17OH-PROGESTERONE ELISA

KAP1401

DIAsource ImmunoAssays S.A. - Rue du Bosquet, 2 - B-1348 Louvain-la-Neuve - Belgium

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en

Read entire protocol before use.

17OH–PROGESTERONE ELISA

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of human 17-α-hydroxyprogesterone (17-OHP) in serum and plasma.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource 17OH–PROGESTERONE ELISA Kit

B. Catalog number : KAP1401: 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
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III. CLINICAL BACKGROUND

A. Biological activities of 17-α-hydroxyprogesterone

17-α-hydroxyprogesterone (17-OHP) is a C-21 steroid hormone (molecular weight 330.3) which is produced from 17-α-hydroxy pregnenolone in the adrenals and also in the ovaries, testes and placenta.
17-OHP is hydroxylated at positions 11 and 21 to produce cortisol via 11-deoxycortisol.

B. Clinical applications of 17-α-hydroxyprogesterone determination

As a rule, serum or amniotic fluid 17-OHP dosages are relevant to diagnose congenital adrenal hyperplasia (CAH). This CAH is due to a specific enzyme defect (six distinct enzyme deficiencies have been described). As a result of these deficiencies, ACTH increases and produces adrenal hyperplasia and the raise of many steroid precursors. But it is also very interesting to know the value of 17-OHP in patients with varicocele (17-OHP and testosterone represent markers of Leydig cell function) and in ageing male patients to detect Benign Prostatic Hypertrophy (BPH) and carcinoma of the prostate (PCA) (plasma17-OHP is significantly lower in PCA and BPH groups than in normal men).
There are other domains for 17-OHP investigations as : male infertility, girls with peripubertal virilization, children with premature adrenache (in these cases, the values of 17-OHP are increased without or after ACTH stimulation).

IV. PRINCIPLES OF THE METHOD

The DIAsource 17OH-PROGESTERONE ELISA kit is a solid phase Enzyme Linked Immunosorbent Assay performed on microtiterplates. During a 1 hour incubation step, at room temperature, with shaking, 17-OHP present in calibrators, controls and samples competes with 17-OHP-HRP conjugate for binding sites of a specific antibody immobilized on the wells of the microtiterplate. After this incubation, microtiterplate is washed to stop the competition reaction. The Chromogenic solution (TMB) is added and incubated for 30 minutes. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is inversely proportional to the 17-OHP concentration.
A calibration curve is plotted and the 17-OHP concentrations of the samples are determined by dose interpolation from the calibration curve.

V. REAGENTS PROVIDED

Reagents	KAP1401	Colour Code	Reconstitution
Microtiterplate with 96 breakable wells coated with anti17-α-OH-progesterone	1 x 96 wells	blue	**Ready** for use
CAL **0** Calibrator 0: human serum with gentamycin and Proclin	1 vial 1 ml	yellow	**Ready** for use
CAL **N** 17-α-OH-progesterone N = 1 to 6 (see exact values on vial labels) in human serum with gentamycin and Proclin	6 vials 0.5 ml	yellow	**Ready** for use
CONTROL **N** Controls N = 2 in human serum with gentamycin and Proclin	2 vials lyophilised	silver	**Add** 0.5 ml distilled water
Ag **HRP** **CONC** Conjugate: HRP labelled 17-α-OH-progesterone	1 vial 0.3 ml	yellow	**Dilute** 100 x with conjugate buffer
CONJ **BUF** Conjugate Buffer with bovine serum albumin	1 vial 30 ml	red	**Ready** for use
WASH **SOLN** **CONC** Wash solution (TRIS-HCl)	1 vial 10 ml	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer).
CHROM **TMB** Chromogenic solution TMB (Tetramethylbenzydine)	1 vial 12 ml	orange	**Ready** for use
STOP **SOLN** Stop solution HCl 1M	1 vial 12 ml		**Ready** for use

Note : Use Calibrator 0 for dilution of samples with values above the highest calibrator.

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:
1. Distilled water
2. Pipettes for delivery of : 25 µl, 100 µl, 200 µl and 500 µl (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Plate shaker (300 to 700 rpm)
6. Washer for microtiterplates
7. Microtiterplate reader capable of reading at 450 nm and 650 (bichromatic reading)

Optional (for extraction of newborns serum samples)
1. Diethyl ether (analytical grade; purity > 98%)
2. Disposable glass tubes (12 x 75 m), with stoppers.
3. DIAsource Reconstitution Solution for 17-OHP -ELISA ref: 4214024

VII. REAGENT PREPARATION

A. Controls: Reconstitute the controls with 0.5ml distilled water.

B. Working 17-OHP-HRP conjugate : Prepare an adequate volume of conjugate solution by adding for example : 20 µl of the 100x concentrated 17-OHP -HRP conjugate to 2 ml of conjugate buffer. Use a vortex to homogenize.
Extemporaneous preparation is recommended.

C. Working Wash solution : Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the label, if kept at 2 to 8°C.
- After reconstitution, controls are stable for 8 weeks at 2 to 8°C. For longer storage periods, aliquots should be made and kept at -20°C for maximum 4 months. Avoid subsequent freeze-thaw cycles.
- Freshly prepared Working Wash solution should be used on the same day.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- This kit is suitable for serum ,heparinized plasma or EDTA plasma samples.
- Plasma samples provide similar results than serum:
  Y(EDTA plasma) = 0.97 X(serum) - 0.01 r = 0.99 n=20
  Y(heparinized plasma) = 0.95 X(serum) – 0.07 r = 0.99 n=20
- Serum or plasma samples must be kept at 2-8°C.
- If the test is not run within 24 hrs, **sampling and storage at -20°C is recommended.**
- Avoid subsequent freeze-thaw cycles.

X. PROCEDURE

A. Handling notes

Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
For the dispensing of the Chromogenic Solution and the Stop Solution avoid pipettes with metal parts.
High precision pipettes or automated pipetting equipment will improve the precision.
Respect the incubation times.
To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section XIII paragraph E (Time delay).
Prepare a calibration curve for each run, do not use data from previous runs.
Dispense the Chromogenic Solution within 15 minutes following the washing of the microtiterplate.
During incubation with Chromogenic Solution, avoid direct sunlight on the microtiterplate.

B. Extraction for NEWBORN SERUM SAMPLES (optional)

1. Label one glass tube for each sample (do not extract calibrators or controls).
2. Pipette 100 µl of serum, followed by 1.5 ml of diethyl ether(analytical grade; purity > 98%) .
3. Vortex all the tubes vigorously (2 x 1 minute)
4. Let stand the tubes for 15 minutes to separate well aqueous phase (lower phase) and organic phase (upper phase).
5. Then place the tubes at -20°C in order to freeze the aqueous phase.
6. Prepare a second series of glass tubes and, for each sample, transfer the organic phase (upper phase) into these new tubes. Avoid contamination by the aqueous phase.
7. Evaporate the organic phase (diethyl ether) completely under a stream of air by placing the tubes at 37°C (water bath). Manipulate under a hood.
8. Dissolve the dry organic extract with 100 µl of Reconstitution solution (not provided, see VI.). Vortex vigorously for 1 minute.
9. Let stand for 10-15 minutes and vortex again for 1 minute. These volumes allow to perform the determination in duplicate.

C. Procedure

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. Pipette 25 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 200 µl of the working 17-OHP-HRP conjugate solution into all the wells.
5. Incubate for 1 hour at room temperature, on a plate shaker (400 rpm)
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   - dispensing 0.4 ml of Wash Solution into each well
   - aspirating the content of each well
8. Pipette 100 µl of the Chromogenic solution into each well within 15 minutes following the washing step.
9. Incubate the microtiterplate for 30 minutes at room temperature, on a plate shaker (400 rpm), avoid direct sunlight.
10. Pipette 100 µl of Stop Solution into each well.
11. Read the absorbances at 450 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI

XI. CALCULATION OF RESULTS

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. Calculate for each calibrator, control and sample:

$$B/B0\,(\%) = \frac{OD\ (Calibrator, Control\ or\ Sample)}{OD\ (Zero\ Calibrator)} x100$$

4. Using either linear-linear of semi-logarithmic graph paper, plot the (B/B0(%)) values for each calibrator point as a function of the 17OH Progesterone concentration of each calibrator point. Reject obvious outliers.
5. Computer assisted methods can also be used to construct the calibration curve. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.
6. By interpolation of the sample (B/B0 (%)) values, determine the 17-OH Progesterone concentrations of the samples from the calibration curve

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

17OH-Progesterone ELISA		**mOD units**
Calibrator	0 ng/ml	2922
	0.08 ng/ml	2768
	0.4 ng/ml	1904
	1.3 ng/ml	1088
	2.5 ng/ml	655
	7.5 ng/ml	241
	15 ng/ml	164

Conversion factor :

From ng/ml to nmol/L : x 3.03
From nmol/L to ng/ml : x 0.33

To the best of our knowledge, no international reference material exists for this parameter.

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty four zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations below the average OD at zero binding, was 0.04 ng/ml.

B. Specificity

The specificity was estimated by spiking a pool of 17-OHP samples (± 0.6 ng/ml) with steroids which might be present in patient samples.

Compound	**Added amount (ng/ml)**	**Cross-Reactivity (%)**
17OH-Progesterone	-	100
Progesterone	1000	0.58
17-α-hydroxypregnenolone	1000	0.5
21-deoxycortisol	1000	4.3
Pregnenolone	1000	0.03
11-deoxycortisol	1000	0.29
Corticosterone	10000	0.01
11-deoxycorticosterone	1000	0.28
Testosterone	5000	0.01
Androstenedione	5000	0.01
Estradiol	10000	ND
17-α-hydroxypregnenolone sulfate	5000	0.44

ND: non detectable

C. Precision

INTRA-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	24	1.16 ± 0.05	4.3
B	24	2.93 ± 0.12	4.1

INTER-ASSAY			
Sample	**N**	**<X> ± SD (ng/ml)**	**C.V. (%)**
A	14	0.99 ± 0.06	6.1
B	14	2.32 ± 0.14	6.0

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST:			
Sample	**added 17-OHP (ng/ml)**	**Recovered 17-OHP (ng/ml)**	**Recovery (%)**
1	10	9.61	96
	5	4.84	97
	1	0.94	94
	0.5	0.43	86
2	10	9.18	92
	5	4.5	90
	1	0.9	90
	0.5	0.5	100

DILUTION TEST:			
Sample dilution	**Theoretical concentration (ng/ml)**	**Measured concentration (ng/ml)**	**Recovery (%)**
1/1	12.01	12.01	100
1/2	6.01	5.51	91.7
1/4	3.00	2.80	93.2
1/8	1.50	1.58	105.2
1/1	10.73	10.73	100
1/2	5.37	4.77	88.9
1/4	2.68	2.68	99.9
1/8	1.34	1.43	106.6

Samples were diluted with the zero calibrator.

E. Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when Working 17-OHP-HRP Conjugate is dispensed 10 and 15 minutes after the calibrator has been added in the coated wells.

TIME DELAY			
	0' (ng/ml)	10' (ng/ml)	15' (ng/ml)
Sample 1	0.68	0.71	0.74
Sample 2	2.81	3.30	3.14

F. Limitations for newborn samples

Very young children (< 3 months old) present high levels of 17OH pregnenolone sulphate. Despite the fact that there is almost no cross reaction with this molecule, we recommend to apply the extraction procedure (X. B.)

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. EXPECTED VALUES

These values are given only for guidance; each laboratory should establish its own normal range of values.

	Concentration range (2.5 to 97.5% percentiles) (ng/ml)	Number of subjects
Normal males	0.36– 2.38	38
Normal Females		
. Follicular phase	0.32 – 0.81	28
. Luteal phase	0.79 - 3.29	34
Pregnancy		
. First and second trimester	1.3 – 3.9	25
. Third trimester	2.2 – 12.4	14
Newborns (0 to 3 months)		
After extraction (cf X B.)	0.3 - 2.3	38

XVI. PRECAUTIONS AND WARNINGS

Safety

For *in vitro* diagnostic use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

XVII. BIBLIOGRAPHY

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Detection of late-onset adrenal hyperplasia in girls with peripubertal virilization.
Acta Endocrinologica (Copenh.) 115, 413-418.

XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	**SAMPLE(S) CONTROLS (µl)**
Calibrators (0-6)	25	-
Controls, Samples or Extracted Samples	-	25
Working 17-OHP HRP Conjugate	200	200
Incubate for 1 hour at room temperature with continuous shaking at 400 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 30 min at room temperature with continuous shaking at 400 rpm		
Stop Solution	100	100
Read on a microtiterplate reader. Record the absorbance of each well at 450 nm (versus 630 or 650 nm).		

DIAsource Catalogue Nr : KAP1401	P.I. Number : 1700922/en	Revision nr : 160921/2

Revision date : 2016-11-09