DRG® Pregnenolone (EIA-4912)

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IVD

INTENDED USE

For the direct quantitative determination of Pregnenolone by enzyme immunoassay in human serum.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows a two-step competitive binding scenario.

During the first incubation, competition occurs between an unlabeled antigen (present in calibrators, control and patient samples) and a biotin-labelled antigen (biotin conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. During the second incubation, the streptavidin-HRP (HRP conjugate) is added and binds to the biotin-conjugate. 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 calibrators is used to plot a calibrator curve from which the amount 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 $P\text{-}450_{SCC}$ 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's 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 calibrator curve must be established for every run.
7. The control provided in the kit 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 control do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells 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, calibrator 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 calibrator 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 control 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. However no test method 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.

1.1 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. Microwell plate reader with a filter set at 450 nm and an upper OD limit of 3.0 or greater* (see assay procedure step 13).

REAGENTS PROVIDED

1. Rabbit Anti-Pregnenolone Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.

Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label.

2. Pregnenolone-Biotin Conjugate Concentrate - X50

Contents: Pregnenolone-Biotin conjugate in a protein-based buffer with a non-mercury preservative.

Volume: 300 µL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label.

Preparation: Dilute **1:50** in *biotin conjugate buffer* before use (e.g. 40 µL of biotin conjugate in 2 mL of that buffer. If the whole plate is to be used dilute 240 µL of biotin conjugate in 12 mL of that buffer). Discard any that is left over.

3. Streptavidin-Horseradish Peroxidase (HRP) Conjugate Concentrate – X50

Contents: Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative.

Volume: 400 µL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label.

Preparation: Dilute **1:50** in *HRP conjugate buffer* before use (e.g. 40 µL of HRP conjugate in 2 mL of that buffer. If the whole plate is to be used dilute 350 µL of HRP conjugate in 17 mL of that buffer). Discard any that is left over.

4. Pregnenolone Calibrators - Ready To Use.

Contents: Six vials containing pregnenolone in a human serum matrix with a non-mercury preservative. Prepared by spiking the matrix with a defined quantity of pregnenolone.

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

Standard	Concentration	Volume/Vial
Standard A	0 ng/mL	2.0 mL
Standard B	0.1 ng/mL	0.5 mL
Standard C	0.4 ng/mL	0.5 mL
Standard D	1.6 ng/mL	0.5 mL
Standard E	6.4 ng/mL	0.5 mL
Standard F	25.6 ng/mL	0.5 mL

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months if properly stored.

5. Control - Ready To Use.

Contents: One vial containing pregnenolone in a human serum matrix with a non-mercury preservative. Prepared by spiking the matrix with a defined quantity of pregnenolone.
Refer to vial label for expected value and acceptable range.

Volume: 0.5 mL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months if properly stored.

6. Biotin Conjugate Buffer - Ready To Use.

Contents: One vial containing a proprietary buffer with a non-mercury preservative.

Volume: 15 mL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label.

7. HRP Conjugate Buffer - Ready To Use.

Contents: One vial containing a proprietary buffer with a non-mercury preservative.

Volume: 20 mL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label

8. Wash Buffer Concentrate - X10

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.

Volume: 50 mL/bottle

Storage: Refrigerate at 2°C - 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.

9. 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°C - 8°C

Stability: 12 months or as indicated on label.

10. Stop Solution - Ready To Use.

Contents: One vial containing 1M sulfuric acid.

Volume: 6 mL/vial

Storage: Refrigerate at 2°C - 8°C

Stability: 12 months or as indicated on label.

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 biotin conjugate, HRP conjugate and wash buffer.
2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 50 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the biotin 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 5 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 HRP conjugate working solution into each well (We recommend using a multi-channel pipette).
8. Incubate on a plate shaker (approximately 200 rpm) for 30 minuets at room temperature
9. Wash the wells 5 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).
10. Pipette 150 µL of TMB substrate into each well at timed intervals.
11. Incubate on a plate shaker for 10-15 minutes at room temperature
12. Pipette 50 µL of stop solution into each well at the same timed intervals as in step 10.
13. Read the plate on a microwell plate reader at 450 nm within 20 minutes after addition of the stop 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 calibrator duplicate.
2. Draw a calibrator 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 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 25.6 ng/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

Standard	OD 1	OD 2	Mean OD	Value (ng/mL)
A	2.558	2.415	2.487	0
B	2.050	1.977	2.014	0.1
C	1.449	1.480	1.465	0.4
D	1.245	1.299	1.272	1.6
E	0.808	0.727	0.768	6.4
F	0.393	0.386	0.390	25.6
Unknown	0.903	0.907	0.905	4.5

PERFORMANCE CHARACTERISTICS

1.2 SENSITIVITY

The lower detection limit is defined as the concentration of Pregnenolone needed to give a B/B0 values equivalent to the point where B is equal to B0 minus 2X the SD of B0. Based on 20 replicate analyses of calibrator A, the sensitivity is 0.054 ng/mL.

1.3 SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross reactivity with the Direct 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.

1.4 INTRA-ASSAY PRECISION

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

Sample	Mean	SD	CV%
1	0.52	0.02	9.8
2	1.88	0.16	8.7
3	3.96	0.36	9.1

1.5 INTER-ASSAY PRECISION

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

Sample	Mean	SD	CV%
1	0.58	0.07	11.7
2	1.76	0.15	8.4
3	4.22	0.41	9.8

1.6 RECOVERY

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

Sample	Obs.Result	Exp.Result	Recovery%
1 Unspiked +6.4(10:1) +6.4(10:2) +25.6(10:2)	0.55 1.26 1.81 5.55	- 1.08 1.53 4.73	- 116.7 118.3 117.3
2 Unspiked +6.4(10:1) +6.4(10:2) +25.6(10:2)	0.83 1.36 1.86 5.57	- 1.34 1.76 4.89	- 101.5 105.7 113.9

1.7 LINEARITY

Two patient serum samples were diluted with calibrator A. The results (in ng/mL) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1	5.57	-	-
1:2	2.48	2.79	88.9
1:4	1.17	1.39	84.2
1:8	0.58	0.70	82.9
2	5.55	-	-
1:2	2.82	2.78	101.4
1:4	1.42	1.39	102.2
1:8	0.66	0.70	94.3

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. Below is a primary study result that should be only used as a guideline

Group	N	Mean (ng/mL)	Abs. Range (ng/mL)
Males	10	0.78	0.38 - 3.5
Females	10	0.84	0.31 - 3.8

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