α -OHP present in the sample and a fixed amount of 17- α -OHP conjugated with horse-radish peroxidase compete for the binding sites of a polyclonal 17- α -OH Progesterone serum coated onto the wells. After one hour incubation the microtiterplate is washed to stop the competition reaction. Having added the substrate solution the concentration of 17- α -OHP is inversely proportional to the optical density measured.

5 REAGENTS

5.1 Contents of the Kit

-  12x8 (break apart) strips, 96 wells
Wells coated with Anti-17- α -OH Progesterone serum
- | | |
|-----|---|
| CAL | N |
|-----|---|

 N=1 to 6, Reference Calibrator Set, 5 vials, 1 ml each
See exact values on vial labels
- | | |
|-----|---|
| CAL | 0 |
|-----|---|

 Zero Calibrator, 1 vial, 1 ml
- | | |
|----|-----|
| Ag | HRP |
|----|-----|

 1 vial, 25 ml
17- α -OH Progesterone conjugated to horseradish peroxidase
- | | |
|-------|-----|
| CHROM | TMB |
|-------|-----|

 (Substrate Solution) 1 vial, 25ml
TMB
- | | |
|------|------|
| STOP | SOLN |
|------|------|

 1 vial, 14 ml
contains 0.5M H₂SO₄

7.

WASH	SOLN	CONC
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 (40x), 1 vial, 30 ml

5.2 Equipment and material required but not provided

1. A microtiterplate reader (450±10 nm).
2. Precision micropipettes with disposable tips for 100 and 200 µl.
3. Standard refrigerator.
4. Absorbent paper.
5. Deionized water.

5.3 Storage conditions

- When stored at 2° to 8°C unbroken reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
- Enzyme-Conjugate, Calibrator Solution, Substrate Solution, Wash Solution and Zero Calibrator must be stored at 2° to 8°C.
- Microtiter wells must be stored at 2° to 8°C. Once the foil bag has been broken, care should be taken to close it tightly again. The immuno-reactivity of the coated microtiter wells is stable for approx. 6 weeks in the broken, but tightly closed bag containing the dessicant.

5.4 Reagent preparation

Wash Solution

Add deionized water to the 40x concentrated Wash Solution (contents: 30 ml) to a final volume of 1200 ml. The diluted Wash Solution is stable for 2 weeks at room temperature.

5.5 Warnings and precautions for users

1. **CAUTION:** Test methods are not available which can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV), or other infectious agents are absent from the reagents in this kit. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists (e.g., USA Center for Disease Control/National Institute of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1984).
2. Avoid contact with Stop Solution, 0.5M H₂SO₄. It may cause skin irritation and burns.
3. Replace caps on reagents immediately. Do not switch caps.
4. Solutions containing additives or preservatives, such as sodium azide, should not be used in the enzyme reaction.
5. Do not pipette reagents by mouth.
6. For in vitro diagnostic use only.
7. Do not mix or use components from kits with different lot numbers.

6 SPECIMEN COLLECTION AND PREPARATION

1. Collect blood by venipuncture, allow to clot, and separate serum by centrifugation at room temperature. No special pretreatment of sample is necessary. The specimen may be stored at 2-8° C for up to 24 hours, and should be frozen at -10° C or lower for longer periods. Do not use grossly hemolyzed or grossly lipemic specimens.
2. Samples suspected to contain 17- α -OH Progesterone concentration higher than the highest calibrator are to be diluted with Zero Calibrator.

Please note: Samples containing sodium azide should not be used in the assay.

7 PERFORMANCE OF THE ASSAY

7.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposable plastic pipette tips for each reagent, calibrator or specimen in order to avoid cross contamination. For the dispensing of the Substrate Solution and the Stop Solution avoid pipettes with metal parts.
- Pipette calibrators and samples onto the bottom of the well. For pipetting of Enzyme Conjugate and Stop Solution it is recommended to hold the pipette in a vertical position above the well and dispense the correspondent solution into the centre of the well so that a complete mixing of Enzyme Conjugate with sample or calibrator and of the Stop Solution with the Substrate Solution is achieved.
- Before starting the assay, it is recommended that all reagents be ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature. This makes interpolation possible for fixed physico-chemical conditions. If in a test run the absorbance of Zero Calibrator is lower than 1.0 or above the upper performance limit of your microtiterplate spectrophotometer you can extend or reduce the incubation time of the final enzymatic formation of color to 45 or 15 minutes accordingly. Since calibrators are assayed in each run, absorbance fluctuations do not affect the result.
- The Substrate Solution should be colourless or slightly blue or green. If the solution is dark blue the reagent is unusable and must be discarded.
- During incubation with Substrate Solution avoid direct sunlight on the microtiter plate.

7.2 Assay Procedure

1. Secure the desired number of coated strips in the holder.
2. Dispense **25 µl** of 17- α -OH Progesterone Calibrators into appropriate wells.
3. Dispense **25 µl** of sample into selected wells. Time between distribution of first Calibrator and last sample can be up to 10 minutes without affecting the results.
4. Incubate plate for **5 minutes** at room temperature.
5. Dispense **200 µl** of Enzyme-Conjugate into each well.
6. Thoroughly mix the plate for 10 seconds. It is important to have complete mixing in this step.
7. Incubate for **60 minutes** at room temperature.
8. Briskly shake out the contents of the wells.

9. Rinse the wells 3 times with diluted Wash Solution (400 µl per well). Strike the wells sharply on absorbent paper to remove residual droplets.
10. Add **200 µl** of Substrate Solution to each well, at timed intervals.
11. Incubate for **30 minutes** at room temperature.
12. Stop the enzymatic reaction by adding **100 µl** of Stop Solution to each well at the same timed intervals as in step 10 and determine the absorbance of each well at **450±10 nm**.

Final Reaction Stability

It is recommended that the wells be read within 10 minutes following step 12.

7.3 Calculation of Results

Any microwell reader capable of determining the absorbance at 450±10nm may be used. The 17- α -OH Progesterone value of each serum sample is obtained as follows :

1. Using linear-linear or semi log graph paper, construct an calibrator curve by plotting the average absorbance (Y) of each Reference Calibrator against its corresponding concentration (X) in ng/ml . For construction of the calibrator curve we recommend a four parameter logistic function.
2. Use the average absorbance of each serum sample to determine the corresponding 17- α -OH Progesterone value by simple interpolation from this calibrator curve, multiplying by the initial sample dilution, if necessary.

Example of a typical calibrator curve

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.

Calibrator	Optical Units
Calibrator 0 (0 ng/ml)	1.92
Calibrator 1 (0.15 ng/ml)	1.53
Calibrator 2 (0.5 ng/ml)	1.17
Calibrator 3 (1.5 ng/ml)	0.85
Calibrator 4 (3.0 ng/ml)	0.60
Calibrator 5 (7.5 ng/ml)	0.35
Calibrator 6 (20 ng/ml)	0.17

8 OPTION II: DETERMINATION OF 17- α -OH PROGESTERONE IN SALIVA

8.1 Sample Preparation

We recommend to freeze the saliva samples at -20° C immediately after sample collection.

Before testing thaw the samples, mix by vortexing and then centrifuge the saliva samples. The supernatant is used for hormone analysis.

8.2 Extraction for the determination of 17- α -OH Progesterone in Saliva:

The saliva samples have to be extracted. Although the calibrators have been prepared in Zero Calibrator and the saliva extracts of the saliva samples are reconstituted with Zero Calibrator, too, we recommend to extract the calibrators as well, to avoid extraction mistakes.

The following extraction method is recommended:

1. Pipet **50 µl** Control-/ Calibrator serum (Test kit) or **250 µl** Saliva Samples in tightly closed glass tubes (Volume approx. 3 ml).
2. Add **1 ml** anaesthetic ether (Hoechst AG, Germany) into each tube.
3. Vortex **1 h**, then freeze **1 h** (or more) at -20° C or lower.
4. Decant the ether phase in a new glass tube and evaporate (this takes ca. 2 hours in an dessicator). After evaporation the tubes should visually look empty.
5. Reconstitute the extract with **250 µl** Zero Calibrator of the test kit.
6. Vortex **15 min**.
7. Continue as described in the instruction manual (see Assay Procedure).

Please note: When using saliva samples the patient results have to be divided by 5 (since you have used the 5-fold volume).

9 PERFORMANCE CHARACTERISTICS

9.1 Expected normal values

Newborn	
5 - 30 day	< 0.7 - 2.5 ng/ml
31 - 60 day	m. 0.8 - 5.0 ng/ml
	f. 0.5 - 2.3 ng/ml
Children	
3 - 14 years	0.07 - 1.7 ng/ml
Reproductive aged women	
Follicular phase	0.1 - 0.8 ng/ml
Luteal phase	0.6 - 2.3 ng/ml
Ovulation	0.3 - 1.4 ng/ml
Post ACTH	< 3.2 ng/ml
Third trimester	2.0 - 12 ng/ml
Postmenopausal women	0.13 - 0.51 ng/ml
Normal men	
	0.5 - 2.1 ng/ml

9.2 Sensitivity

The lowest detectable level of 17- α -OH Progesterone that can be distinguished from the Zero Calibrator is 0.05 ng/ml at the 95 % confidence limit.

9.3 Specificity

The following materials have been checked for cross reactivity. The percentage indicate cross reactivity at 50% displacement compared to 17-OH Progesterone.

Steroid	% Cross Reaction
17- α -OH Progesterone	100.0
Estriol	< 0.01
Estradiol 17 β	< 0.01
Testosterone	< 0.01
Dihydrotestosterone	< 0.01
DOC	0.05
11-Desoxicortisol	1.4
Progesterone	1.2
DHEA	< 0.01
DHEAS	< 0.001
Cortisol	< 0.01
Corticosterone	< 0.05
Aldosterone	< 0.01
Androstendione	< 0.01
Dehydroepiandrosten sulfate	< 0.01
Prednison	< 0.01

9.4 Precision

Serum	Intraassay			Interassay		
	n	<X> \pm SD ng/ml	CV %	n	<X> \pm SD ng/ml	CV %
1	10	0.99 \pm 0.08	8.1	10	0.95 \pm 0.09	9.5
2	10	2.34 \pm 0.10	4.3	10	2.24 \pm 0.18	8.0
3	10	6.75 \pm 0.34	5.0	10	6.52 \pm 0.49	7.5

9.5 Accuracy

The accuracy of the assay was evaluated by recovery and dilution tests.

9.5.1 Recovery test

Serum	Endogenous 17- α -OHP ng/ml	Added 17- α -OHP ng/ml	Recovery %
1	3.1	5	105
		2	103
		1	97
2	1.9	5	102
		2	105
		1	99
		0.3	101

9.5.2 Dilution test

Serum	Dilution factor	Measured conc. ng/ml	Recovery %
1	Undiluted	1.23	
	1:2	0.62	101
	1:4	0.29	94
	1:8	0.15	98
2	Undiluted	6.21	
	1:2	2.93	94
	1:4	1.62	104
	1:8	0.76	98
	1:16	0.37	95
	1:32	0.17	88
3	Undiluted	7.35	
	1:2	3.72	101
	1:4	1.91	104
	1:8	0.93	101
	1:16	0.44	96
	1:32	0.24	104

10 METHOD COMPARISON: 17- α -OH PROGESTERON ELISA VS. RIA-CT

17- α -OH Progesteron ELISA was compared with data obtained from a commercially available 17- α -OH Progesteron RIA (n= 33). A correlation coefficient of $r = 0.96$ was found between the two tests.

11 QUALITY CONTROL

Good laboratory practice requires that controls are run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. We recommend to use BIO RAD Lyphochek Immunoassay Control Sera.

12 LIMITATION OF PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.
3. Complete mixing of Conjugate with calibrator or sample (step 5) and of Stop Solution with Substrate Solution (step 12) is critical. Insufficient mixing will result in poor precision.

13 REFERENCES

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