Neonatal G-6-PD Deficiency Screening Assay

BornSafe™
G6PD

Enzymatic assay for the quantitative determination of Glucose-6-phosphate dehydrogenase (G6PD) deficiency in new born dried blood spots

(FOR IN VITRO DIAGNOSTIC USE ONLY)

1.INTENDED USE

Born Safe™ Neonatal G-6-PD Screening Assay is an enzymatic colorimetric assay for the quantitative determination of glucose-6-phosphate dehydrogenase (G6PD) activity in blood specimens dried on Whatman S&S 903 filter paper. This test is intended for use as a screening method for red cell glucose-6-phosphate dehydrogenase deficiency in new-borns.

2. SUMMARY AND EXPLANATION OF THE ASSAY

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common of all clinically significant enzyme defects.[1] It is an X-linked recessive hereditary disease characterised by abnormally low levels of glucose-6-phosphate dehydrogenase (G6PD), a cytoplasmic enzyme in the pentose phosphate metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione in these cells that helps protect the red blood cells against oxidative damage.

Glucose-6-phosphatase dehydrogenase deficiency is the most common disease-producing enzymopathy in humans. It affects 400 million people worldwide. The prevalence ranges from 5 to 25% in endemic areas, such as Africa, the Middle East, Asia, the Mediterranean and Papua New Guinea. The highest incidence is found in Kurdish Jews: 65%. Incidences ranging from 0.5 to 6.9% have been reported in North and South America. Around 400 mutations have been reported so far. G6PD is remarkable for its genetic diversity. Many variants of G6PD, mostly produced from missense mutations, have been described with wide ranging levels of enzyme activity and associated clinical symptoms.[2,3,4,5,6,7,8,9,10,11,12] Four G6PD variants have been classified, listed here in order of severity: class 1. hereditary no spherocytic hemolytic anemia; class 2. severe deficiency; class 3. mild deficiency; class 4. not deficient variant.[13] Patients with G6PD deficiency are sensitive to certain chemicals such as 6-aminoquinoline antimalarial drugs, fava beans (G6PD deficiency is closely linked to favism, a disorder characterized by a hemolytic reaction to consumption of broad beans), sulfa drugs, some types of infection, and large doses of vitamin C, all of which have been shown to induce hemolysis.[1] Sufferers of G6PD deficiency, who have been subjected to oxidative stress due to contact with precipitating factors, often show signs of jaundice, fatigue, paleness, shortness of breath, lethargy, tachycardia, and an enlarged spleen.[14] Management of G6PD deficiency is essentially the avoidance of precipitating factors. G6PD deficiency in neonates and young children warrants extra attention, as the build-up of unconjugated bilirubin can lead to kernicterus,[15,16] a leading cause of mental retardation and death in neonates. The incidence of jaundice is much higher in new born populations with higher levels of class 2 variants than in other population [17,18] but, if detected, is easily preventable through phototherapy [19,20] and phenobarbital treatment [21]. Most individuals with G6PD deficiency are asymptomatic.

3. PRINCIPLE OF THE ASSAY

The Neonatal G-6-PD Screening Assay uses dried blood spots specimens (cellulose paper) eluted in a buffer. After the elution step, the eluate containing G-6-PD is incubated with a reagent containing glucose-6-phosphate, which in the presence of NADP, catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate. The NADPH produced reacts with a colour reagent in which a tetrazolium salt gets reduced producing a distinct colour.

This colour is measured at 550 nm and is directly proportional to the concentration of Glucose-6-phosphate dehydrogenase present in the sample. The results are calculated by evaluating the increase in OD per minute for unknowns against the calibrator with known G-6-PD activity. The reduced tetrazolium salt can be measured by a two-point measurement mode using two measurements one at time = 0 min, and the second one at time = 15 min.

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6-PG} + \text{NADPH}^* + \text{H}^+$$

G-6-P = Glucose-6- phosphate
$NADP^+$ = Nicotinamide adenine dinucleotide phosphate
G6PD = Glucose-6-phosphate dehydrogenase
6-PG = 6-phosphogluconate
NADPH = Nicotinamide adenine dinucleotide phosphate (reduced)

4. PRESENTATION

REF	Pack Size
1122020096	96 Assays

5. KIT COMPONENTS:

Reagents: (96T Pack size)

1. **Elution Buffer**: 1 x 8.0 ml of buffered solution. Ready to use. Preservative NaN_3 (< 0.1%).
2. **Reagent** :For 96T: 4 x 2.0 ml of Glucose-6-phosphate solution. Lyophilized. Reconstitute with 2.0 ml of distilled water. After reconstitution, the reagent can be stored at 2-8° C for one month. Preservative: NaN_3 (< 0.1%).
3. **Colour Reagent**: 1 x 8.0 ml. of tetrazolium salt. Ready to use. Preservative NaN_3 (< 0.1%).
4. **Colour Booster**: 1 x 1.0 ml of intermediate electron acceptor. Ready to use. Preservative NaN_3 (< 0.1%).
5. **Calibrator and Controls blood spots**: 1 +1 set of dried blood spots cards of human whole blood spotted onto Whatman S&S 903 paper containing 1 calibrator and 2 controls.

Reagents	Quantity (96T)	Physical State
Elution Buffer	1 x 8.0 ml	Ready to use
Reagent	4 x 2.0 ml	Lyophilized
Colour Reagent	1 x 8.0 ml	Ready to use
Colour Booster	1 x 1.0 ml	Ready to use
Calibrator and Controls	1 set each	Dried blood spots

Accessories:

1. **Round bottom microtiter plates** (Elution Plates).
2. **Flat-bottom microtiter plate**s 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. Ensure that all reagents are equilibrated to room temperature before use.
4. 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 nm filter along with other standard filters.

To be Procured Separately

4. Blood spot puncher 3.2 mm.
5. Orbital plate shaker (900 rpm).
6. Blood spots collection cards [Whatman Schleicher & Schuell 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 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. 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 of, 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 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.

6. 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.
7. 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.
8. **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 fifth days 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). Bilirubin (≤ 685 µmol/L) and hemoglobin (≤ 250 g/L) do not interfere with the assay. 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, [22] 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.
- Store in sealed paper envelopes or containers that will provide protection from moisture, light, heat and contact with other materials
- Samples should be stored at 2-8°C in zip lock, moisture resistant bags with desiccants. There is significant decline in G6PD activity in blood spot samples stored at room temperature. Specimens should be tested as soon as possible after receipt. Samples that have lost G6PD activity may test as false positives.
- Due to the loss of enzyme activity of the sample, if the test is not run within 3-4 days, storage at –20°C is recommended.
- 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), 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.

Note: Transport the specimen to the laboratory within 24 hours after collection. Specimens that are analysed 3 to 4 weeks or longer after collection have shown an increased tendency to float in solutions contained in the wells.

10. REAGENT PREPARATION

REAGENT:

Reconstitute each vial with 2 ml of distilled or de-ionized water. Mix gently to aid reconstitution. After reconstitution, the reagent can be stored at 2-8°C for one month. Preservative: NaN_3 (< 0.1%).

COLOUR REAGENT MIXTURE:

Prepare the mixture by adding 1 part of Colour Booster to 10 parts of Colour reagent.

The following table gives the volumes required for each component to run specific number of tests (volumes in µl).

No. of tests	Colour Booster (µl)	Colour Reagent (µl)	Colour Reagent mixture Total volume (µl)
10	80	800	880
20	160	1600	1760
40	320	3200	3520
80	640	6400	7040
100	800	8000	8800

After reconstitution keep the Colour Reagent mixture away from the direct light (i.e. wrapped in aluminium foil); stable for 4 hours at 2-8°C. Not to be left out of the refrigerator longer than needed. Take the colour reagent out of the refrigerator just prior to use. Take out just the quantity you are going to use for the day. Return the rest of the colour reagent in the refrigerator.

11. ASSAY PROCEDURE

A. ELUTION STEP:

1. Bring all reagents (except the colour reagent) to room temperature before pipetting.
2. Punch 2 blood spots (3.2 mm diameter) from the **Blood Spot cards** of **Calibrator** and **Controls** in U bottom microtiter plate. After that, punch 2 blood spots from each patient specimens into the remaining wells of the plate.
3. Add **75 µl** of **Elution Buffer** into each well.
4. Incubate the microtiter plate on an orbital plate shaker (900 rpm) for **30 minutes** at room temperature. Ensure that each disk is fully immersed in the Elution Buffer.
5. During the elution, reconstitute the **Reagent** and prepare the **Colour Reagent mixture** and a flat bottom microtiter plate for the assay.

B. SAMPLE TRANSFER AND ASSAY:

1. After the incubation, remove the plate from the plate shaker and transfer **15 µl** of the eluate from each well to the corresponding wells of the new flat bottom microtiter plate.
2. Add **75 µl** of the **Reagent** to each well and mix well.
3. Add **75 µl** of **Colour Reagent mixture** prepared by mixing 1 part of Colour Reagent Booster with 10 parts of Colour reagent.
4. Place the plate in a plate reader and read the absorbance of the plate at 550 nm using two measurements one at time = 0 and the second one 15 minutes later.

C. CERTIFICATION/TRACEABILITY TO REFERENCE MATERIAL

Currently there is no Reference Material available for G6PD

12. CALCULATION OF RESULTS

The optical densities for the calibrator and samples are obtained at '0' minute and at '15' th minute respectively. The Δ OD/minute can be calculated as per following formulas.

RESULTS EXPRESSED IN U / g Hb.

$$\textbf{Sample Value (Activity in U/g Hb)} = \frac{(\Delta\,\textbf{OD sample}_{550nm}\textbf{/min})}{\Delta\,\textbf{OD calibrator}_{550nm}\textbf{/min}} \times \textbf{Calibrator value}$$

$$(\Delta\,\textbf{OD sample}_{550nm}\textbf{/min}) = \frac{OD\,sample_{550nm}/15\,min - OD\,sample_{550nm}/0\,min}{15}$$

$$(\Delta\textbf{OD calibrator}_{550nm}\textbf{/min}) = \frac{OD\,calibrator_{550nm}/15\,min - OD\,calibrator_{550nm}/0\,min}{15}$$

- ΔOD sample is the change in optical density (per minute) for the sample, 550 nm is the wavelength at which optical density is measured
- ΔOD calibrator is the change in optical density (per minute) of a calibrator measured at a particular wavelength (550 nm)

13. QUALITY CONTROL

Internal controls {Levels L1 and L2} included in the kit should be routinely monitored to check that measured activities 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 and temperature control devices. [23]

14. EXPECTED VALUES AND INTERPRETATION CRITERIA

The normal range of G6PD activity and incidence of clinically significant G6PD deficiency, is known to vary based on the population. The determination of presumptive positives for G6PD deficiency is based on a predetermined cut-off point. The incidence

of G6PD deficiency will be inherently higher in some populations than others.
A review of various NBS programs studies and numerous recommended guidelines suggests that Cut-off points are determined based on minimizing the number of false positive and false negative results. Because the cut-off values derived in those studies were based on the comparison with each lab's current method, laboratories that do not have a method in place should start by using a cut-off of 2 U/g Hb. A re-evaluation of the cut-off value should be made after at least 1000 samples have been run of G6PD deficiency will be inherently higher in some populations than others.

Suggested Cut off Values:

Interpretation	Concentration (U/g Hb)
Deficiency	<1.5 U/g Hb
Partial Deficiency	1.5 – 2 U/g Hb
Normal	>2 U/g Hb

Please note that the values mentioned in this section should only be used as a guideline. They are not intended to represent the true cut-off value of any given population of specimens. It is strongly recommended that each laboratory establish its own normal range and cut-off value based on specimens from the laboratory's routine population.
Some specimens, including true Deficient may give values which are below the Analytical Sensitivity of the method. These specimens should be classified and reported as 'presumptive deficient'.

15. LIMITATIONS OF THE PROCEDURE
As with any other in vitro screening test, the data obtained using the Neonatal G6PD 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 glucose-6-phosphate dehydrogenase 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 faecal material
- samples stored in warm and/or humid conditions lose activity and may cause false positive results

Specimen collection and storage have a significant effect on the test results. Refer Specimen Collection & Storage for details.

Blood Collection Paper:
Born Safe™ Neonatal G6PD Deficiency Screening Assay is designed for use with specimens collected on Whatman S&S 903 specimen collection paper. The use of other non CLSI NBS01-A6 compliant collection papers may affect results, hence not recommended.
Heat and Humidity:
Specimens which have been exposed to elevated temperatures and/or humidity may exhibit low G6PD activity due to enzyme inactivation, increasing the numbers of specimens classified as presumed Deficient and Indeterminate during hot and humid months of the year.
Transfusions:
The G6PD activity measured in specimens from infants who have received transfusions may not provide an accurate assessment of the true status of the new born from which the specimen was received.
Confirmatory Testing:
Patient diagnosis should not be considered solely from a blood spot test. Supplemental testing according to the established protocol of the screening facility should be used to verify the true status of all specimens classified as presumptive Deficient.

16. 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.

17. REFERENCES

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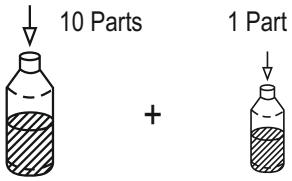
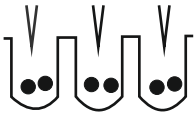
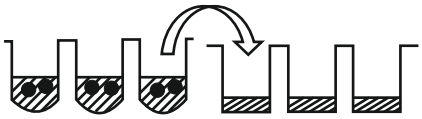
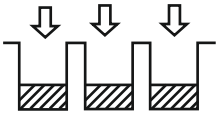
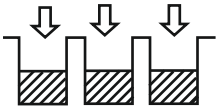
Summary Protocol

BornSafe™
G6PD

REAGENT PREPARATION

Reconstitute each vial of G6PD Substrate Reagent		2 ml Distilled/Deionized water
Colour Reagent Mixture		10 parts Colour Reagent + 1-part Colour Booster

ASSAY PROCEDURE

1. Punch out Calibrators, Controls and Unknowns in 'U' bottom microtiter plate		2 blood spots into each wells of round bottom microtiter plate
2. Add Elution Buffer		Add 75µl. Ensure that each disk is fully immersed in Elution Buffer
3. Incubate		30 min at RT (20°-26°C) on an orbital plate shaker (900rpm)
4.Transfer to corresponding well of flat bottom microtiter plate		Add 15µl of the eluate from each well
5. Add Substrate reagent		75µl to respective well and mix
6. Add Colour Reagent Mixture		75µl to respective well and mix
7. Read/Measure		Place the plate in a microplate reader and read at 550nm at '0' minute and at '15' th minute

SYMBOL KEYS

Temperature Limitation	Consult Instructions for use	Date of Manufacture	Batch Number / Lot Number
Manufacturer	*In vitro* Diagnostic Medical Device	This side up	
Use by	Catalogue Number	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.

1218/VER-01