Instructions for use

For *in vitro* diagnostic use only

Neonatal G6PD

Fluorometric determination of glucose-6-phosphate dehydrogenase activity from blood specimens dried on filter paper.

Product no. 6199 860 (S&S 903, 960 wells)

CE

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INTENDED USE

Ani Labsystems' Neonatal G6PD test is designed for the quantitative *in vitro* determination of glucose-6-phosphate dehydrogenase activity in blood specimens dried on filter paper intended for newborn screening of glucose-6-phosphate dehydrogenase deficiency

INTRODUCTION

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is the most common human enzyme deficiency; an estimated 400 million people worldwide are affected by this enzymopathy (6). With regards to the demographics of G6PD deficiency, most of the affected individuals reside in Africa, the Middle East, and Southeast Asia. The incidence can be one in four people for given populations (6).

An important gene located at the q28 locus (5) on the X-chromosome is the gene for the G6PD enzyme (6). All X-linked genetic conditions, such as G6PD deficiency, are more likely to affect males than females. Severity of the disease varies with the type of mutation (1, 6).

The G6PD enzyme catalyzes an oxidation/reduction reaction. The G6PD enzyme functions in catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconolactone while concomitantly reducing nicotinamide adenine dinucleotide phosphate (NADP+) to NADPH.

This is the first step in the pentose phosphate pathway and is essential for the synthesis of both DNA and RNA (7). G6PD is also responsible for maintaining adequate levels of NADPH inside the cell. NADPH is a required cofactor in many biosynthetic reactions (6). This has a profound effect on the stability of red blood cells since they are especially sensitive to oxidative stresses in addition to having only one NADPH-producing enzyme to remove these harmful oxidants. When the red blood cells can no longer transport oxygen effectively throughout the body (7), a condition called hemolytic anemia arises. An anemic response can be induced in affected individuals by certain oxidative drugs, fava beans, or infections (2). Death ensues if the hemolytic episode is not properly treated.

In addition to hemolytic anemia, G6PD deficient individuals may expect neonatal jaundice, abdominal and/or back pain, dizziness, headache, dyspnea (irregular breathing), and palpitations (3). In some cases, the neonatal jaundice is severe enough to cause death or permanent neurologic damage (2).

Treatments for neonatal jaundice and hemolytic anemia have existed for many years (3, 4). Naturally the patients first need to be identified as carrying the deficiency which makes the diagnostic so critical.

PRINCIPLE OF THE TEST

The principle of the Ani Labsystems' Neonatal G6PD assay is based on an enzymatic method intended for the quantitative determination of glucose-6-phosphate dehydrogenase activity from dried blood spots. $NADP^+$ is reduced by G-6-PD (glucose-6-phosphate dehydrogenase) in the presence of G-6-P (glucose-6-phosphate), and the rate of formation of NADPH is proportional to the G-6-PD activity, and is determined fluorometrically (see below). Cold copper reagent is added to stop the reaction and stabilize the fluorescent complex. Fluorescence is measured (λex 355 nm, λem 460 nm).

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PD}} \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

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 exposure of calibrators and controls to moisture.
- Open the substrate bottle just before use.

1 SUBSTRATE — 10 bottles
Lyophilized reaction mixture contains G-6-P and $NADP^+$

2 SUBSTRATE BUFFER — 4 x 50 ml

3 STOP SOLUTION — 4 x 50 ml
Copper containing solution.

4 CALIBRATORS A –F — 1 sheet
Ready to use calibrators, 5 sets.
The values (IU/gHb) of calibrators are lot specific. For exact values refer to the calibrator/control sheet included in each kit. Potential biohazardous material.

5 CONTROLS C1 – C2 — 1 sheet
Ready to use controls, 5 sets.
The values (IU/gHb) of controls are lot specific. For exact values refer to the calibrator/control sheet included in each kit. Potential biohazardous material.

6 CLEAR MICROPLATES — 10 pcs
Solid microtiter plates (96 wells) meant for the enzymatic reaction and measurement.

INCUBATION COVERS, 10 pcs

REAGENT BASINS, 10 pcs

REAGENT PREPARATION

Reagent	Preparation	Stability of opened / diluted reagents (at +2°C to +8°C)
1 Substrate	Ready for use for reconstituted substrate	.
2 Substrate buffer	Ready for use for reconstituted substrate	12 months *)
Reconstituted substrate	Solubilize the lyophilized Substrate (reagent 1) with 16 ml of Substrate buffer (reagent 2).	Discard unused reconstituted substrate.
3 Stop solution	Ready for use	12 months *)
4-5 Calibrators / Controls	Ready for use	12 months *)

*) Open the substrate bottle just before use. Once the calibrators and controls foil-packages are opened, they should be resealed tightly with the desiccant. The stability of the opened reagents is the maximum only if they are stored properly at +2°C to +8°C. High environmental temperature, moisture and contamination may decrease the stability.

MATERIALS REQUIRED BUT NOT PROVIDED

- Graduated cylinders for reagent dilution.
- Vials to store the diluted reagents.
- Precision pipettes (one channel eg. 5-50 µl, 20-200µl, 100-1000 µl ranges and multi-channel 50-300 µl)
- Timer, 90 min range.
- Microplate incubator/shaker eg. iEMS Incubator/Shaker with (cat.no. 5112 200 or cat. no. 5112250)
- Disc puncher with a diameter of 3 mm to cut off paper discs of dried blood controls, calibrators and samples or Woodpecker disc processor to punch discs (cat. no. 5600 200)
- Disc holders for Woodpecker, disposable (100 pcs, cat. no. 9700300X)
- Microplate fluorometer, (λex 355 nm, λem 460 nm), eg. Fluoroskan Ascent Neonatal (100-240V) (cat. no. 5210530).
- Disposable gloves.

PRECAUTIONS
For *in vitro* diagnostic use only.

Warning - POTENTIAL BIOHAZARDOUS MATERIAL:

Each donor unit used in the preparation of the calibrators/controls in the kit has 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 specimens should be handled at the Biosafety level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes for Health Manual, “Biosafety in Microbiological and Biomedical Laboratories,” 1999 (10).

Discard solutions into a waste drainage network and flush with large volumes of water.

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

SPECIMEN 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 specimen in a separate paper envelope and mail it to the laboratory. Blood spot specimens received in the laboratory should be stored at +2°C ... +8°C protected against moisture.

The specimen collection technique is described in detail in NCCLS document LA4-A3 (8).

TEST PROCEDURE

PRELIMINARY PREPARATIONS

– **Bring the reagents to room temperature (+20°C to +25°C) before starting the assay, except the stopping solution which should be kept at +4°C.**

Prestep. Solubilize the substrate vial with 16 ml of substrate buffer [1)]

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

Punch out single **3 mm** discs from patient specimens into microplate wells.

Add **150 µl** of reconstituted substrate into each well. Make sure that **THE DISCS ARE COMPLETELY SOAKED IN THE LIQUID BEFORE INCUBATION**.

Cover the plate and incubate **30 min** (+/- 5 min) at **RT** (+20°C - +25°C) **in dark** with **shaking speed of 1150 rpm** (or shaking speed of 650rpm if Woodpecker is used).

STEP II
Add **150 µl** of cold (+4 °C) stop solution. Make sure that **THE DISCS ARE COMPLETELY SOAKED IN THE LIQUID, OR REMOVE THE FLOATING DISCS BEFORE MEASUREMENT**.

Measure immediately the fluorescence **(λex 355 nm, λem 460 nm)**

NOTES:
1) 16 ml is needed for the re-suspension of one lyophilized substrate vial and sufficient for 96 well.

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

RESULTS

Quality Control Values

Before calculating the results, make sure that the fluorescence ratios obtained for the calibrators and the concentration of controls fall within the Quality Control guidelines indicated below.

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

Quality control values

QC Sample	Expected value
RFU Ratio (4D-4A) / (4B-4A) RFU Ratio (4F-4A) / (4B-4A)	2.7 – 5.1 6.0 – 11.2
Controls C1 and C2	see attached control sheet

Calculation of the Results

Manual calculation:

1. Draw a standard curve on graph paper with fluorescence values on the ordinate and G6PD enzyme concentrations of the calibrators on the abscissa.
2. Read the G6PD enzyme concentrations of controls and unknowns from the calibration curve.

Automatic calculation:

If automatic data processing can be used, cubic spline curve fitting with lin-lin axis scaling is recommended. See figure 1 for an example of the typical calibration curve (obtained with Fluoroskan Ascent with normal beam scaling factor of 5,18)

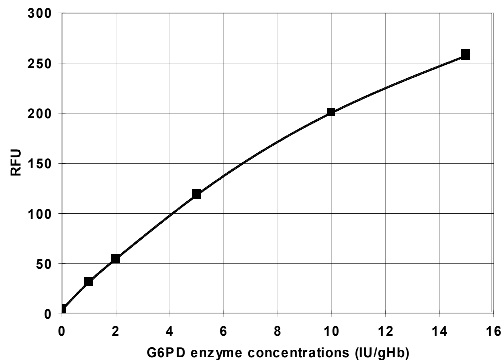


Figure 1: Typical calibration curve using cubic spline curve fitting

Interpretation of the Results

The test will give quantitative values for G6PD activity present in specimen. The discrimination between normal subjects and presumptive positive for G6PD deficiency is based on the predetermined cut-off point. **It is recommended that each laboratory sets its own cut-off value** based on the reference range of the given normal population.

PERFORMANCE CHARACTERISTICS

Reproducibility

Sample no.	Mean concentration (U/gHb)	Standard deviation (U/gHb)	CV%
Within-run imprecision (10 replicates)			
4B 4D 4E	1,0 5,0 10,0	0,058 0,126 0,257	5,8 2,5 2,6
Between-run imprecision (16 successive runs, averages of 2-10 replicates)			
Ref1 Ref2 Ref3	7,1 4,2 1,2	0,28 0,19 0,09	3,9 4,6 7,5

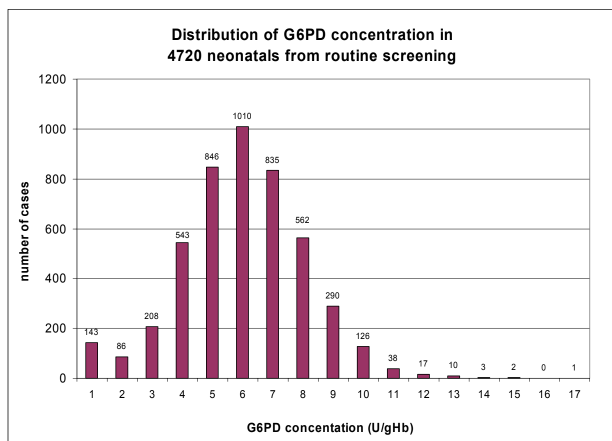
Detection limit

The detection limit of G6PD calculated as a mean + 3SD of the near-to-zero blank was 0.05 U/gHb.

CLINICAL EVALUATIONS

Limited clinical evaluation is performed at Guangzhou Neonatal Screening Center, Guangzhou China. Routine samples from normal screening program are screened with Ani Labsystems G6PD kit. Among samples (n=4720) that were analyzed, 231 cases were determined to be initially positive when the cut-off is set at 2.0 U/gHb. Among these initial positive cases, 203 cases were confirmed positive by G6PD/6PGD ratio method which is the routine confirmatory method from Guangzhou Neonatal Screening Center, suggesting the incidence of 4.3% (203/4720). 28 cases were false positive giving false positive rate of 0.59%. We are not aware of missing positive cases. See figure 2 for the distribution of G6PD concentration among all the samples tested.

Figure 2. distribution of G6PD enzyme activities among all the samples tested (N=4720)



LIMITATIONS OF THE PROCEDURE

The Neonatal G6PD is designed for screening of newborns for G6PD deficiency. For exact G6PD enzyme activity values use serum as a specimen.

Because no single method leads to the definitive diagnosis, the results of the present method should be interpreted in conjunction with the clinical condition and other laboratory methods.

TROUBLE SHOOTING

DECREASED SLOPE OF THE CALIBRATION CURVE AND LOW FLUORESCENCES	
Cause/Error	**Remedy**
1. Reagents are deteriorated * due to contamination of all reagents * due to improper storage * opened calibrators and controls are not sealed tightly	1. To prevent deterioration use aseptic technique when pipetting reagents repeatedly from the same vial. 2. To avoid deterioration, see instructions for reagent storage. 3. Seal tightly the opened calibrators and controls after use
2. Incubation temperature is too low	Incubate at 20 – 25°C
3. Incubation time is too short	Incubate 30min
4. Shaking speed is too low	Shake at 1150rpm for manual punch or 650rpm for Woodpecker punch
5. Fluoroskan Ascent Neonatal wavelength settings are not correct	Fluoroskan Ascent Neonatal excitation/emission wavelengths are 355/460

POOR PRECISION	
Cause/Error	**Remedy**
Only calibration curve	
1. Calibrators and controls are deteriorated due to improper storage	Protect calibrators and controls from excessive light and moisture by resealing the foil package tightly
2. blood discs are not completely soaked in the liquid	Make sure the discs are completely soaked in the liquid before incuation and measurement
Whole plate (including calibration curve)	
1. Liquid handling devices are not properly calibrated	Check calibration of your pipetting device
2. Air bubbles when pipetting	Pipette carefully
3. Contamination of wells by particles from air.	Perform assay in clean environment
4. Shaking speed is too low	Shake at 1150rpm for manual punch or 650rpm for Woodpecker punch
5. Blood discs are not completely soaked in the liquid	Make sure the discs are completely soaked in the liquid before incubation and measurement
Only patient samples	
1. Uneven distribution of blood in the sample	When possible, use samples fully impregnated with blood
2. Blood discs are not completely soaked in the liquid	Make sure the discs are completely soaked in the liquid before incubation and measurement

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RELATED PRODUCTS AND OTHER INFORMATION

Product no	Product description	Kit size
61 99 860	Neonatal G6PD	960 wells
61 99 896	Neonatal Phenylalanine	960 wells
61 99 897	Neonatal Phenylalanine	4800 wells
61 90 930	Neonatal Phenylalanine Controls	5 sets of 3 levels
61 90 940	Neonatal Phenylalanine Calibrators	5 sets of 6 levels
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

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