Neonatal Biotinidase Deficiency Screening Assay

BornSafe™ Biotinidase

Enzymatic assay for the quantitative determination of Biotinidase deficiency in new born dried blood spots

(FOR IN VITRO DIAGNOSTIC USE ONLY)

1. INTENDED USE

Born Safe™ Neonatal Biotinidase Screening Assay is an enzymatic assay for the quantitative determination of biotinidase activity in neonates using blood spot samples dried on Whatman S&S 903 filter paper. This kit is particularly suitable for use in a neonatal screening program as an aid in identifying biotinidase deficiency in new-borns. The kit should not be used for confirmatory testing or to monitor therapy.

2. SUMMARY AND EXPLANATION OF THE ASSAY

Biotinidase deficiency, also known as late-onset multiple carboxylase deficiency, is an autosomal recessively inherited disorder of biotin recycling. The disorder is caused by absent or markedly deficient activity of biotinidase, a cytosolic enzyme that liberates free biotin from biocytin during the normal proteolytic turnover of holocarboxylases and other biotinylated proteins. As a result, the biotinidase deficiency is associated, in patients, with neurologic and cutaneous symptoms.[1,2,3] Individuals lacking biotinidase activity exhibit a variety of symptoms which are frequently not present at birth, thus making it difficult to diagnose the disease by clinical observation.[7] Symptoms and the time of onset vary greatly. There are two types of Biotinidase deficiency, profound and partial, with profound deficients having <10% of normal activity and partial deficients having 10 - 37% of normal activity.[11,12] Partial deficients are clinically asymptomatic unless stressed by infection or insufficient biotin dietary intake[11]. The most common clinical signs observed in profound deficient infants usually develop hypotonia, ataxia, seizures, breathing difficulties, and developmental delay between two and six months of age.

Cutaneous abnormalities (skin rash, alopecia) may or may not manifest.[8]

Once diagnosed, the clinical features of the disorder can be improved or prevented by administering pharmacological doses of the vitamin biotin. Treatment with biotin is effective; however, if the therapy is delayed, neurological damage may not be completely reversed and the disorder can rapidly lead to coma and death.[4,5,6,8,12]

3. PRINCIPLE OF THE ASSAY

The biotinidase from cellulose paper (dried blood spot samples) is extracted with a buffer containing the artificial substrate biotin 4-amidobenzoic acid (B-PAB). The biotinidase enzyme catalyses the release of free 4-amidobenzoic acid (PABA) from B-PAB. The reaction is stopped by trichloroacetic acid which denatures the biotinidase. Then the colour reaction begins: the PABA produced reacts with sodium nitrite to form a diazoted compound (the nitrite in excess is neutralized with the ammonium sulfamate); after that the diazoted compound reacts with the NED to form an azo-dye. The purple colour produced can be measured colorimetrically with a photometer at 550 nm and is directly proportional to the activity of biotinidase present in the sample.

Reaction Sequence

B-PAB (catalysed by biotinidase) $\longrightarrow$ Biotin + PABA

PABA + H^+ + HNO_2 (diazotation reaction) $\longrightarrow$ $PABA^+$ (diazonium ion) + H_2O

$PABA^+$ + NED (measured at 550 nm) $\longrightarrow$ Azo dye (purple) + H^+

4. PRESENTATION

REF	Pack Size
1122050096	96 Assays

5. KIT COMPONENTS:

Reagents: (96 T Pack size)

1. **Calibrators and Controls blood spots:** 1+1 set of blood spot cards of porcine whole blood spotted onto Whatman S&S 903 paper containing 6 calibrators (with known concentrations of PABA) and 2 controls.
   Refer to the quality control sheet for the exact concentrations of the Calibrators and acceptable range values of the Controls.
2. **Substrate buffer**: 4 x 2.5 ml of lyophilized substrate buffer containing substrate (B-PAB),a preservative and a stabilizer. Reconstitute each vial with 2.5 ml of distilled water.
   The reconstituted substrate is stable for 3 months at 2-8 °C.
3. **Stop solution**: 1 x 10.0 ml of trichloroacetic acid 30% (w/v). Ready to use.
4. **Colour Reagent 1**: 1 x 3.0 ml of sodium nitrite with a preservative. Ready to use.
5. **Colour Reagent 2**: 1 x 3.0 ml of ammonium sulfamate with a preservative. Ready to use.
6. **Colour Reagent 3**: 1 x 3.0 ml of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED). Ready to use.

Reagents	Quantity	Physical State
Calibrators & Controls	1 set each	Dried blood spots
Substrate buffer	4 x 2.5 ml	Lyophilized
Stop solution	1 x 10.0 ml	Ready to use
Colour reagent 1	1 x 3.0 ml	Ready to use
Colour reagent 2	1 x 3.0 ml	Ready to use
Colour reagent 3	1 x 3.0 ml	Ready to use

Accessories:

1. **Microtubes**
2. **Flat-bottom microtiter plates** with superior optical quality (Assay Plates).

6. STORAGE AND STABILITY OF THE KIT

1. Store all reagents at 2-8°C when not in use. Calibrators and Controls should be stored protected from moisture and light in the original bag with desiccant. Stable at 2-8°C until expiry date stated on the label. Make sure that the plastic bag remains sealed during storage.
2. **We recommend that the Blood Spots (Calibrators & Controls) should be preferably stored at -20°C with desiccant when not in use for prolonged period**.
3. Unopened reagents will retain reactivity until expiration date shown on the label. Do not use reagents beyond this date.

7. MATERIALS REQUIRED BUT NOT PROVIDED

1. Distilled or de-ionized water.
2. Adjustable, automatic micropipettes with disposable tips.
3. Microtiter plate reader equipped with 550 and 630 nm filter in endpoint reading mode.

To be Procured Separately

4. Blood spot puncher 3.2 mm.
5. Blood spots collection cards [Whatman Schleicher & Schuell 903 recommended; CLSI NBS01-A6 compliant].
6. Incubator (37°C).
7. Centrifuge with rotor adapted for microtubes.

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 labware.
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 foil pouch containing desiccant when not in use, but ensure that spots are equilibrated to 18-25°C before use.

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. 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 3%. 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.
5. Avoid splashing and aerosol formation; in case of spilling, wash carefully with a 3% sodium hypochlorite solution and dispose of this cleaning liquid as potentially infectious waste.
6. Some reagents contain sodium azide as preservative; to prevent build-up of explosive metal azides in lead and copper plumbing, reagents should be discarded by flushing the drain with large amounts of water.
7. **Caution**: Elution Buffer containing trichloroacetic acid (TCA), is highly acidic and

corrosive. Protective gloves and safety glasses should be worn while using this reagent.

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 [9] 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 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.
- Given the lack of stability of biotinidase activity on dried blood, we recommend to store the dried blood spot at –20°C or lower (the activity is stable for a period of at least 1 month at –20°C).
- 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:
- 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), birth weight 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. ASSAY PROCEDURE

1. Bring all reagents and calibrators/controls to room temperature before pipetting.
2. Reconstitute each vial of the **Substrate buffer** with **2.5 ml** distilled water and prepare the microtubes for the tests.
3. Punch 2 blood spots of **Calibrators (C0-C5), Controls (L1, L2)** and **Samples** (each 3.2 mm diameter). Put 2 discs into the respective microtubes.
4. Pipette **100 µl** of **Substrate Buffer** into each microtube. Ensure that each disk is fully immersed in the liquid.
5. Eluate the spots for **10 minutes** on a shaker at 900 rpm.
6. Incubate the microtubes overnight for (**16 – 18 hours)** at **37°C**.

Note: The same incubation time and temperature must be adhered strictly across all your tests run to maintain assay consistency.

7. After the incubation, remove the microtubes from the incubator and pipette **60 µl** of **Stop Solution** into each microtube. A precipitate is formed in seconds. We recommend to centrifuge the microtubes to settle down all precipitates (**10 min** at **4000 rpm**). Transfer **60 µl** of the eluate from each microtube to the corresponding well of the flat bottom microtiter plate (be careful not to resuspend or transfer the precipitate).
8. Pipette quickly **30 µl** of the **Colour Reagent 1** into each well.
9. Incubate **3 minutes** (± 1 min) at room temperature (**20-26°C**).
10. Add **30 µl** of **Colour Reagent 2** to each well.
11. Incubate **3 minutes** (± 1 min) at room temperature (**20-26°C**).
12. Add **30 µl** of **Colour Reagent 3** to each well
13. After **10 minutes** (± 1min) of incubation at room temperature measure the optical density at 550 nm with a reference at 630 nm. There is no need to wait longer than 10 minutes.

Please note the following:
A too long exposition to Stop solution could affect the linearity of the curve and the stability of the samples. Make sure this step is as short as possible.

11. CALCULATION OF RESULTS

Draw a calibration curve, by plotting the calibrators activity in µmol/dl (x-axis) against the absorbance obtained for each calibrator (y-axis). The standard curve is calculated by a linear regression function. Using computer programs, the curve is best described by a 2-point linear regression fit with linear axes. Corresponding Biotinidase activity are obtained by interpolating the absorbance of each sample on the calibration curve.

Note: Measuring Unit: 1 µmol of p-aminobenzoic acid produced from Biotin-PAB per dL per (16-18hrs) of incubation at 37°C.

12. EXPECTED VALUES AND INTERPRETATION CRITERIA

CLASSIFICATION

	Activity	µmol/dl
No deficiency	100%	~ 70 µmol/dl
Partial deficiency	10 to 37% normal activity	7 – 26 µmol/dl
Total deficiency	< 10% normal activity	< 7 µmol/dl

NB: these levels were established from a Caucasian population. We recommend each laboratory to establish their own classification based on specimens from the lab routine population.

13. QUALITY CONTROL

Internal controls (L1-L2) included in the kit should be routinely monitored to check that measured concentrations are within the stated values. These controls provide valuable information regarding the validity of the test according to manufacturer. If the precision of the assay does not correlate with this standard and repetition excludes errors in technique, check the pipetting and timing devices, instrument calibration, expiration dates on reagent labels and prepared working solutions, storage conditions, temperature control devices.[10]

14. LIMITATIONS OF THE PROCEDURE

As with any other in vitro screening test, the data obtained using **Born Safe™** Neonatal Biotinidase kit should be used as an aid to other medically established procedures and results interpreted in conjunction with other clinical data available to the clinician. A diagnostic procedure should be used to confirm a diagnosis of biotinidase deficiency. Conditions which are known to cause anomalous analytical assay results are:

- sample spot not uniformly saturated with blood
- sample spots punched too close to the edge of the blood spot
- poorly collected and improperly dried specimens
- non-eluting blood spot due to deterioration of sample caused by exposure to heat and humidity contamination of blood spot filter paper with fecal material.

15. 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.

16. REFERENCES

1. Cowan TM, Blitzer MG, Wolf B; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. Technical standards and guidelines for the diagnosis of biotinidase deficiency. Genet Med. 2010 Jul;12(7):464-70.
2. Heard GS, Secor McVoy JR, Wolf B. A screening method for biotinidase deficiency in newborns. Clin Chem. 1984 Jan;30(1):125-7.
3. Wolf B, Grier RE, Allen RJ, Goodman SI, Kien CL. Biotinidase deficiency: the enzymatic defect in late onset multiple carboxylase deficiency. Clin Chim Acta. 1983 Jul 15;131(3):273-81.
4. Wolf B. Worldwide survey of neonatal screening for biotinidase deficiency. J Inherit Metab Dis.1991;14(6):923-7.
5. Wolf B.,Cowan T., Blitzer M., Techincal standards and guidelines for the diagnosis of biotinidase deficiency. Genet Med. 2010 Jul;12(7):464-70.
6. Yamaguchi A, Fukushi M, Arai O, Mizushima Y, Sato Y, Shimizu Y, Tomidokoro

K, Takasugi N. A simple method for quantification of biotinidase activity in dried blood spot and its application to screening of biotinidase deficiency. Tohoku J Exp Med. 1987 Aug;152(4):339-46
7. Wolf B., Heard, G.S., Jefferson, B.S. et al. (1985): Clinical findings in four Children with biotinidase deficiency detected through a newborn statewide neonatal screening program. N Engl J Med 313, 16-19.
8. Wolf, B., Grier, R.E., McVoy, J.R.S. and Heard, G.S. (1985): Biotinidase Deficiency: A novel vitamin recycling defect. J Inherit Metab Dis 8 suppl.1, 53-58.
9. Clinical and Laboratory Standards Institute (2007): Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard – Fifth Edition; CLSI Document LA4-A5. CLSI, Wayne, Pennsylvania 19087-1898, USA.
10. Westgard, J.O. et al.(1981): A multi-rule chart for quality control. Clin. Chem. **27**, 493–501.
11. Wolf B. (1991). J. Inherit. Metab. Dis. 14 (6) 923
12. Hymes J, Wolf B. (1996). Clin. Chim. Acta 15 (1) 1

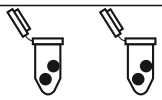
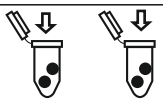
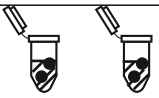
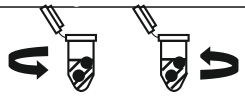
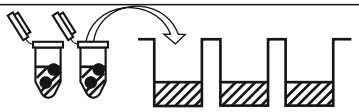
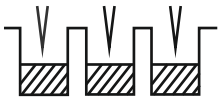
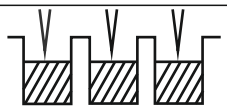
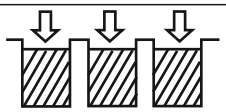
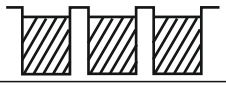
Summary Protocol

BornSafe™
Biotinidase

REAGENT PREPARATION

Reconstitute Biotinidase Substrate		Each vial with 2.5 ml distilled/ deionized Water

ASSAY PROCEDURE

1. Punch out Calibrators, Controls and Unknown		2 blood spots into microtubes
2. Add Biotinidase Substrate Reagent		Add 100µl. Ensure that each disc is fully immersed in substrate buffer
3. Extraction & release of PABA from B-PAB		Elute for 10 min on an orbital plate shaker (900rpm)
4. Incubate		16-18 hrs at 37°C
5. Add Stop Solution		Add 60µl into each microtubes
6. Centrifuge		10 mins at 4000 rpm
7. Transfer to corresponding wells of flat bottom microtiter plate		Pipette 60µl of the eluate from each microtubes
8. Pipette Colour reagent 1		Dispense 30µl, gently mix
9. Incubate		3 minutes (±1min) at RT (20-26°C)
10. Pipette Colour reagent 2		Dispense 30µl, gently mix
11. Incubate		3 minutes (±1min) at RT (20-26°C)
12. Pipette Colour reagent 3		Dispense 30µl, gently mix
13. Incubate		10 minutes (±1min) at RT (20-26°C)
14. Read/Measure		Measure the OD at 550nm with a reference at 630nm

SYMBOL KEYS

Temperature Limitation	Consult Instructions for use	Date of Manufacture	LOT Batch Number / Lot Number
Manufacturer	IVD *In vitro* Diagnostic Medical Device	This side up	
Use by	REF Catalogue Number	Σ Contains sufficient for <n> tests	Caution

Manufactured by:
Coral Clinical Systems
A Division of Tulip Diagnostics (P) Ltd.
Bldg. 'D', Plot No. M-46, Phase III B, Verna Industrial Estate, Verna, Goa - 403 722, INDIA.
Regd. Office: Gitanjali, Tulip Block, Dr. Antonio Do Rego Bagh, Alto Santacruz, Bambolim Complex P.O., Goa - 403 202, INDIA.

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