

# Neonatal 17-OH- Progesterone EIA

CE



480 / 960 tests



**Instructions for use**

IVD



Ani Labsystems Ltd. Oy  
Tiilitie 3, FIN-01720 Vantaa, Finland  
Tel. +358-20-155 7523, Fax +358-20-155 7521  
E-mail: [sales@anilabsystems.com](mailto:sales@anilabsystems.com)  
[www.anilabsystems.com](http://www.anilabsystems.com)

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# Instructions for use

## For *in vitro* diagnostic use only

Enzyme immunoassay with colorimetric detection for the determination of human 17-hydroxyprogesterone from blood samples dried on filter paper.

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## INTENDED USE

Ani Lab systems Neonatal 17-OH-Progesterone EIA test is a simple competition immunoassay enabling the quantification of 17-hydroxyprogesterone steroid present in blood samples dried on filter paper.

## INTRODUCTION

Congenital Adrenal Hyperplasia (CAH) includes a group of disorders in which the cortisol production by adrenals is lowered. Cortisol is a steroid hormone necessary to maintenance of blood sugar level, partial maintenance of body fluids and electrolytes and protection of the body against stress. There are several different forms of CAH, each related to one of the enzymes necessary to transform precursors to cortisol [1-2]. When one of these enzymes is deficient, this leads to a hyperfunction and increased size (hyperplasia) of the adrenals, hence the name Congenital Adrenal Hyperplasia. Three types of adrenal steroids hormones are produced in abnormal amounts in people with CAH, glucocorticoids ("sugar hormones"), mineralocorticoids ("salt-water hormones") and androgens ("male hormones") [1].

Among the various forms of CAH, the 21-hydroxylase deficiency is by far the most frequent, representing more than 90% of all cases. 21-hydroxylase deficiency results in high level of circulating 17-hydroxyprogesterone. Various mutations of the 21-hydroxylase gene result in various degrees of CAH severity.

The Salt-Losing Form of CAH (most severe) is the result of a total or near-total deficiency of the 21-hydroxylase enzyme. This results in the complete inability to produce cortisol and aldosterone. The body's total inability to produce cortisol leads to a build-up of cortisol precursors (i.e., 17-hydroxyprogesterone and androgens) which leads to hypoglycemia, water loss, salt-losing tendency and masculinization. Cortisol and aldosterone are necessary to

life. Infants with complete 21-hydroxylase deficiency (salt-losing form) die shortly after birth as a result of a salt-losing crisis.

The Simple-Virilizing Form of CAH (moderate severity) refers to a partial deficiency of the 21-hydroxylase enzyme. Similar to the salt-losing patients, simple-virilizing patients experience an increase in the production of 17-hydroxyprogesterone as well as adrenal androgens and a salt-losing tendency.

Excess androgen production during fetal life, associated with salt-losing and simple-virilizing CAH, masculinizes the external genitalia of female infants. At puberty, this includes the early appearance of body hair, acne and increased musculature in both sexes, normal feminization of girls fails to occur (no breast development, no menstruation) [3]. For women left untreated, fertility is compromised due to the absence of ovulation. In both sexes, the large amount of androgens produce rapid growth in height, but also early bone maturation resulting in short stature at adulthood [4].

Non-classic or Late-Onset Form of CAH (least severe) refers to a mild deficiency of the 21-hydroxylase enzyme. People with late-onset CAH start to exhibit symptoms related to excess androgen production in childhood or adolescence. In boys and girls, this results in rapid growth and early virilization. In girls, this can also result in masculinization and abnormal menses. A study based on Italian population showed that the incidence on new borns is about 1 for 20 000 [5]. In complex atypical CAH, associated genetic mutations are direct precursor of endometrial cancer [6].

CAH cannot be cured, but it can be effectively treated by steroid substitution therapy if detected in early stage of life and disease course. Because of large individual variation in cortisol secretion, it is also important to monitor treatment very carefully. The means to determine an appropriate replacement dose includes measurement of serum/plasma 17-hydroxyprogesterone. Ani Lab systems' Neonatal 17-OH-Progesterone EIA test allows quantification of 17-hydroxyprogesterone in dried blood spot samples.

## PRINCIPLE OF THE TEST

The principle of the Ani Lab systems' Neonatal 17-OH-Progesterone EIA kit is based on competition solid-phase enzyme immunoassay with horseradish peroxidase as a marker enzyme. The assay proceeds according to the following reactions.

17-OH-Progesterone from the patient sample competes with the horseradish peroxidase 17-OH-Progesterone conjugate for the binding to the specific antibody attached to the polystyrene surface of the Microplate® wells. Residual patient sample and unbound conjugate are removed by washing. Residual patient sample and unbound conjugate are removed by washing. A colorless enzyme substrate (H<sub>2</sub>O<sub>2</sub>) containing the chromogen (TMB, Tetramethylbenzidine, a non-mutagenic chromogen for horseradish peroxidase) is added. The enzyme reaction with the chromogen results in a colored end product. The color formation reaction is terminated by adding acid (H<sub>2</sub>SO<sub>4</sub>). The color intensity is conversely proportional to the concentration of 17-hydroxy-Progesterone in a patient sample.

**KIT CONTENTS**

- Reagents are stored between +2°C and +8°C.
  - The expiration date is printed on each component label and on the package. Do not use reagents after the expiration date.
  - Avoid unnecessary exposure to light. This is merely a precaution. The light sensitive reagents are the conjugate and the TMB-substrate solution, the latter one is packaged in non-transparent plastic vial for protection.
- 1 MICROPLATES, 96x5 wells/ 96x10 wells  
Coated Microplates.
  - 2 CONJUGATE CONCENTRATE, 1.1 ml/ 2.2 ml   
100x concentrate 17-OH-Progesterone conjugated to horseradish peroxidase containing 0.02% Thimerosal as preservative. R20/21/22, R33
  - 3 CONJUGATE DILUENT, 125 ml/ 250 ml   
Buffer saline containing 0.02% Thimerosal as a preservative. R20/21/22, R33
  - 4 CALIBRATORS and CONTROLS, 1 aluminium bag/  
2 aluminium bags  
Ready to use calibrators C0-C7 and controls L1-L2 (dried blood spots on grade 903 filter paper).
  - 5 WASHING SOLUTION, 125 ml/ 250 ml  
15x concentrated containing 0.01% Thimerosal as preservative. R20/21/22, R33
  - 6 TMB CHROMOGEN, 2 ml/ 4ml   
3,3',5,5'-Tetramethylbenzidine. Xi, T (Irritant, Toxic) R25, R 36/38, S26.
  - 7 SUBSTRATE BUFFER, 2x50 ml/ 200ml  
Buffer containing hydrogen peroxide and 0.05% Bromonitrodioxane as a preservative.
  - 8 STOPPING SOLUTION 80 ml/ 160 ml  
0.45 M H<sub>2</sub>SO<sub>4</sub>
- PLASTIC COVERS, 5 pcs/ 10 pcs  
Plastic incubation covers for microplates
- REAGENT BASINS, 10 pcs/ 20 pcs
- SHEET WITH VALUES FOR CALIBRATORS AND CONTROLS

**REAGENT PREPARATION**

Reagent	Preparation	Stability of opened / diluted reagents (at +2°C to +8°C)
1 Coated Microplates®	Ready for use	2 months (*.
2 17-OHP-HRP conjugate		3 months.
3 Conjugate diluent		3 months.
Conjugate solution	Dilute 17-OHP-HRP conjugate (vial 2) 1:100 with conjugate diluent (vial 3) just prior to use. Mix carefully.	Discard unused conjugate solution
4 Calibrators and Controls	Ready for use	3 months (*. In very humid climatic environments opened calibrators and controls are recommended to be stored in an desiccator.
5 Washing solution	Dilute concentrated solution 1:15 with distilled or deionised water, e.g. 100+1400ml=1500ml.	Conservation of concentrated washing solution at +2-8C may lead to crystal formation. These crystals dissolve upon heating (37C). Stability after dilution: 1 week at 2-8C.
6 TMB chromogen		6 months
7 Substrate buffer		6 months
Substrate solution	Dilute 1+49 (1:50) TMB (vial 6) with buffer (vial 7) just before use.	Discard unused substrate solution. A deep blue colour present in the substrate solution indicates that the solution has been contaminated and must be discarded.
8 Stopping solution	Ready for use	6 months

*Relevant R-phrases:*

- 20 Harmful by inhalation
- 21 Harmful in contact with skin
- 22 Harmful if swallowed
- 25 Toxic in contact with the skin or if swallowed
- 33 Danger of cumulative effects
- 36/38 Irritating to eyes, respiratory system and skin

*Relevant S-phrases:*

- 26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
- 37 Wear suitable protective clothes and gloves

\*) When air-tightly closed in the foil package with the desiccant after opening and stored properly at +2°C to +8°C.

**MATERIALS REQUIRED BUT NOT PROVIDED**

- Distilled or deionized water, preferably sterile.
- Graduated cylinders for reagent dilution.
- Vials to store the diluted reagents.
- Precision pipettes (multi-channel 50-300 µl)
- Paper towels or absorbent paper.
- Timer, 120 min range.
- Microplate incubator / shaker
- Microplate photometer, 450nm
- Microplate washer (not compulsory)
- Disk puncher with a diameter of 3 mm to cut off paper disks of dried blood controls, calibrators and samples
- Sodium hypochlorite solution, free available chlorine 50-500 mg/l.
- Disposable gloves.

**PRECAUTIONS**

For *in vitro* diagnostic use only.

**Warning - POTENTIAL BIOHAZARDOUS MATERIAL:**

All human materials used in the preparation of the calibrators/controls in the kit have been tested for the presence of the antibodies to HIV (Human Immunodeficiency Virus) and HCV (Hepatitis C Virus) as well as Hepatitis B surface antigen (HBsAg) and found to be non-reactive. Because no test method can offer complete assurance that HIV, hepatitis B virus, HCV, or other infectious agents are absent, these calibrators and controls as well as samples should be handled at the Biosafety level 2 as recommended for any potentially infectious human serum or blood sample in the Centers for Disease Control/National Institutes for Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1999.

Discard all materials and samples as if capable of transmitting infection. The preferred method of disposal is autoclaving for a minimum of one hour at 121°C. Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 50-500 mg/l free available chlorine. Allow 30 minutes for decontamination to be completed. Spills should be wiped off thoroughly using either an iodophor disinfectant or sodium hypochlorite solution. Materials used to wipe off spills should be added to biohazardous waste matter for proper disposal. Reusable glassware must be disinfected, washed out and rinsed free of detergents.

Avoid contact with skin and eyes when handling conjugate, conjugate diluent, substrate and stopping solutions.

Wear disposable gloves while handling samples and kit reagents. Afterwards wash hands carefully. Never pipette by mouth.

Accurate and precise pipetting, as well as following the exact time and temperature requirements, is essential.

**SAMPLE COLLECTION AND HANDLING**

A blood spot on the filter paper is obtained by one application of the filter paper onto a drop of blood from the pricked heel of the baby 3-5 days after birth. Schleicher & Schuell 903 filter paper is recommended for collection of blood spots. Make sure that the filter paper sample is fully covered and soaked through. The blood spot is dried for at least 3 hours. Once dry, place each sample in a separate paper envelope and mail it to the laboratory. Blood spot samples received in the laboratory should be stored at +2°C ... +8°C protected against moisture.

The sample collection technique is described in detail in NCCLS document LA4-A5 [7].

**TEST PROCEDURE****PRELIMINARY PREPARATIONS**

- **Bring the reagents to room temperature (+20°C to +25°C) at least 30 min before starting the assay.**

**STEP I**

Punch out **3 mm** disks containing blood calibrators and controls in duplicates into the microtiter plate.

Punch out single **3 mm** disks from patient samples into microplate wells.

Add **200 µl** of 17-OHP-HRP conjugate. Make sure that the disks are properly soaked.

Cover the plate and incubate <sup>1)</sup> :

**1 hour (+/- 5 min) at RT (+20°C - +25°C) in dark** with shaking speed of **900 rpm** and then overnight (**18h +/- 2h**) at **+ 4°C without shaking**

**STEP II**

Remove the disks and wash 4 x 300 µl

**STEP III**

Add **100 µl** of Substrate solution

Incubate **20 min** at **+RT** in the **dark**

**STEP II**

Add **100 µl** of Stop solution.

Measure absorbance at 450 nm

**NOTES:**

1) To avoid high background it is important to cover the plate.

The use of an 8-channel pipette device is recommended for improved efficiency and precision.

It is recommended to use **calibrators and controls in duplicates on each plate.**

**Avoid contamination:** When removing aliquots from the reagent vials, use aseptic technique to avoid contamination. The kit includes 10 disposable reagent basins that can be used for pipetting the reconstituted substrate.

Use **only clean glassware** when preparing and dispensing of substrate solution. Use preferably disposable tubes to mix the substrate components and disposable reagent basin when pipetting the substrate solution.

## RESULTS

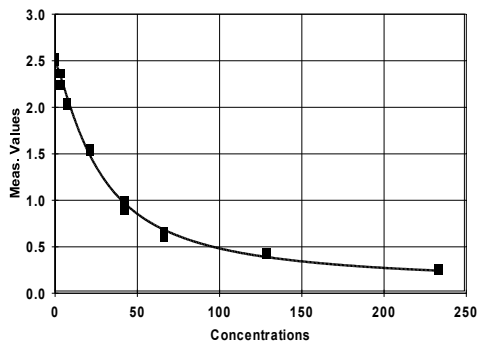
### Calculation of the Results

If automatic data processing can be used, four parameter logistic curve fitting with lin-lin axis scaling is recommended. **Do not use** curve extrapolation since erroneous results can be returned from extrapolated cubic spline or four parameter logistic curve outside the range of calibrators.

Manual calculation:

1. Draw a standard curve on graph paper with absorbance values on the ordinate and 17-OH progesterone concentrations of the calibrators on the abscissa.
2. Read the 17-OH progesterone concentration of controls and samples from the calibration curve.

Figure below shows a typical calibration curve.



### Quality Control Values

The **values of calibrators and controls** are **lot specific**. For exact values refer to the sheet included in each kit

If the calibrators and the controls do not give expected values, the results are invalid and the samples should be retested.

### Conversion:

To convert ng/ml serum to nmol/L blood, multiply by 1.36.

### Interpretation of the Results

The test will give quantitative values for 17-OH progesterone concentration present in sample. The discrimination between normal subjects and presumptive positives is based on a predetermined cut-off value for 17-OH progesterone which is set empirically based on the reference range of the given normal population. An example of cut-off determination is given in Chapter Evaluation Studies . **It is recommended that each laboratory sets its own cut-off value** based on the reference range of the given normal population and to retest samples giving values above or near the cut-off value.

## PERFORMANCE CHARACTERISTICS

### Sensitivity

Sensitivity is defined as the minimum concentration of 17-OHP which can be statistically distinguished from calibrator 0 ng/ml. This concentration is 1.82 ng/ml.

### Precision

#### Intra-assay

The intra-assay imprecision has been tested with 2 samples containing various amounts of 17-OHP in 8 replicates.

Samples	Replicates n	Mean Conc. ng/ml serum	CV %
Sample 1	8	7,5	10,8
Sample 2	8	52,8	8,6

#### Inter-assay

The inter-assay imprecision has been tested with in 3 separated assays performed with 4 sample containing various amounts of 17-OHP was tested in 8 replicates.

Samples	Runs n	Repli-cates n	Mean Conc. ng/ml serum	CV %
Sample 1	3	8	21	7,4
Sample 2	3	8	107	7,6
Sample 3	3	8	137	7,0
Sample 4	3	8	183	9,5

### Correlation

Ani Labsystems' Neonatal 17-OHP EIA was tested with Ani Labsystems' Neonatal 17-OHP FEIA (fluorometric enzyme immunoassay) on a range of 36 internal and external standards and showed a correlation factor of 0.99.

### Recovery

Known amounts of 17-OH progesterone were added to determine recovery of the assay. 2 samples were tested in 4 replicates.

17-OHP added (ng/ml)	17-OHP recovered (ng/ml) in FEIA test	Recovery (%)
200	214	107
100	110	110
25	23	92
50	55	110

### Method comparison

The performance of 17-OHP EIA test has been assessed by determination of 17-OHP concentration in 169 samples in comparison with another commercially available kit. The correlation was 0.94.

### Cross reactivity

The cross reactivity for a number of related steroids have been calculated on a weight/ weight basis. No significant cross-reactivity was detected.

Compound	% Cross Reactivity
17 $\alpha$ -hydroxypregnenolone	0.3
11-deoxycortisol	0.5
21-deoxycortisol	0.5
11-deoxycorticosterone	0.035
Progesterone	0.7
Pregnenolone	0.022
Cortisol	0.008
Corticosterone	Not detectable
Testosterone	Not detectable
Estradiol	0.001
Androstenedione	0.001

### EVALUATION STUDIES

The external evaluation was performed in two reference hospitals. Hospital A tested 100 suspected samples and 190 negative samples with Ani LabSystems Neonatal 17-OH-Progesterone FEIA test and a commercial test. Hospital B tested 3800 random samples with Ani LabSystems Neonatal 17-OH-Progesterone FEIA test. The results are described below:

Cut off set ng/ml serum (nmol/L blood)	Hospital A		Hospital B
	Sensitivity	Specificity	Negative Prevalence on random population
22 (30)	96,5 % (80/83 positive)	99,5 % (206/207 negative)	94,1 % (3577 / 3800)
29 (40)	95,2 % (79/83 positive)	99,5 % (206/207 negative)	97,5 % (3705 / 3800)

17-OHP concentration increases during pregnancy in the maternal and fetal blood. After birth, values declines rapidly to reach normal adult values in 2 to 7 days. Thus, it is advisable not to collect samples before 3 days of life. Premature and sick infants exhibit 2 to 3 fold 17-OHP values with no CAH disorder. It is suggested that a different cut off be adopted for pre-term and sick infants.

The normal range for 17-OHP in 5 days term neonates has been established internally using the Neonatal 17-OHP kit.

	Weight >2.5kg	Weight 0.5-1.9kg
	N=265	N=42
Median	13.2	66.5
P99	60	205.6
P95	35	195.5
P5	6	33.9

Pre-term infants have 17-OHP concentrations much higher than normal full term babies. The suggested normal range and cut-off is a guideline only. Each lab should establish specific thresholds and range based on performance of the assay in their lab and with their constituent demographic population.

### LIMITATIONS OF THE PROCEDURE

The Neonatal 17-OHP screening kit is a screening method for measuring the 17-OHP concentration in newborn blood spot samples. It is not to be used for confirmatory testing, monitoring therapy or prenatal testing. Blood spots with elevated 17-OHP values should be confirmed with an extracted 17-OHP assay using serum samples.

The Neonatal 17-OHP kit detects only CAH caused by 21-hydroxylase deficiency with accounts for approximately 90% of the disorder. It will not detect CAH caused by deficiency of other enzymes, notably 11-beta hydroxylase deficiency.

Premature and infants with low birth weights tend to have higher 17-OHP values. Do not use cord blood. Samples collected prior to the second day of life tend to have higher 17-OHP values due to the placental cross over.

Elevated results are not necessarily diagnostic of primary congenital adrenal hyperplasia, but indicate the need for further study of the newborn .

To ensure accurate and reliable results, make sure that all blood spot disks are within the conjugate solution during the incubation period.

Strict adherence to the protocol is advised to obtain reliable results. Any modification or change made to the kit or the assay procedures are under the responsibility of the user.

This assay is designed to be used with dried blood samples that are exclusively collected on grade 903 filter paper. The use of other collection papers may affect results.

Population variations, infant weight, age, prematurity and twinning can affect the 17-OHP concentrations. Laboratories should be aware of all these factors.

**REFERENCE**

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6. Ellenson, L.H. (2005) Early molecular changes in endometrial cancer. *Int. J. Gynecol. Cancer*, 2, 399-400.
7. National Committee for Clinical Laboratory Standards (NCCLS): Blood collection on filter paper for newborn screening programs, Approved standard, 5<sup>th</sup> Ed. NCCLS Document LA4-A5, ISBN Number 1-56238-644-1 (2007).

**RELATED PRODUCTS:**

Product number	Product description	
6199870	Neonatal 17-OHP FEIA	480 wells
6199871	Neonatal 17-OHP FEIA	960 wells
6199875	Neonatal 17-OHP EIA	480 wells
6199876	Neonatal 17-OHP EIA	960 wells
61 99 896	Neonatal Phenylalanine	960 wells
61 99 897	Neonatal Phenylalanine	4800 wells
61 90 930	Neonatal Phenylalanine	5 sets of 3 levels Controls
61 90 940	Neonatal Phenylalanine	5 sets of 5 levels Calibrators
61 99 880	Neonatal hTSH FEIA Plus	960 wells
61 99 881	Neonatal hTSH FEIA Plus	4800 wells
61 99 892	Neonatal hTSH EIA	960 wells
61 99 8923	Neonatal hTSH EIA	4800 wells
61 99 802	Neonatal Toxoplasma gondii IgM FEIA	480 wells
61 99 804	Neonatal Toxoplasma gondii IgM EIA	480 wells

**MANUFACTURER:**

Ani Lab systems Ltd. Oy  
 Tiilitie 3, FIN-01720 Vantaa, Finland  
 Tel. +358-20-155 7530, Fax +358-20-155 7531  
 E-mail: [sales@anilabsystems.com](mailto:sales@anilabsystems.com)  
[www.anilabsystems.com](http://www.anilabsystems.com)