Instructions for Use

17-OH Progesterone Neonatal ELISA

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REF EIA-1429

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Please use only the valid version of the Instructions for Use provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Gebrauchsanweisung.
Si prega di usare la versione valida delle istruzioni per l'uso a disposizione con il kit.
Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit.
Utilisez seulement la version valide des Instructions d'utilisation fournies avec le kit.

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1 INTRODUCTION

Intended Use:

The quantitative determination of 17-hydroxyprogesterone concentration in human whole blood by a microplate enzyme immunoassay, colorimetric.

2 SUMMARY AND EXPLANATION OF THE TEST

The results are used to screen newborns for CAH (congenital adrenal hyperplasia). CAH is a genetic disorder, 90% of which is caused by 21-hydroxylase deficiency. The incidence is roughly estimated to be 1 in 15,0000 newborns and can reach as high as 1 in 1480 in native Alaskans. Early diagnosis is valuable to detect CAH in newborns afflicted with the disease, not clinically recognizable but which will lead to life threatening adrenal crisis in the neonatal period and to determine the cause of infants with ambiguous genitalia. Delayed diagnosis may also lead to further virilization in female children, acceleration of skeletal maturation and premature development of secondary sex characteristics in male children. Prompt treatment can save the life of infants and allow afflicted children to attain normal growth.

17-OHP is a steroid produced in the adrenal cortex and the gonads. It is the immediate precursor to 11-desoxycortisol (CpS) which is converted to cortisol. Because CpS is produced by 21-hydroxylation of 17-OHP, measurement of 17-OHP is an indirect indicator of 21- hydroxylase activity. CAH occurs where there is a deficiency of this enzyme. The result is a decrease in the conversion of 17-OHP to CpS which blocks the normal synthesis of cortisol. Due to the feedback mechanism, a decrease in cortisol causes an increase in ACTH secretion resulting in adrenal hyperplasia. As 17-OHP is not being converted, increased concentrations of this steroid will be found.

17-OHP concentration increases during pregnancy in the maternal and fetal blood. After birth, values decline rapidly to reach normal adult values in 2 to 7 days. Thus it is advisable not to collect samples before the 3rd day of life. Premature and sick term infants exhibit 2 to 3 fold 17-OHP values with no CAH disorder.

It is suggested that a different cut-off be adopted to pre-term and sick infants.

3 PRINCIPLE

Delayed Equilibrium Enzyme Immunoassay:

The essential reagents required for an enzyme immunoassay include antibody, enzyme-antigen conjugate and native antigen. Upon mixing the biotinylated antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation:

$$Ag + Ab_{(Btn)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} AgAb_{(Btn)}$$

$Ab_{(Btn)}$ = Specific Biotinylatedl Antibody (Constant Quantity)

Ag = Native Antigen (Variable Quantity)

$AgAb_{(Btn)}$ = Antigen-Antibody complex (Variable Quantity)

k_a = Rate Constant of Association

k_{-a} = Rate Constant of Dissociation

$K = k_a / k_{-a}$ =Equilibrium Constant

After a short period, the enzyme conjugate is added. (This delayed addition permits an increase in sensitivity for low concentration samples and better precision). Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of anibody finding sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{(Btn)} \rightleftharpoons AgAb_{(Btn)} + {}^{Enz}AgAb_{(Btn)}$$

$Enz_{AgAb_{(Btn)}}$ = Enzyme-Antigen (Constant Quantity)

$rAb_{(Btn)}$ = Remaining biotinylated antibody not consumed

Simultaneously, the immune complex is immobilized through the interaction with streptavidin coated to the well. Unbound reactants (17-OHP and 17-OHP-HRP) are removed at the end of the incubation time.

$$AgAb_{(Btn)} + {}^{Enz}AgAb_{(Btn)} + \underline{Streptavidin}_{CW} \Rightarrow Immobilized\ Complex\ (IC)$$

$Streptavidin_{CW}$ = Streptavidin immobilized on well

Immobilized complex (IC) = Ag-Ab bound to the well

A substrate is then reacted with the enzyme bound on the wall of the microwells. The enzymatic reaction is terminated with an acid. The end product is measured at 450 nm. The 17-OHP of the unknown sample is determined using a calibration curve generated with known concentration of 17-OHP:

$$^{Enz}AgAb_{(CW)} + Substrate \longrightarrow Color\ (450nm)$$

$$^{Enz}AgAb_{(IC)} = immobilized\ enzyme\ bound\ reactant$$

4 REAGENTS

4.1 Material Provided

A) **N-17-OHP Calibrators – Dried Blood Spots (Two rows by six dots levels – 2 x 6)**
Six (6) levels of N-17-OHP calibrators in dried blood spots (adjusted to 55% hematocrit) at approximate concentrations of 0 (A), 6 (B), 13.5 (C), 26 (D) 53 (E) and 105 (F) ng/mL placed on WHATMAN type 903 filter paper.
Store at 2 °C - 8 °C. A preservative has been added.

A	B	B	D	E	F
0 ng/mL	6 ng/mL	13.5 ng/mL	26 ng/mL	53 ng/mL	100 ng/mL

B) **N-17-OHP Controls – Dried Blood Spots (Two rows by three dots – 2 x 3)**
Three (3) levels of N-17-OHP controls in dried blood spots (adjusted to 55% hematocrit) with different concentrations placed on WHATMAN type 903 filter paper in circles C1, C2, and C3.
Store at 2 °C - 8 °C. A preservative has been added.

Note 1: ***Calibrator values and control values are Lot Specific*** and were manufactured to fall within significant clinical ranges. The exact values are printed on the outside of the aluminum pouch used for storage.

Note 2: The Lot Specific calibrators, whole human blood based, were verified with the N-17-OHP blood spots supplied by CDC.

Note 3: Do not use blood spots with appearance of caking, clotting, or moisture.

C) **N-17-OHP Biotin Reagent – 2 x 13 mL vial**
Anti-N-17-OHP polyclonal IgG labeled with biotin in buffer with green dye. A preservative has been added.
Store at 2 °C - 8 °C.

D) **NN17-OHP Enzyme Reagent – 2 x 7 mL/vial**
N-17-OHP horseradish peroxidase (HRP) conjugates in a proteinstabilizing matrix with red dye. A preservative has been added. Store at 2 °C - 8 °C.

E) **Streptavidin Coated Plate – 2 x 96 wells**
96-well microplates coated with streptavidin and packaged in an aluminum bag with a drying agent.
Store at 2 °C - 8 °C.

F) **Wash Solution Concentrate – 1 x 20 mL/vial**
One vial containing a surfactant in buffered saline. A preservative has been added. Store at 2 °C - 8 °C.

G) **Substrate Solution – 2 x 14 mL/vial**
Tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) in buffer. Store at 2 °C - 8 °C.

H) **Stop Solution – 2 x 8 mL/vial**
A strong acid (0.5M H_2SO_4). Store at 2 °C - 8 °C.

Note 1: Do not use reagents beyond that of expiration date.

Note 2: Avoid extended exposure to heat and light. ***Opened reagents are stable for sixty (60) days when stored at 2 °C - 8 °C. Kit and component stability are identified on the label.***

Note 3: Above reagents are for a two 96-well plate kit

4.2 Required But Not Provided

1. Laboratory shaker capable of 150 rpm rotation.
2. Dispenser(s) for repetitive deliveries of 0.050 mL, 0.100 mL, and 0.350 mL (50, 100 & 350 µL) volumes with a precision of better than 1.5%.
3. Adjustable volume (20 - 200 µL) and (200 - 1000 µL) dispenser(s) for conjugate dilutions.
4. 1/8 inch hole punch.
5. Tweezers to pick up the punched spots
6. Microplate washer or a squeeze bottle (optional).
7. Microplate reader capable of absorbance readings at 450 nm and 620 nm.
8. Absorbent paper for blotting the microplate wells.
9. Plastic wrap and microplate cover for incubation steps.
10. Vacuum aspirator (optional) for wash steps.
11. Timer
12. External quality control.

5 PRECAUTIONS

For In Vitro Diagnostic Use

Not for Internal or External Use in Humans or Animals

All products that contain human blood have been found to be non- reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA required tests. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, “Biosafety in Microbiological and Biomedical Laboratories,” 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

Safe disposal of kit components must be according to local and regulatory statutory requirements.

6 SPECIMEN COLLECTION AND PREPARATION

Follow the guidelines in the NCCLS publication LA4T (7) for collecting blood samples in the neonatal screening program; copies of which can be obtained from: NCCLS, 771 E. Lancaster Ave., Villanova, PA 19085.

Use WHATMAN type 903.

For samples screening for CAH, collect samples 3 to 5 days after birth.

Use disposable lancets with tips less than 2.5 mm to prick the medial or lateral sides of the bottom of the heel. Allow a drop of blood to form with sufficient volume to fill a 5/8 inch diameter spot on filter paper.

Gently touch the drop of blood with the filter paper. DO NOT PRESS AGAINST THE SKIN. DO NOT TOUCH SPOTTED AREA.

Suspend spotted papers horizontally and allow to dry at room temperature for a minimum of 3 hours.

Avoid spots touching other surfaces and keep away from direct light. The samples should be transported (13) to the laboratory within 24 hours after collection in appropriate storage container. The laboratory should store the specimens at 2 °C - 8 °C protected from moisture and direct light.

Blood spots are stable for at least 3 weeks at 2 °C - 8 °C protected from light and moisture.

Reject samples with the following conditions:

1. Specimens not collected in WHATMAN type 903 paper.
2. Blood spots not completely saturated on both sides.
3. Blood spots with appearance of caking or clotting.
4. Blood spots with appearance of moisture.

7 QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal, and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicated unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8 REAGENT PREPARATION

Wash Buffer

Dilute contents of wash solution to 1000 mL with distilled or deionized water in a suitable storage container.
The reagent can be stored at 2 °C - 30 °C for up to 60 days.

Note 1: Do not use the substrate if it looks blue.
Note 2: Do not use reagents that are contaminated or have bacteria growth.

9 TEST PROCEDURE

Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 °C - 27 °C).
** Test procedure should be performed by a skilled individual or trained professional**

1. Assemble the required number of microwells for each calibrator, control, and patient sample to be assayed in duplicate. *Replace any unused microwell strips back into the aluminum bag, seal and store at 2 °C - 8 °C*.
2. Punch out 1/8" blood dot out of each calibrator, control, and specimen into the assigned wells.
   (NOTE: Do not punch blood dots from areas that are printed or that are near the edge of the blood spot).
3. Add 0.100 mL (100 µL) of N-17-OHP Biotin Reagent to all the wells.
4. Shake the microplate gently for 20-30 seconds to mix.
   (NOTE: Make sure that all blood dots are fully submerged in the liquid and not stuck to the walls of the microwells).
5. Cover with a microplate cover and rotate for **30 minutes at ambient temperature** using a laboratory rotator set at 150 rpm.
6. Remove from shaker and add 0.050 mL (50 µL) of N-17-OHP Enzyme Reagent directly to each well.
   Do not remove the reactants (DBS) in the well.
7. Shake the microplate gently for 20-30 seconds to mix.
   (NOTE: Make sure that all blood dots are fully submerged in the liquid and not stuck to the walls of the microwells).
8. Cover with a microplate cover and rotate for **90 minutes at ambient temperature** using a laboratory rotator set at 150 rpm.
9. Discard the contents of the microplate by decantation or aspiration.
   If decanting, blot the plate dry with absorbent paper.
   NOTE: Make sure all the blood spot are removed at this point. There should be no dots left in the microwells.
10. Add 0.350 mL (350 µL) of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat four (4) additional times for a total of five (5) washes.
    An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat four (4) additional times.
11. Add 0.100 mL (100 µL) of substrate solution (color developer) to each well.
    Do not shake plate after substrate addition.
12. Cover the microplate and incubate for 15 minutes at ambient temperature.
13. Add 0.050 mL (50 µL) of stop solution to each well and gently mix until a uniform color is obtained.
    NOTE: Always add reagents in the same order to minimize reaction time differences between wells.
14. Read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader.
    The results should be read within fifteen (15) minutes of adding the stop solution.

Note:
Calibrators and controls are provided in the kit and should be assayed n duplicates.
Each plate is limited to 38 patient samples when calibrators and controls are included. Should there be more than 38 samples needed to assay at the same time, it is advisable to assay 1 set of calibrator and control in singlets per plate and take the average O.D from each plate for calculation. Assay no more than 3 plates at a time. This means the maximum patient sample load is 129 samples.

10 CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of Neonatal 17OHP in unknown specimens.

1. Record the absorbance obtained from the printout of the microplate reader as outlined in Example 1.
2. Plot the absorbance for each duplicate serum reference versus the corresponding N-17-OHP concentration in ng/mL on semi-log graph paper (average the duplicates of the serum references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of N-17-OHP for an unknown, locate the average absorbance of the duplicates for each unknown on the vertical axis of the graph, find the intersecting point on the curve, and read the concentration (in ng/mL) from the horizontal axis of the graph (the duplicates of the unknown may be averaged as indicated). In the following example, the average absorbance (0.521) intersects the dose response curve at (47.0 ng/mL) 17-OHP concentration.

NOTE: Computer data reduction software designed for ELISA assays may also be used for the data reduction. If such data reduction software is utilized, the validation of the software should be ascertained.

EXAMPLE 1 (30+90+15 min Procedure)

Sample I.D	Well Number	Abs (A)	Mean Abs (B)	Value (ng/mL)
Cal A	A1	2.330	2.324	0.0
	B1	2.318		
Cal B	C1	1.602	2.606	6.0
	D1	1.609		
Cal C	E1	1.098	1.099	13.5
	F1	1.100		
Cal D	G1	0.789	0.786	26.0
	H1	0.783		
Cal E	A2	0.483	0.466	53.0
	B2	0.452		
Cal F	C2	0.309	0.290	105.0
	D2	0.270		
Control 1	E2	1.223	1.204	11.4
	F2	1.185		
Control 2	G2	0.687	0.701	31.9
	H2	0.715		
Patient	A3	0.516	0.521	47.0
	B3	0.526		

*The data presented in Example 1 is for illustration only and should not be used in lieu of a dose response curve prepared with each assay.

11 QC PARAMETERS

In order for the assay results to be considered valid, the following criteria should be met:

1. The absorbance (OD) of calibrator 0 ng/mL should be ≥ 1.3.
2. Four out of six quality pools should be within the established ranges.

12 RISK ANALYSIS

12.1 Assay Performance

1. It is important that the time of reaction in each well is held constant to achieve reproducible results.
2. Pipetting of reagents should not extend beyond ten (10) minutes to avoid assay drift.
3. If more than one (1) plate is used, it is recommended to repeat the dose response curve.
4. The addition of substrate solution initiates a kinetic reaction, which is terminated by the addition of the stop solution. Therefore, the substrate and stop solution should be added in the same sequence to eliminate any time-deviation during reaction.
5. Plate readers measure vertically. Do not touch the bottom of the wells.
6. Failure to remove adhering solution adequately in the aspiration or decantation wash step(s) may result in poor replication and spurious results.
7. Use components from the same lot. No intermixing of reagents from different batches.
8. Accurate and precise pipetting, as well as following the exact time and temperature requirements prescribed are essential. Any deviation from the IFU may yield inaccurate results.
9. All applicable national standards, regulations, and laws, including, but not limited to, good laboratory procedures, must be strictly followed to ensure compliance and proper device usage.
10. It is important to calibrate all the equipment e.g. Pipettes, Readers, Washers and/or the automated instruments used with this device, and to perform routine preventative maintenance.

12.2 Interpretation

1. **Measurements and interpretation of results must be performed by a skilled individual or a trained professional.**
2. Laboratory results alone are only one aspect for determining patient care and should not be the sole basis for therapy, particularly if the results conflict with other determinants.
3. For valid test results, adequate controls and other parameters must be within the listed ranges and assay requirements.
4. If test kits are altered, such as by mixing parts of different kits, which could produce false test results, or if results are incorrectly interpreted, DRG shall have no liability.
5. If computer controlled data reduction is used to interpret the results of the test, it is imperative that the predicted values for the calibrators fall with 10% of the assigned concentrations.
6. This assay is intended solely to screen CAH in newborns. It is not to be used for confirmatory testing, monitor therapy, or prenatal testing.
7. The N-17-OHP blood spot screening detects only CAH caused by 21-hydroxylase deficiency which accounts for approximately 90% of the disorder (1).
   It will not detect CAH caused by deficiency of other enzymes, notably 11-β-hydroxylase deficiencies.
8. Premature and infants with low birth weights tend to have higher 17-OHP values (6).
9. Samples collected prior to the second day of life tend to have higher 17-OHP values due to placental cross over (5).
10. This is a screening test. Blood spots with elevated 17-OHP values should be confirmed with an extracted 17-OHP assay using serum samples.

13 EXPECTED RANGE OF VALUES

REPORTABLE RANGE: Analytical Range = 5 – 105 ng/mL

Samples that fall within the calibration curve should be reported as such.

Samples that fall outside the calibration curve should be reported less than <5 ng/mL or greater than >105 ng/mL. Detection limit for the assay is 0.6 ng/mL.

The following is a guideline from screening programs reported in literatures (2&10). For assays that meet the individual lab Q.C. criteria, report results less than 22 ng/mL. Request a second specimen for values 22 – 35 ng/mL. Values greater than 35 ng/mL should be confirmed with an extracted 17-OHP assay using serum samples.

Since pre-term infants have 17-OHP concentrations much higher than normal full term babies, cut off level of 80 pg/disk (11) or 57 ng/mL serum equivalence (assuming each 3 mm disk contains 1.4 µL of serum) has been suggested.

One hundred forty eight normal neonatal blood spot samples free of steroid treatments obtained from a public health screening laboratory were assayed in this 17-OHP kit.

Calculated mean for the assay is 12.2 ng/mL with 69% of the values falling between 5 – 15 ng/mL.

14 PERFORMANCE CHARACTERISTICS

14.1 Precision

The within and between assay precisions of the Neonatal 17-OHP ELISA kit were determined by analyses on three different levels of dried blood controls. The number (N), mean values, standard deviation (S.D.), and coefficient of variation (C.V.) for each of these controls are presented in Table 1 and Table 2.

Table 1: Within Assay Precision (Values in ng/mL)

Sample	N	Mean	S.D.	C.V.
Low	18	12.2	1.02	8.4%
Mid	18	34.3	2.67	7.8%
High	18	67.2	3.58	5.3%

Table 2: Between Assay Precision (Values in ng/mL)

Sample	N	Mean	S.D.	C.V.
Low	10	13.2	1.19	9.0%
Mid	10	35.4	3.05	8.6%
High	10	73.2	5.23	7.1%

*** As measured in duplicate.

14.2 Sensitivity

The Neonatal 17-OHP ELISA kit has a sensitivity of 0.6 ng/mL.

The sensitivity was ascertained by determining the variability of the 0 ng/mL calibrator and using the 2 S.D. (95% certainty) statistic to calculate the minimum dose.

14.3 Accuracy

The Neonatal 17-OHP ELISA kit was compared with a predicate 17-OHP method. Dried blood spots with concentrations from 5 – 80 ng/mL were used. The total number of such specimens was 54.

The least square regression equation and the correlation coefficient were computed for this NN17-OHP ELISA method in comparison with the reference method. The data obtained is displayed in Table 3.

Table 3

Method	Least Squares Regression Analysis	Correlation Coefficient
This Method (y)	y = 1.03(x) + 0.95	0.987

Reference (x)

Only slight amounts of bias between this method and the reference method are indicated by the closeness of the values.

The least square regression equation and correlation coefficient indicates good method agreement.

14.4 Specificity

The antiserum used in the assay is highly specific for the detection of 17α-hydroxyprogesterone. The following naturally occurring steroids are spiked in a pooled human whole blood adjusted to 55% hematocrit at different concentrations. The preparation is spotted on WHATMAN type 903 filter paper, dried and assayed. The percentage indicated is the cross reactivity at 50% intercept.

Compound	In ng/mL Concentration	% Cross-Reactivity
11-desoxycortisol	78 - 20,000	5.2
Progesterone	78 - 20,000	4.6
17α-hydroxypregnenolone	78 - 20,000	3.7
17α-hydroxypregnenolone sulfate	600 - 10,000	1.4
Pregnenolone sulfate	78 - 20,000	<0.1
Desoxycorticosterone	20,000	<0.1
Aldosterone	20,000	<0.1
Cholesterol	20,000	<0.1
Corticosterone	20,000	<0.1
Cortisol	20,000	<0.1
Dehydroepiandrosterone	20,000	<0.1
Dihydrotestosterone	20,000	<0.1
17α-estradiol	20,000	<0.1
17β-estradiol	20,000	<0.1
Estriol	20,000	<0.1
Estrone	20,000	<0.1
Testosterone	20,000	<0.1

15 REFERENCES / LITERATURE

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SYMBOLS USED

Symbol	English	Deutsch	Italiano	Español	Français
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes
	Consult instructions for use *	Gebrauchsanweisung beachten *	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation
IVD	*In vitro* diagnostic medical device *	*In-vitro*-Diagnostikum *	Dispositivo medico-diagnostico in vitro	Producto sanitario para diagnóstico In vitro	Dispositif médical de diagnostic in vitro
REF	Catalogue number *	Artikelnummer *	Numero di Catalogo	Nûmero de catálogo	Référence de catalogue
LOT	Batch code *	Chargencode *	Codice del lotto	Codigo de lote	Numéro de lot
	Contains sufficient for <n> tests *	Ausreichend für <n> Prüfungen *	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos	Contenu suffisant pour "n" tests
	Temperature limit *	Temperaturbegrenzung *	Temperatura di conservazione	Temperatura de conservacion	Température de conservation
	Use-by date *	Verwendbar bis *	Utilizzare prima del	Establa hasta	Utiliser jusque
	Manufacturer *	Hersteller *	Fabbricante	Fabricante	Fabricant
	Caution *	Achtung *			
RUO	For research use only	Nur für Forschungszwecke	Solo a scopo di ricerca	Sólo para uso en investigación	Seulement dans le cadre de recherches
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