



# Neonatal 17-OH Progesterone Screening Assay (ELISA)



## 17-OHP

### Enzyme immunoassay for the quantitative determination of 17-OH Progesterone (17-OHP) in New born dried blood spots

(FOR IN VITRO DIAGNOSTIC USE ONLY)

#### 1. INTENDED USE

**Born Safe™** Neonatal 17-OHP ELISA is an enzyme immunoassay for the quantitative determination of 17 Alpha-Hydroxyprogesterone in blood samples dried on Whatman S&S 903 filter paper. This test is intended as a screening method for the determination of 17-OHP concentrations in newborn dried blood spot specimens as an aid to the diagnosis Congenital Adrenal Hyperplasia (CAH).

#### 2. SUMMARY AND EXPLANATION OF THE ASSAY

There are various congenital enzyme defects of the steroid biosynthesis, which cause congenital adrenal hyperplasia (CAH). They are genetically different, but are all transmitted in an autosomal recessive mode. The most frequent types are the 21-hydroxylase deficiency<sup>1</sup>. Complete or partial deficiency of 21-hydroxylase accounts for 90 to 95% of all cases and the 11β-hydroxylase deficiency (about 10% of all cases) whereas other types (lipoid adrenal hyperplasia or 20α-hydroxylase deficiency and 3β-hydroxysteroid dehydrogenase deficiency) are very rare. CAH results from a recessively inherited defect in any of the five enzymatic steps required to synthesize cortisol from cholesterol<sup>2</sup>. 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. Persistently high concentrations of 17-OHP, due to blockage of the pathway converting the precursor steroids to cortisol, are considered presumptively diagnostic of CAH resulting from 21-hydroxylase deficiency<sup>2</sup>. CAH exists in three forms: salt wasting (SW), simple virilizing (SV) and non-classical (NC). The SW and SV forms of the disorder result in excessive adrenal androgen secretion early in fetal life, resulting in virilization of genitals in females. Aldosterone biosynthesis is defective in the SW form but is apparently normal in the SV forms. If left untreated, the SW form can result in life-threatening adrenal crises within the first weeks of life and precocious growth in both sexes. Non-classical CAH may result in persistent slight elevations of 17-OHP from birth with clinical manifestations occurring later in life<sup>3</sup>.

The overall frequency of CAH in the general population varies ethnically. In Europe it is estimated to be 1:10,000 to 1:15,000 newborns. The SW and SV forms have been found to occur at a frequency of 1 in 15,000 live births<sup>4</sup>. The non-classical form has been shown to have an extremely high frequency in the general white population (1:100) with further increased incidence in certain ethnic groups (1:27 in Ashkenazi Jews, 1:53 in Hispanics, 1:63 in Yugoslavs and 1:333 in Italians)<sup>5</sup>. In India, based on the review of two different population studies CAH prevalence is 1:2575 & 1:5762 respectively<sup>7</sup>. 17α-OH-progesterone, a precursor of cortisol, is increased in both 21- and 11β-hydroxylase deficiency, but not in the other types. Its determination is thus useful as a screening method for the two most frequent types of CAH or about 95% of all cases. 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<sup>8</sup>. 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<sup>9</sup>. It is suggested that a different cutoff be adapted to pre-term and sick infants.

The principal application of the 17-OHP is in the screening for and diagnosis of CAH in newborn children with ambiguous genitalia and in girls who become virilized during adolescence. Since 17-OHP is the immediate precursor to 11-desoxycortisol, basal 17-OHP concentrations are sharply elevated in patients with 21-hydroxylase deficiency and to a lesser degree in patients with 11-hydroxylase deficiency. Because 17-OHP concentrations are so markedly elevated in newborns and adolescent girls afflicted with CAH, a single basal measurement is ordinarily all that is required to make the diagnosis<sup>9</sup>.

Prompt treatment can save the life of infants and allow afflicted children to attain normal growth.

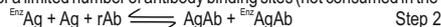
#### 3. PRINCIPLE OF THE ASSAY

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



Ab = Specific Antibody (Constant Quantity)  
Ag = Native Antigen (Variable Quantity)

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 antibody binding sites (not consumed in the first incubation).



<sup>Enz</sup>AgAb = Enzyme-Antigen (Constant Quantity)  
rAb = remaining antibody not consumed in Step 1

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



ARA<sub>CW</sub> = Anti-Rabbit antibody immobilized on well  
<sup>Enz</sup>AgAb - ARA<sub>CW</sub> = Immobilized enzyme bound reactant  
AgAb - ARA<sub>CW</sub> = Immobilized non-enzyme bound reactant

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:



#### 4. PRESENTATION

REF	▽
525020096	96 Assays

#### 5. KIT COMPONENTS: Reagents (96T Pack Size)

Material Provided:

- 17-OHP Calibrator Level C0 to C5** – Dried Blood Spots in Aluminium pouch (One row of six spots levels-1x6): Six (6) levels of 17-OHP calibrators in dried blood spots (adjusted to 55% hematocrit) at approximate concentrations of 0 (C0), 5 (C1), 12 (C2), 24 (C3), 53 (C4) and 120 (C5) ng/ml spotted on S&S Whatman 903 filter paper. Store at 2-8°C. Desiccant is included. Preservative has been added.  
**Note 1:** The Lot Specific calibrators are whole human blood based, are calibrated against the 6th ISNS Reference Preparation for Neonatal Screening (6th ISNS-RPNS), RIVM, Bilthoven, Netherlands.  
**Note 2:** The exact 17-OHP values are printed on the lot specific quality control certificate included in the kit. (Above values are for illustration purpose only.)
- 17-OHP Control Level L1 and L2** – Dried Blood Spots in Aluminium pouch (One row of two spots – 1 x 2) Two levels of whole human blood controls with different concentrations of 17-OHP spotted on S&S type 903 filter paper. Store at 2-8°C. Desiccant is included Preservative has been added.  
**Note 1:** The controls are whole human blood based, are manufactured to fall within significant clinical ranges.  
**Note 2:** The exact values are printed on the lot specific quality control certificate included in the kit.
- 17-OHP Enzyme Reagent** – 14.0 ml/vial  
One (1) vial contains enzyme 17-OHP horseradish peroxidase (HRP) conjugate in buffer, and preservative. Store at 2-8°C.
- 17-OHP Antibody Reagent** – 14.0 ml/vial  
One (1) vial containing Antibody to 17-OHP in buffer and preservative. Store at 2-8°C.
- Coated Microwells:** One 96-well microplate coated with Anti-rabbit antibody and packaged in an aluminium pouch with a desiccant. Store at 2-8°C
- Wash Buffer** – 60.0 ml Concentrated (20X)  
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-8°C.
- Substrate Solution** – 15.0 ml/vial  
One (1) vial containing tetramethylbenzidine (TMB) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in buffer. Store at 2-8°C.
- Stop Solution** – 7.0 ml/vial
- Plate Sealers** – 3 nos.
- Product Instructions** – Details of the Kit and testing methodology  
**Note 1:** Above reagents are for a single 96-well microplate.

Materials	Quantity (96T)	Physical State
17-OHP Calibrator Level C0 to C5	1 set (6 levels)	Ready to use
17-OHP Control Level L1 and L2	1 set (2 levels)	Ready to use
17-OHP Enzyme Reagent	1 vial (14.0 ml)	Ready to use
17-OHP Antibody Reagent	1 vial (14.0 ml)	Ready to use
Coated Microwells	96 (12 x 8) wells	Ready to use
Wash Buffer	60.0 ml	Concentrated (20X)
Substrate Solution	1 vial (15.0 ml)	Ready to Use
Stop Solution	1 vial (7.0 ml)	Ready to use
Plate Sealers	3 Nos.	Ready to use
Pack Insert	1 No.	
Protocol Sheet	1 No.	
Microwell Holder	1 No.	

#### 6. STORAGE AND STABILITY OF THE KIT

- Store the kit and reagents at 2-8°C. Calibrators and Controls should be stored protected from moisture and light in the aluminium pouch with desiccant. Stable at 2-8°C until expiry date stated on the label. Make sure that the aluminium pouch remains sealed during storage.
- We recommend that the Blood Spots (Calibrators and Controls) should be preferably stored at -20°C with desiccants when not in use for prolonged periods.**
- Unopened reagents will retain reactivity until expiration date shown on the label. Do not use reagents beyond this date.
- Unopened test kits should be stored at 2-8°C upon receipt. The test kit may be used throughout the expiration date of the kit. Refer to the package label for the expiration date.
- Microtiter wells must be stored at 2- 8 °C. Once the pouch has been opened care should be taken to seal it tightly again.
- Equilibrate all kit components to ambient temperature (18-25°C) before use. We recommend you use up all wells within 4 weeks after initial opening of the pouch.
- Avoid extended exposure to heat and light.

#### 7. MATERIALS REQUIRED BUT NOT PROVIDED

- Dispensers for repetitive deliveries of 0.050ml, 0.100ml, and 0.350ml volumes with a precision of better than 1.5% (optional)
- Microplate washer or a squeeze bottle (optional)



3. Microplate reader with 450nm and 620nm wavelength absorbance capability (The 620nm filter is optional)
4. Absorbent paper for blotting the microplate wells.
5. Aluminium foil wrap or microplate cover for incubation steps.
6. Timer
7. Storage container for storage of wash buffer
8. Distilled water or deionized water
9. 1/8" Blood spot puncher for dispensing 3.2 mm dried blood spots.
10. Orbital plate Shaker (100 to 1100 rpm) with orbital diameter 2mm
11. Blood collection cards [Whatman S&S 903 recommended; CLSI NBS01-A6 compliant]

## 8. WARNINGS AND PRECAUTIONS

A thorough understanding of the pack insert is mandatory before performing the test for the first time. Adherence to the protocol specified herein is necessary to ensure optimal performance of the product. Any deviation from the assay procedure may affect the results.

**Operating: In order to obtain reproducible results, the following rules must be observed:**

1. Do not mix reagents of different lots.
2. Do not use reagents beyond their expiry date.
3. Use thoroughly clean glassware.
4. Use distilled water, stored in clean containers.
5. Avoid any contamination among samples; for this purpose, disposable tips should be used for each sample and reagent.
6. Keep all reagents at normal refrigerator temperature (2-8°C) in closed containers when not in use, but ensure that all reagents are equilibrated to 18-25°C before use. Keep Blood Spot Standards and Controls at normal refrigerator temperature (2-8°C) in the original aluminium pouch containing desiccant when not in use, but ensure that spots are equilibrated to 18-25°C before use.
7. The strips, calibrators and internal controls are packed in an pouch containing a desiccant. Immediately after removal of strips, the remaining strips should be resealed or closed with a Scotch tape in the pouch along with the desiccant and stored at 2-8° C. It is important to ensure the desiccant remains in the pouch. **If the desiccant has turned pink, ensure to replace the pink coloured desiccant (inactivated) with blue coloured desiccant (activated).**
8. Do not use reagents from other manufacturers along with the kit reagents for a given test run.
9. Do not interchange reagent vials and their screw caps to avoid cross-contamination. Use a clean, fresh, disposable pipette tip for each reagent or specimen manipulation.
10. Close reagent vials tightly immediately after use to avoid microbial contamination.

**Safety: In order to avoid personal and environmental contamination, the following precautions must be observed:**

1. Use disposable gloves while handling potentially infectious material and performing the assay.
2. Do not pipette reagents by mouth.
3. Do not smoke, eat, drink or apply cosmetics during the assay.
4. All material of human origin used for the preparation of this kit tested negative for HBsAg, anti-HIV and anti-HCV. Since no test at present can guarantee complete absence of these viruses, all samples and reagents used for the assay must be considered potentially infectious. Therefore, the assay waste must be decontaminated and disposed off, in accordance with established safety procedures.
5. Disposable ignitable material must be incinerated; disposable non-ignitable material must be sterilized in autoclave for at least 1 hour at 121°C. Liquid wastes must be added with sodium hypochlorite at a final concentration of 2%. Let the hypochlorite act for at least 30 minutes. Liquid wastes containing acid must be neutralized with appropriate amounts of base, before treating with sodium hypochlorite.
6. Avoid splashing and aerosol formation; in case of spilling, wash carefully with a 2% sodium hypochlorite solution and dispose of this cleaning liquid as potentially infectious waste.

## 9. SPECIMEN COLLECTION AND HANDLING

Blood samples should ideally be collected between the third and the fifth day of life (48 to 120 hours after birth) and should be taken directly from a heel prick onto filter paper. Neonatal screening programs differ from one another in the type of specimen required, the recommendation is a blood spot, approximately 12.7 mm (½ inch) in diameter, collected by heel prick and spotted onto filter paper (Whatman Schleicher & Schuell 903). The specimen collection device must comply with national regulations. A method based on dried blood samples requires skillful collecting, handling and transport of samples. The collection technique is described in detail in CLSI document LA4-A5, and the main points are listed below.

- Blood from the new-born's heel should be collected **ONLY** from the medial (closest to the body center-line) or lateral portion (furthest from the body center-line) of the planter surface (walking surface).
- Blood collection from other areas of the infant's foot, e.g. arch, may result in nerve, tendon or cartilage injury.
- Clean the skin with an alcohol swab and allow to air-dry.
- Puncture the infant's heel with a sterile lancet or with a heel incision device to the depth of approximately 2.0 mm. Puncturing deeper than 2.0 mm on small infants may cause bone damage.
- Wipe away the first drop of blood. Gently touch the filter paper against a large drop of blood and, in one step, allow a sufficient quantity of blood to soak through to completely fill a pre-printed circle on the filter paper. Examine both sides of the filter paper to make sure that the blood has penetrated and saturated the paper. Excessive milking or squeezing the puncture may cause haemolysis of the specimen or an admixture of tissue fluids with the specimen. Do not layer successive drops of blood in the collection circle (this causes caking).
- Allow the blood specimen to air-dry in a horizontal position for at least 4 hours at ambient temperature (18-25°C). Do not heat or stack the specimens during the drying process.
- Arrange transport of the collection card to the screening laboratory within 24 hours of collection.
- Store in sealed paper envelopes or containers that will provide protection from moisture, light, heat and contact with other materials

- The sample discs should be punched from similar areas on each individual blood spot. Do not punch sample discs from areas that include printed marks or that are near the edges of the blood spot.
- Be sure that the required information on the specimen collection card has been completed. The minimum pre-printed information required on the collection device includes:
  - ◆ last name (and first, if available), sex, birth date (optional: time of birth), birthweight and age of the infant; (indicate if < 24 h), and patient identification number
  - ◆ the first and last name of the mother
  - ◆ date of specimen collection (optional: time of collection)
  - ◆ the name and address of the submitter (optional: birth facility)
  - ◆ the name and phone number of the physician (health care provider)
  - ◆ the name of the new born screening program and address
  - ◆ each card should have a unique serial number and an expiration date.
- Specimens should not be placed in hermetically sealed containers (e.g. plastic or foil bags). If required, sufficient desiccant packages must be included. Humidity and moisture are detrimental to the dried blood spot specimen.
- Before placing the specimens in a container for transport, the dried blood spots on the collection cards should be separated by a physical barrier from the blood spots on the cards in the stack immediately above and below. The blood spots can also be protected by a fold-over cover attachment or by placing glassine paper between the specimens.

## 10. REAGENTS PREPARATION

### Wash Buffer

Dilute wash buffer 20 times (for example add 50 ml concentrated buffer to 950 ml distilled or deionized water). Mix well before use. Diluted Wash Buffer is stable upto one week when stored at 2-8°C.

**Note 1: Do not use reagents that are contaminated or have bacterial growth.**

**Note 2: Do not use the substrate if it looks blue.**

## 11. ASSAY PROCEDURE

**A. Before proceeding with the assay, bring all reagents and patient samples to room temperature (18-25° 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 pouch, seal and store at 2-8°C with activated desiccant.
2. Punch out a 3.2 mm blood dot out of each calibrator, control and specimens 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 80 µl of 17-OHP Antibody 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 the microplate/ wells using Plate Sealers of appropriate size and rotate for 60 minutes at ambient temperature using a laboratory rotator set at 900rpm.
6. Remove from shaker and add 100 µl of 17-OHP Enzyme Reagent directly to each well. **Do not remove the reactants (DBS) from 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 wells using Plate Sealers and rotate for 60 minutes at ambient temperature using a laboratory rotator set at 150rpm.
9. Discard the contents of the microplate by decantation, blot and tap the microwell dry with absorbent paper. **Note:** Make sure all the blood dots are removed at this point. There should be no dots left in the microwells.
10. Add 350 µl of diluted wash buffer (see Reagent Preparation Section) to each well, decant (tap and blot) or aspirate. Repeat the same four (4) additional times for a total of five (5) washes. Follow the manufacturer's instruction while using automatic or manual plate washer for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash buffer. Decant the wash and repeat four (4) additional times
11. Add 100 µl of substrate solution (color developer) to each well.

### DO NOT SHAKE PLATE AFTER SUBSTRATE ADDITION

12. Cover the microplate using aluminium foil and incubate for 15 minutes at ambient temperature (in dark without shaking).
13. Add 50 µl of stop solution to each well and gently mix for 15-20 seconds till 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 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results should be read within fifteen (15) minutes of adding the stop solution.

## B. CERTIFICATION/TRACEABILITY TO REFERENCE MATERIAL

The Lot Specific Blood Spot Standards and Controls provided with the Neonatal 17-OHP Screening Assay kit is traceable to the 6th ISNS Reference Preparation for Neonatal Screening (6th ISNS-RPNS), RIVM, Bilthoven, Netherlands.

## 12. CALCULATION OF RESULTS

A dose response curve is used to ascertain the concentration of 17-OHP 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 blood reference versus the corresponding 17-OHP concentration in ng/ml on semi-log graph paper (average the duplicates of the blood references before plotting).
3. Draw the best-fit curve through the plotted points.
4. To determine the concentration of 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).

**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

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

Calibrator	Concentration (ng/ml)	Absorbance
C0	0	2.364
C1	3	1.682
C2	7	1.38
C3	20	0.712
C4	50	0.307
C5	110	0.187

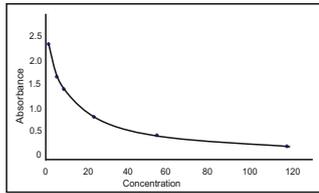


Fig 1

Parameters of four parameter logistics were a=2.364, b=1, c=8.6, d=0

**Note:** 17-OHP values are expressed in whole blood units. To convert ng/ml blood units to ng/ml serum units, multiply by 2.2. To convert nmol/L blood units to ng/ml serum units, multiply by 0.73 or the factor for converting ng/ml whole blood to nmol/L whole blood is: 0.33 ng/ml whole blood = 1 nmol/L whole blood

### 13. QUALITY CONTROL

Internal controls (L1-L2) included in the kit should be routinely monitored to check that measured concentrations stay within the stated values. These controls provide valuable information regarding the validity of the test according to manufacturer specifications.

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 indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

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. Do not use reagents beyond the kit expiration date.
3. Avoid extended exposure to heat and light. Kit and component stability are identified on label.
4. Values in this kit are expressed in ng/mL whole blood.

### 14. EXPECTED VALUES AND INTERPRETATION CRITERIA

Please note that the values mentioned in this section should only be used as a guideline, and each laboratory should establish its own specific cut-off and reference range based on the specimens from the laboratory routine population and also a procedure for the follow up of newborns from which a 'presumptive positive' specimen was received as the concentration of 17-OHP in newborns depends on demographic variations, age, weight, prematurity and twinning. 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 cutoff be adopted for pre-term and sick infants. Pre-term infants have 17-OHP concentrations much higher than normal full-term babies. Periodic review and adjustment of reference ranges are suggested.

A review of various published NBS programs study outcomes and referring recommended guidelines of national and international organizations such as ICMR, IAP, ISPAE, AAP & CDC & Working Group on Neonatal Screening of the European Society for Pediatric Endocrinology following guidelines are suggested;

For term neonates of 2 to 6 days old, following cut-off values are suggested:

Suggested Cut off Values:

Interpretation	Concentration (whole blood) (ng/ml)
Presumptive Negative	<12 ng/ml
Borderline (Repunch)	12 – 35 ng/ml
Presumptive Positive	>35 ng/ml

Elevated concentrations of 17-OHP are not per se diagnostic of CAH the circulating 17-OHP concentration may be elevated in infants who are pre-term, under stress, have respiratory disorders or other severe illness. Many screening programs, therefore, use cut off values that are adjusted for birth weight. **Values in this kit are expressed in ng/mL whole blood.** The factor for converting ng/mL whole blood to ng/mL serum is 2.22. The factor for converting ng/mL whole blood to nmol/L whole blood is: 0.33 ng/mL whole blood = 1 nmol/L whole blood.

### 15. ANALYTICAL PERFORMANCE CHARACTERISTICS

**Precision:** The intra assay and inter assay precision were determined as per NCCLS Evaluation Protocol (EP5-A2). Within day, within operator, Within Lot run Precision was determined by using 3 different samples spiked with different concentration of 17-OHP and run in 5 replicates each respectively by three operators each for 5 days using 2 Lots of reagent.

N=150	Within Day		Within Operator		Within Lot		Reproducibility		
	Mean	SD	CV%	SD	CV%	SD	CV%	SD	CV%
Sample # 1	10.441	0.915	8.77	0.966	9.26	0.934	8.95	0.915	8.77
Sample # 2	22.341	1.822	8.16	1.979	8.86	2.043	9.15	1.822	8.16
Sample # 3	44.442	2.596	5.84	2.426	5.46	2.596	5.84	2.596	5.84

### Analytical Sensitivity:

Analytical Sensitivity of **BornSafe™** Neonatal 17-OHP Screening assay was assessed following NCCLS approved guidelines EP17-A. The limit of detection was calculated as 1.6 ng/ml.

### Interference/Cross Reactivity:

Steroids	Cross Reactivity
Corticosterone	Not detectable (0 to 1.25 mg/ml)
Beta Estradiol	Not detectable (0 to 1.25 mg/ml)
Testosterone	Not detectable (0 to 12.5 ug/ml)
Cholesterol	Not detectable (0 to 12.5 mg/ml)

Pregnenolone	0.025%
Progesterone	0.66%
17-OH Pregnenolone	1.6%
Cortisol	Not detectable (0 to 1.25 mg/ml)
11-decortisol	0.025%
Androsterone	Not detectable (0 to 1.25 mg/ml)
Alpha estradiol	Not detectable (0 to 1.25 mg/ml)

**Method Comparison:** **BornSafe™** Neonatal 17-OHP Screening Assay was compared with a CE certified commercial Neonatal 17-OHP Assay kit using routine new born screening dried blood spots samples. Total 300 no of samples were tested in comparison in both assays. The range of 17-OHP concentration was 0.85 ng/ml to 10.98 ng/ml. Excellent Co-relation was achieved between the two 17-OHP Screening Assays. (r=0.975); n=300.

**Recovery:** CDC-QC samples were used to establish the recovery of 17-OHP at several concentrations. These samples were run in triplicates each in five different runs (n=15)

Sample	N	Mean 17-OHP Target Value (ng/ml) in blood	Mean 17-OHP Recovered (ng/ml) in blood	Mean Recovery %	Range Recovery %
Low A	15	10.13	10.30	101	91%-103%
Medium B	15	20.95	20.67	98.66	94%-107%
High C	15	39.72	40.73	102	92%-108%

### 16. LIMITATIONS OF THE PROCEDURE

As with any other in vitro screening test, the test results themselves should not be the only reason for any therapeutic consequences. They have to be correlated to other clinical observations and diagnostic tests. Results obtained using the **BornSafe™** Neonatal 17-OHP ELISA should be used as an aid to other medically established procedures and results interpreted in conjunction with other clinical data available to the clinician.

#### Interpretation:

1. **BornSafe™** Neonatal 17-OHP Screening ELISA is a screening method for measuring the 17-OHP concentration in newborn blood spot specimens. It is not to be used for confirmatory testing, monitoring therapy or prenatal testing.
2. **BornSafe™** Neonatal 17-OHP Screening ELISA detects only CAH caused by 21-hydroxylase deficiency which accounts for approximately 90% of the disorder. It will not detect CAH caused by deficiency of other enzymes, notably 11-beta hydroxylase deficiency.
3. Premature and infants with low birth weights tend to have higher 17-OHP values.
4. 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.
5. Elevated results are not diagnostic per se of primary congenital adrenal hyperplasia, but indicate the need for further study of the newborn from which a presumptive positive specimen was received.
6. To ensure accurate and reliable results, make sure that all blood spot discs are within the reagent during the incubation period.
7. Strict adherence to the protocol is advised to obtain reliable results. Any modification or change made to the kit or the assay procedure are under the responsibility of the user.
8. This assay is designed to be used with dried blood specimens that are exclusively collected on Schleicher & Schuell's Filter Paper #903. The use of other collection papers may affect results.
9. Demographic variations, infant weight, age, prematurity and twinning can affect the 17-OHP concentrations. Laboratories should be aware of all these factors.
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.

#### 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 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. Deviation from kit instructions may yield inaccurate results.

### 17. COMPLAINTS

Complaints can be accepted in written format (preferably on the manufacturer's complaint form). All details of the test kit, as well as the test results, can be included. A copy of the complaint form is available from Tulip Diagnostics Pvt Ltd. upon request.

### 18. REFERENCES

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9. New MI. (1987) Basic and clinical aspects of congenital adrenal hyperplasia. J. Steroid Biochem. 27: 1-7.

# Summary Protocol

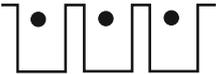
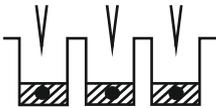
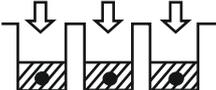
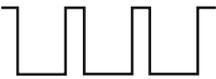
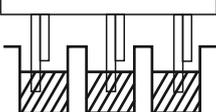
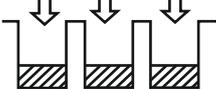
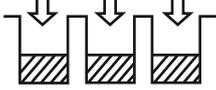


## REAGENT PREPARATION

### 17-OHP

Reconstitution of Wash Buffer	 950 ml D/W + 50 ml (Wash Buffer)	Dilute 50 ml of Wash Buffer in 950 ml of Deionized/Distilled water.
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## ASSAY PROCEDURE

1. Punch out Calibrators, Controls and patient's sample in coated microwells		One blood spot into each well of coated microwell strips
2. Add 80 µl 17-OHP Antibody Reagent and shake gently for 20-30 secs		Ensure that each disc is fully immersed in 17-OHP Antibody Reagent and not stuck to the microwell walls
3. Incubate		Cover with Plate Sealers and incubate for 60 min at RT (18-25°C) on an orbital plate shaker (900 rpm)
4. Add 100 µl 17-OHP Enzyme Reagent directly into each well and shake gently for 20-30 secs. Do not remove DBS from the wells		Ensure that each disc is fully immersed in the reagent and not stuck to the microwell walls
5. Incubate		Cover with Plate Sealers and incubate for 60 min at RT (18-25°C) on an orbital plate shaker (150 rpm)
6. Discard the contents and remove the discs		Decant the well content to remove blood dots completely, blot and tap the microwell dry with absorbent paper
7. Wash		Add 350 µl wash buffer. Decant or Aspirate. Repeat 5 times.
8. Add 100µl Substrate solution.		Cover the microwells with aluminium foil or store in dark to incubate for 15 min at RT (18-25°C)
9. Add 50µl Stop Solution NOTE: Add reagents in the same order to minimize reaction time differences between wells		Gently mix for 15-20 secs for uniform colouration
10. Read/ Measure. Results should be read within 15 min of adding the stop solution.		Place the plate in a microplate reader and read at 450 nm and using reference wavelength of 620nm-630nm

### SYMBOL KEYS

Store at 2-8°C	Consult Instructions for use	Date of Manufacture	LOT Batch Number / Lot Number
Manufacturer	IVD In vitro Diagnostic Medical Device	This side up	Caution
Use by	REF Catalogue Number	Contains sufficient for <n> tests	EC REP Authorised Representative in the European Community

  
 Manufactured by:  
 Zephyr Biomedicals

**A Division of Tulip Diagnostics (P) Ltd.**

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**EC REP**

CMC Medical Devices & Drugs S.L., Spain.