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Instructions for use

Pregnenolone ELISA

REF FR E-2700

2°C – 8°C Σ 96 IVD CE

Pregnenolone ELISA

INTENDED USE

For the direct quantitative determination of Pregnenolone by an enzyme immunoassay in human serum.
For *in vitro* diagnostic use only.

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 patient 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 pregnenolone in the sample. A set of standards is used to plot a standard curve from which the concentration of pregnenolone in patient samples and controls can be directly read.

CLINICAL APPLICATIONS

Pregnenolone (3β-hydroxypregn-5-en-20-one) is the first steroid to be derived from cholesterol in the pathway of steroidogenesis, and it is the common precursor for all of the adrenal and gonadal steroids. Its production occurs in the mitochondrion by cleavage of the C-20 side chain of cholesterol by the P-450SCC enzyme. Once produced, pregnenolone may be utilized by two pathways of steroidogenesis. Pregnenolone may either be converted to 17-OH pregnenolone via the enzymatic action of 17α-hydroxylase or to progesterone via the enzymatic action of 3β-hydroxysteroid dehydrogenase.
Elevated pregnenolone levels occur in forms of congenital adrenal hyperplasia (CAH), due to 3β-hydroxysteroid dehydrogenase deficiency or 17α-hydroxylase deficiencies. Higher levels have also been reported in women with idiopathic hirsutism. Studies on pregnenolone levels in regard to sex and age differences indicate that maximum levels occur at approximately 17 and 16 years of age for women and men, while minimum levels occur at approximately 37 and 38 years of age for women and men, respectively. In general, women were found to have slightly higher values when compared to men.
Many areas of pregnenolone physiology remain to be investigated. Current research indicates that the determination of pregnenolone in serum may be useful for studying its metabolite, pregnenolone sulfate, which has been reported to have various effects in the mammalian brain and central nervous system.

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. It is recommended to all customers to prepare their own control materials or serum pools that 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 standard curve must be established for every run.
7. The controls provided in the kit should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, in- complete 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 pregnenolone in human serum. The kit is not calibrated for the determination of pregnenolone in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only Standard A may be used to dilute any high serum samples.
   The use of any other reagent may lead to false results.
5. The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, 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 clinical diagnosis should include all aspects of a patient's 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 specimen.

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.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.2 ml of serum is required per duplicate determination. Collect 4 – 5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store 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.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 50, 100, 150 and 300 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. 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. AA E-0030 WASH-CONC 10x **Wash Buffer Concentrate** – X10

Content:	One vial containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume:	50 ml/vial
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.

2. AA E-0055 SUBSTRATE **TMB Substrate** – Ready To Use

Content:	One vial containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume:	16 ml/vial
Storage:	Refrigerate at 2 – 8 °C
Stability:	12 months or as indicated on label.

3. AA E-0080 STOP-SOLN **Stopping Solution** – Ready To Use

Content: One vial containing 1 M sulfuric acid.

Volume: 6 ml/vial

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

Hazards identification:

H315 Causes skin irritation.
H319 Causes serious eye irritation.

4. Standards and **Controls** – Ready To Use

Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Symbol	Standard	Concentration	Volume/Vial
FR E-2701	STANDARD A	Standard A	0 ng/ml	2.0 ml
FR E-2702	STANDARD B	Standard B	0.1 ng/ml	0.5 ml
FR E-2703	STANDARD C	Standard C	0.4 ng/ml	0.5 ml
FR E-2704	STANDARD D	Standard D	1.6 ng/ml	0.5 ml
FR E-2705	STANDARD E	Standard E	6.4 ng/ml	0.5 ml
FR E-2706	STANDARD F	Standard F	25.6 ng/ml	0.5 ml
FR E-2751	CONTROL 1	Control 1	Refer to vial labels for acceptable range!	0.5 ml
FR E-2752	CONTROL 2	Control 2		0.5 ml

Content: Pregnenolone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of pregnenolone.

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.

5. FR E-2713 ASSAY-BUFF **Assay Buffer** – Ready To Use

Content: One vial containing a protein-based buffer with a non-mercury preservative.

Volume: 15 ml/vial

Storage: Refrigerate at 2 – 8 °C

Stability: 12 months or as indicated on label.

6. FR E-2731 96 **Rabbit Anti-Pregnenolone Antibody Coated Microwell Plate – Break Apart Wells** – Ready To Use

Content: 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.

7. FR E-2740 CONJUGATE-CONC 50x **Pregnenolone-Horseradish Peroxidase (HRP) Conjugate Concentrate** – X50

Content: Pregnenolone-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 1:50 in assay buffer before use (e.g. 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12 ml of assay buffer. Discard any that is left over.

ASSAY PROCEDURE

Specimen Pretreatment: **None.**

All reagents must reach room temperature before use. Standards, controls and specimen 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 **pregnenolone-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 **standard, control and specimen 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.
8.	Incubate on a plate shaker for **10 – 15 minutes** at **room temperature** *(or until Standard 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 patient / control samples.*

CALCULATIONS

1. Calculate the mean optical density of each standard duplicate.
2. Draw a standard curve on semi-log paper with the mean optical densities on the Y-axis and the standard 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 standard curve.
5. If a sample reads more than 25.6 ng/ml then dilute it with Standard A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

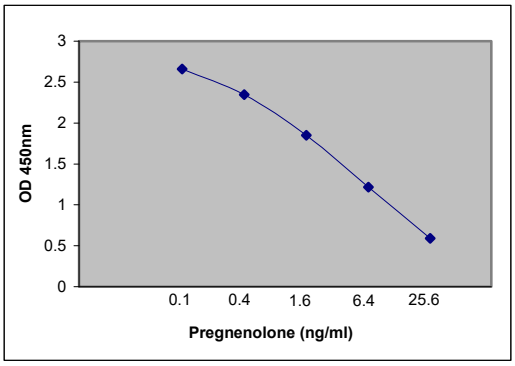
TYPICAL TABULATED DATA

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

Standard	OD 1	OD 2	Mean OD	Value (ng/ml)
A	2.891	2.808	2.850	0
B	2.613	2.651	2.632	0.1
C	2.350	2.343	2.347	0.4
D	1.823	1.879	1.851	1.6
E	1.237	1.197	1.217	6.4
F	0.589	0.594	0.591	25.6
Unknown	1.431	1.451	1.441	4.0

TYPICAL STANDARD 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 Standard A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Pregnenolone ELISA kit is **0.05 ng/ml**.

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with the Pregnenolone ELISA kit with pregnenolone cross-reacting at 100%.

Steroid	% Cross Reactivity
Pregnenolone	100
Progesterone	6.0
Dehydroisoandrosterone	5.2
5α-Androstandiol	4.7
Epiandrosterone	1.0
Pregnenolone Sulfate	0.4
Androstandione	0.3
5α-Androsterone	0.3
DHEAS	0.2
Etiocholanolone	0.1

The following steroids were tested but cross-reacted at less than 0.1%: Adrenosterone, Aldosterone, Androstenedione, Cholesterol, Corticosterone, 5α-DHT, 17β-Estradiol, Estriol and Testosterone.

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same standard curve. The results (in ng/ml) are tabulated below:

Sample	Mean	SD	CV %
1	0.19	0.02	10.6
2	1.04	0.85	8.2
3	4.77	0.37	7.8

INTER-ASSAY PRECISION

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

Sample	Mean	SD	CV %
1	0.22	0.03	14.5
2	1.14	0.14	12.3
3	4.56	0.44	9.6

RECOVERY
Spiked samples were prepared by adding defined amounts of pregnenolone to four patient serum samples. The results (in ng/ml) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1 Unspiked + 4.14	0.37 5.31	– 4.51	– 117.7
2 Unspiked + 4.01	0.77 5.69	– 4.78	– 119.0
3 Unspiked + 3.98	0.85 5.18	– 4.83	– 107.2
4 Unspiked + 3.78	1.47 6.31	– 5.25	– 120.2

LINEARITY
Three patient serum samples were diluted with Standard A. The results (in ng/ml) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1 1:2 1:4 1:8	5.31 2.89 1.26 0.71	– 2.66 1.33 0.66	– 108.6 94.7 107.6
2 1:2 1:4 1:8	6.51 2.75 1.54 0.80	– 3.26 1.63 0.81	– 84.4 94.5 98.8
3 1:2 1:4 1:8	8.34 3.78 2.15 1.05	– 4.17 2.09 1.04	– 90.6 102.9 101.0

EXPECTED NORMAL VALUES
As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Group	N	Mean (ng/ml)	Abs. Range (ng/ml)
Males	30	0.50	0.1 – 3.4
Females	50	0.55	0.1 – 3.8

REFERENCES

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CHANGE HISTORY

Previous Version:	7.0	**New Version:**	7.0a
Changes:	***REAGENTS PROVIDED*** Hazard labelling for component AA E-0080 updated		

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date	LOT	Batch code	IVD	For in-vitro diagnostic use only!
	Consult instructions for use	CONT	Content		CE marking of conformity
	Caution	REF	Catalogue number		Distributor
	Date of manufacture				