Read entire protocol before use.

PROG-EASIA

I. INTENDED USE

Enzyme Immunoassay for the quantitative measurement of Progesterone in serum and plasma (96 determinations).

II. GENERAL INFORMATION

A. Proprietary name : DIAsource PROG-EASIA Kit

B. Catalogue number : KAP1451 : 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue de l'Industrie, 8, B-1400 Nivelles, Belgium.

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III. INTRODUCTION

A. Progesterone

Progesterone is a C-21 steroid hormone (molecular weight: 314.5) which is synthesized from cholesterol via pregnenolone in the granulosa and theca cells of the corpus iuteum under the influence of LH. The major production sites are ovary and placenta and somewhat the adrenal cortex in both men and women.
Progesterone is rapidly metabolized in the liver. Blood levels are very low during the follicular phase whereas one does observe a sharply increase during the luteal phase of menstrual cycles reaching a maximum 5 to 10 days after the midcycle LH peak.

B. Clinical applications of the dosage of Progesterone

Serum Progesterone levels, which are low during the follicular phase, increase during the luteal phase of menstrual cycle. Unless pregnancy occurs, the Progesterone level declines 4 days before the next menstrual period. Thus, the measurement of Progesterone levels constitutes a well-established method for detection of ovulation.
But there are many cases where the progesterone measurements are also of Interest :

- to check the effectiveness of ovulation induction;
- to monitor the embryon transfer and Progesterone replacement therapy;
- to detect the patients at risk for abortion during the beginning of pregnancy;
- to aid in the diagnostic of ectopic pregnancy;
- to detect all ovarian tumor (benign and malign) in postmenopausal women;
- to diagnose luteinized unruptured follicule by the dosage of 17 beta.-Estradiol and Progesterone levels in peritoneal fluid;
- the steroid profiles of follicules fluids and the ratio of E2IPROG allow to detect a normal or a dysfunctional ovulation Induction. (The empty follicular syndrome may reflect a dysfunctional ovulation induction).

C. Principle of the test

DIAsource PROG-EASIA is a direct Enzyme Immunoassay performed in microtiter plate. A fixed amount of Progesterone labelled with horseradish peroxydase (HRP) competes with unlabelled Progesterone present in calibrators or samples for a limited number of specific antibody binding sites.
After 3 hours Incubation at RT, the plate is washed to stop the reaction.
The revelation solution (tetramethylbenzydine (TMB)-H202) is added and incubated for 30 min. The reaction is stopped with H2S04 and the microtiter plate is read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which Is Inversely proportional to the Progesterone concentration. Progesterone levels of the samples are extrapolated from the plotted calibration curve.

V. REAGENTS PROVIDED

Reagents	Quantity	Color Code	Reconstitution
Microtiterplate with 96 anti-progesterone coated wells	96 wells	blue	**Ready** for use
CAL 0 Calibrator 0 ng/ml in human serum and preservatives	1 vial	yellow	**Reconstitute** with 2 ml reconstitution solution
CAL N Calibrator in human serum and preservatives (see exact values on vial labels)	5 vials	yellow	**Reconstitute** with 0.5 ml reconstitution soultion
CONTROL N Controls 1 and 2 in human serum and preservatives	2 vials	silver	**Reconstitute** with 0.5 ml reconstitution solution
REC SOLN Reconstitution solution	1 vial 8 ml	yellow	**Ready** for use
Ag HRP CONC Concentrated Progesterone-HRP conjugate in phosphate buffer with preservatives	1 vial 1 ml	red	**Add** 0.2 ml into 1 vial of conjugate buffer
CONJ BUF Conjugate buffer for dilution of Progesterone Conjugate	3 vials 21 ml	red	**Ready** for use
WASH SOLN CONC Washing solution	1 vial 10 ml	brown	**Dilute** 2 ml in 400 ml distilled water or the vial content in 2000 ml distilled water
CHROM TMB CONC Chromogen TMB (Tetramethylbenzidine)	1 vial 1 ml	green	**Add** 0.20 ml into 1 vial of substrate buffer
SUB BUF Substrate buffer H2O2 in acetate/citrate buffer	3 vials 21 ml	white	**Ready** for use
STOP SOLN H2SO4 1.8 N Stopping reagent	1 vial 6 ml	black	**Ready** for use

Note: Calibrator 0 ng/ml is recommended for samples dilutions.

VI. STORAGE AND STABILITY

- Store the kit at 2°C to 8°C until expiration date mentioned on the kit label
- Once opened, the HRP-Progesterone vial must be stored at 2° to 8°C.
- After reconstitution with the special solution provided, calibrators and controls may be stored at 4°C for one week. For longer storage, they have to be frozen. Three freezing-thawing cycles do not affect calibrators quality.
- Store the unused strips at 2°C to 8°C in the closed bag containing the dessicant until expiration date.
- In order to avoid obstructions of the washerheads, it is recommended to prepare every day a fresh diluted washing solution.

VII. MATERIAL REQUIRED BUT NOT PROVIDED

- Distilled water
- Pipettes : 50 µl, 100 µl, 200 µl, 500 µl and 2 ml
- Vortex mixer and magnetic stirrer
- Shaker at 700 rpm

VIII. WARNINGS

Safety

- For In vitro diagnostic use only
- The human blood components included in this kit have been tested and found non reactive for HBsAg and anti-HIV. Nevertheless, 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, e.g.CDC/HIH Health Manual : 'Biosafety in Microbiological and Biomedical Laboratories" l984.
- Avoid any skin contact with H2S04, H202 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipet liquids by mouth.

Handling

- Do not use kit components beyond the expiration date.
- Do not mix materials from different kit lots.
- Do not mix strips from different plates.
- Bring all the reagents and specimens to room temperature (18°C to 30°C) prior to use.
- Thoroughly mix the reagents and samples before use by gentle agitation or swirling.
- Use a clean disposable plastic pipette tip for each reagent, calibrator, control or specimen addition in order to avoid cross-contamination; for the dispensing of H2SO4 and the substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- The TMB solution in substrate buffer should be colourless. If a dark blue colour develops within a few minutes after preparation, this indicates that the reagent is unusable and must be discarded.
- During incubation with revelation solution, avoid direct sunlight on the microtiter plate.
- Respect the incubation times described in the assay procedure.
- Dispense the revelation solution immediately after the washing of the microtiter plate.

IX. SAMPLE COLLECTION AND PREPARATION

- No special pretreatment of the sample is necessary. Samples suspected to contain Progesterone concentration higher than the highest calibrator are to be diluted with the zero calibrator.
- Prior to use, all the samples and reagents must be at room temperature. It's recommended to vortex the samples before use.
- Hemolysis has to be avoided.
- Samples may be stored for 24 hours at 2° to 8°C prior to testing.
  Samples held for longer time must be frozen at -2O"C prior to assaying.
- Serum, heparinized plasma or EDTA plasma provide similar results.
  Y (serum) = 0.93 x (EDTA plasma) + 0.11 R=0.97 n=57
  Y (serum) = 0.94 x (hep. plasma) + 0.27 R=0.97 n=57

X. REAGENTS PREPARATION

- HRP-Progesterone conjugate : pipette 0.2 ml of the concentrated HRP-Progesterone conjugate into one vial of conjugate buffer. Extemporaneous preparation is mandatory. Maximum stability is 4 hours at room temperature or 24 hours at 2°C to 8°C., avoiding direct exposure to sunlight.
- Washing solution : dilute 2 ml in 400 ml distilled water or all the content of the vial in 2000 ml distilled water (use a magnetic stirrer).
  The washing solution is common to all DIAsource EASIA kits.
- Revelation solution: pipet 0.2 ml of the chromogen TMB into one of the vials of substrate buffer. Extemporaneous preparation is recommended. Maximum stability before use is 15 min. at room temperature avoiding direct exposure to sunlight and the excess must be discarded afterwards.

XI. ASSAY PROCEDURE

It's recommended to perform the assays in duplicate and to follow the Instruction of the assay procedure to obtain reliable results.

1. Select sufficient strips to accommodate calibrators, controls and samples.
2. Fit the strips into the holding frame.
3. Dispense 50 µl of each calibrator, control or sample into the appropriate wells. Time between distribution of the first calibrator and the last sample can be up to 40 minutes without affecting the results. Vertical alignment is recommended.
4. Dispense 200 µl of Progesterone-HRP-conjugate in each well.
5. Incubate for 3 hours at 18-26°C on an horizontal shaker set at 700 +/- 100 RPM.

6. Wash the plate by :
   a) aspirating the liquid from each well,
   b) dispensing 0.4 ml of washing solution into each well,
   c) aspirating the contents of each well.
   Repeat steps b) and c) 2 times.
7. Dispense 200 µl of the freshly prepared revelation solution in each well. Immediately after the washing step is completed.
8. Incubate the plate for 30 min. at 18-26°C, avoiding direct sunlight, on an horizontal shaker set at 700 +/- 100RPM.
9. Dispense 50 µl of stopping reagent into each well.
10. Read the absorbances within 1 hour at 450 nm and calculate the results as described in the section 9.

XII. READING AND RESULTS INTERPRETATION

- Read the microtiter plate at 450 nm (reference filter : 630 or 650 nm).

- Calculate the mean of duplicate determinations, rejecting obvious outlyers.
- For each calibrator or sample calculate

  $$B/BO \cdot 100 = \frac{OD \text{ (calibrator or samples)}}{OD \text{ (zero calibrator)}} \times 100$$

- Using either linear-linear or semi log graph paper, plot the (B/BO x 100) values for each calibrator point as a function of the Progesterone concentration of each calibrator point.
- By interpolation of the samples (B/BO x 100) values, determine the Progesterone concentration of the samples from the reference curve.

The 4 parameters logistic curve-fit is recommended.

XIII. EXAMPLE OF TYPICAL REFERENCE CURVE

The following data are for demonstration purpose only and cannot be used in place of data generated at the time of assay.

CALIBRATORS	OD units	B/BO x 100
0 ng/ml	1.767	100
0.20 ng/ml	1.569	88.8
0.75 ng/ml	1.236	69.9
2.00 ng/ml	0.813	46.1
7.50 ng/ml	0.442	25.1
20.00 ng/ml	0.233	13.2

XIV. EXPECTED VALUES

The normal values provided below are given for guidance. Each laboratory should establish its own normal range of values.

	Range (ng/ml)		Number of subjects
MALES	0.18	0.90	51
FEMALES			
- follicular phase	0.19	0.90	57
- luteal phase	1.6	18.6	57
- menopause	0.09	0.73	50
- pregnancy 1st trimester	4.4	33	40
- pregnancy 2nd trimester	18.5	107	40
- pregnancy 3rd trimester	50	224	43

Conversion factor : ng/ml x 3.18 = nmol/L
nmole/L x 0.314 = ng/ml

XV. PERFORMANCE CHARACTERISTICS

- Minimum detectable concentration (MDC)
  Minimum detectable Concentration of Progesterone in 20 different assays was 0.08 +/- 0.03 ng/ml.

- Specificity
  The percentage of cross-reactivity was estimated under physiological conditions in serum by comparison of the concentration yielding a 50 % binding inhibition :

Compound	Cross-reactivity (%)
Progesterone	100
17-α OH-Progesterone	0.95
20-α OH-Progesterone	0.056
5β-Pregnan-3α, 20α diol	0.046
5β-Pregnan-3,20 dione	0.30
Pregnenolone	0.16
Cortisol	0.005
Deoxycorticosterone	1.08
DHEA-SO4	0.009
Estrone	0.013
Norethisterone	0.011
Norgestrel	0.007

- Precision

INTRA ASSAY				INTER ASSAY			
Serum	**N**	**<X> ± SD (ng/ml)**	**CV (%)**	**Serum**	**N**	**<X> ± SD (ng/ml)**	**CV (%)**
1	16	1.51 ± 0.16	10.7	1	12	0.60 ± 0.05	12.5
2	16	6.43 ± 0.68	10.5	2	12	3.3 ± 0.29	8.8
3	16	10.30 ± 1.0	9.7	3	12	8.3 ± 0.90	15.5
				4	12	16.2 ± 1.54	9.6

- Accuracy

RECOVERY				DILUTION TEST			
Sample	Added (ng/ml)	Recovered (ng/ml)	Recovery (%)	Serum Dilution	Theoritical Conc. (ng/ml)	Measured Conc. (ng/ml)	Recovery (%)
Serum	20.0	22.3	111	1/1	16.8	16.8	100
	10.0	12.1	121	½	8.4	8.3	99
	5.0	5.3	106	¼	4.2	4.3	102
	1.67	1.9	114	1/8	2.1	2.2	100
				1/16	1.05	1.13	108
	20.0	17.2	86	1/1	4.0	4.0	100
	10.0	11.2	112	1/2	2.0	2.3	115
	5.0	4.8	96	1/4	1.0	1.1	110
	1.67	1.83	110	1/8	0.5	0.55	110

XVI. BIBLIOGRAPHY

1. ALPER M. et al (1987) Comparison of follicular fluid hormones in patients with one or two ovaries participating in a program of in vitro fertilization. Fertil. and Steril., 48, 1, 94-97.

2. GIDLEY-BAIRD A. et al. (1986) Failure of implantation in human in vitro fertilization and embryo transfer patients : the effects of altered progesterone/estrogen ratios in humans and mice. Fertil. Steril., 45, 1, 69-74.

3. HEINONEN P. et al (1985) Elevated progesterone levels in serum and ovarian venous blood in patients with ovarian tumors. Acta Obstet. Gynecol. Scand., 64, 649-652.

4. JANSSEN-CASPERS H. et al. (1986) Diagnosis of luteinized unruptured follicle by ultrasound and steroid hormone assays in peritonial fluid : a comparative study. Fertil. Steril., 46, 5, 823-827.

5. KO-ENHUANG et al. (1986) Serum progesterone levels in women treated with human menopausal gonadotropin and human chorionic gonadotropin for in vitro fertilization. Fertil. Steril., 46, 5, 903-906.

6. KRUITWAGEN R. et al. (1987) Oestradiol-17b and progesterone level changes in peritoneal fluid around the time of ovulation. British Journal of Obstetrics and Gynecology, 94, 548-553.

7. MATTHEWS C. (1986) Serum progesterone levels as an aid in the diagnosis of ectopic pregnancy. Obstetrics and Gynecology, 68, 390-394.

8. OSKOWITZ S. et al (1986) Luteal phase serum progesterone levels after follicle aspiration with and without clomiphene citrate treatment. Fertil. Steril., 46, 3, 461-465.

SUMMARY OF ASSAY PROCEDURE

	Calibrators (µl)	Controls-Samples (µl)
Calibrators (0-5)	50	-
Controls-samples	-	50
Progesterone-HRP	200	200
Incubate for 3 hours at 18-26°C with continuous shaking (700 RPM) Aspirate the content of each well Wash 3 times with 0.4 ml of wash solution and aspirate		
Substrate solution	200	200
Incubate 30 min. at 18-26°C with continuous shaking (700 RPM)		
HdSO4	50	50
Read the microtiter plate at 450 nm (versus 630 or 650 nm)		

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