

G6PD Quantitative Kit A SCREENING KIT FOR DRIED BLOOD SPOTS

This kit is particularly suitable for screening for G-6-PD deficiency in newborns

KIT SIZE: 75 TESTS

1. Intended Use

G-6-PD Quantitative assay is an enzymatic colorimetric method for the quantitative determination of G-6-PD activity in dried blood spots specimens. The test is intended for use as a screening method for red cell glucose-6-phosphate dehydrogenase deficiency in newborns.

2. Summary and Explanation of the Test

Glucose-6-phosphate dehydrogenase (G-6-PD) is a cytoplasmic enzyme that is distributed in all cells. It catalyses the first step in the hexose monophosphate pathway, producing NADPH (Picture 1). This coenzyme is required as hydrogen donor for reactions of various biochemical pathways as well as for the stability of catalase and the preservation and regeneration of the reduced form of glutathione. Catalase and glutathione are both essential for the detoxification of hydrogen peroxide, therefore the defense of cells against H_2O_2 ultimately and heavily depends on G-6-PD. The red cells are exquisitely sensitive for oxidative damage and lack other NADPH-producing enzymes. The defense against oxidizing agents, epitomized by H_2O_2 , is mainly realized by glutathione, which converts H_2O_2 to H_2O stoichiometrically via glutathione peroxidase. NADPH is the hydrogen donor for the regeneration of reduced glutathione. An alternative pathway of H_2O_2 detoxification is via catalase, but this route is regarded ineffective under normal conditions (1), because of the lower affinity of catalase for H_2O_2 compared to that of glutathione peroxidase. G-6-PD deficiency is the most common known enzymopathy with around 400 million people affected worldwide. The prevalence ranges from 5 to 25% in endemic areas, such as Africa (2,3), the Middle East, Asia (4,5,6,7), the Mediterranean (8,9,10) 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 (11,12,13,14).

The clinical manifestations associated with G-6-PD deficiency are:

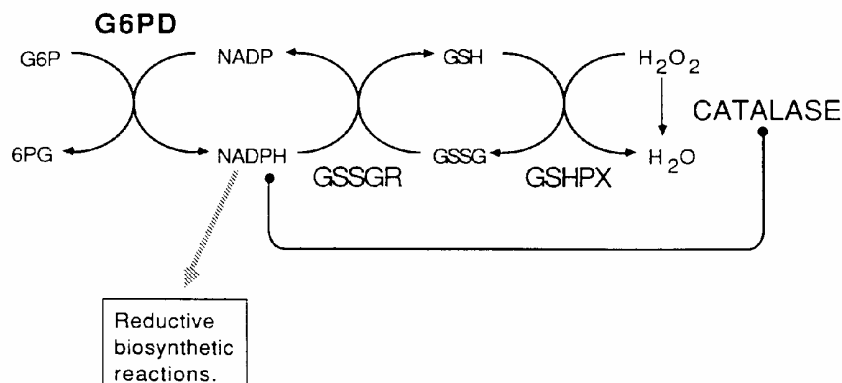
Drug induced hemolysis: certain anti-malarials, sulfonamides, sulfones and other drugs or chemicals are associated with significant hemolysis in subjects (15,16,17).

Infection induced hemolysis: numerous bacterial, viral and rickettsial infections have precipitated hemolysis, but the most important are infectious hepatitis, pneumonia and typhoid fever (18,19,20).

Favism: sudden onset of acute hemolytic anemia within 24 to 48 hours of ingesting fava beans (21,22, 23, 24, 25, 26, 28).

Neonatal jaundice: jaundice usually appears by 1 to 4 days of age (29,30,31,32).

Chronic nonspherocytic hemolytic anemia (33).



The main metabolic role of G-6-PD in red cells is the defense against oxidizing agents, epitomized by hydrogen peroxide. NADPH, a product of the G-6-PD reaction, is both the hydrogen donor for regeneration of reduced glutathione and a ligand for catalase (see text). GSSGR = glutathione reductase; GSHPX = glutathione peroxidase; G-6-P = glucose-6-phosphate; 6PG = phosphogluconate.

3. Principle of the Assay

This assay utilizes glucose-6-phosphate-dehydrogenase, which in the presence of NADP, catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate. The NADPH produced reacts with a color reagent in which a tetrazolium salt gets reduced producing a distinct color. This color is measured colorimetrically at 550 nm (500-570 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 (slope) for unknowns against the slope for a Standard with known G-6-PD activity. Alternatively, the reduced tetrazolium salt can be measured in endpoint mode using two measurements one at zero time and a second one after 12-15 minutes. During the measurements, the microplate should remain in the incubator. This protocol is strongly encouraged if your microplate reader is not equipped with a kinetic software or a heating plate. In this case the total ΔOD / sample should be used for calculations. See the attached "Benchtop" instructions for the formulas.

4. Kit Contents

- 1X 6 ml ELUTION/DILUTION BUFFER: Liquide. Contains 0.15% (w/v) sodium azide as preservative. (CAUTION: See safety section 6).
- 3 X 2 ml REAGENT VIALS: Lyophilized. CAUTION: Very toxic to humans.
- 1 X 1 ml COLOR REAGENT BOOSTER: liquid.
- 1 X 8 ml COLOR REAGENT: bright yellow liquid. CAUTION: Keep away from strong light sources.
- Filter paper.

Stability of the Chemicals

The minimum shelf-life of the chemicals contained in the kit are as follows:

ELUTION/ DILUTION BUFFER	Stable for at least 24 months at room temperature. See Exp. date on Label
REAGENT VIAL	After reconstitution, the reagent is stable for 5 days at 2-8°C, and 10 days if frozen, plus an additional 5 days following thawing. Stable for at least 18 months lyophilized in unopened vial. See Exp. date on label.
COLOR REAGENT BOOSTER	Stable for at least 24 months refrigerated. See Exp. date on label.
COLOR REAGENT	Stable for at least 24 months refrigerated. See Exp. date on label.

5. Reagents / Materials required but not provided

- U - well micro titration plates (elution plates)
- Water for injection (de-ionized water).
- Flat-bottomed micro titration plates (assay plates)
- Single or multichannel automatic pipettes to deliver volumes in the range of 15 to 75 μ l with an accuracy of +/- 1.5% over this range.
- A micro titration plate reader capable of reading absorbencies at 340 nm in KINETIC reading mode.
- A hole punch which produces 3/16" or 1/8" diameter discs.
- A plate incubator set at 37°C with an accuracy of +/- 1°C
- Blood collection cards.

6. Precautions - Safety and Operating

All reagents must be stored according to the instructions on the label and instructions for use.

The assay-performance can be seriously affected if the instructions as outlined in this package insert and on the labels are not strictly adhered to.

The Elution Agent contains 0.15% sodium azide as anti microbial preservative. This substance has a toxic effect if absorbed or indigested. They should be discarded with an adequate water flow.

All blood samples of human origin must be regarded as a potential biohazards and normal laboratory precautions must be taken whilst handling these samples.

Do not use kit components after the expiry date stated on the label.

Do not mix components of different lot numbers

Do not use any solutions that have become turbid or discolored.

7. Sample Collection

Collect from the infant's heel. After the sample is taken and the blood has dried, the cards must be stored at 2-8°C. Spots not stored under these conditions gradually lose the enzyme activity due to heat inactivation, causing potential risk of misclassifying samples as screen-positive (34,35).

8. Reagent Preparation

The reagents must be warmed up slowly to 30 or 37°C prior to use if these are the temperatures the assay is going to be performed at. No pre-warming needed for temperatures 22-29°C.

Reconstitute 1 VIAL of REAGENT with **2 ml** of WATER FOR INJECTION (or de-ionized water). This reagent is stable for 5 days at 2-8°C or 10 days at -20°C.

Prepare a **working mixture** of color reagent. To do this mix 1 part of Color Reagent Booster with 10 parts of Color Reagent. Return the original components in the refrigerator. *The **Color Reagent Mixture** prepared this way is stable for at least 8 hours.*

9. ASSAY PROCEDURE

1. Punch blood spots 3/16 inch diameter (or 2x 1/8") and place it in U bottom micro titer plate. Alternatively, you can use 5 microliters of whole blood (with anticoagulant). Use position A1, A2 for the Normal Standard, (not provided with the kit). Optionally, you may use 2 wells for the Intermediate and Deficient Controls (not provided with the kit.)
2. Add **75 µl** of ELUTION AGENT to each well.
3. Place micro titer plate on an orbital plate shaker for 30 minutes at room temperature (8 minutes for whole blood samples). This step can be performed in an incubator at 30 or 37°C.
4. During the elution, reconstitute and prepare Reagent Mixture. The reagents must be warmed up slowly to 30 or 37°C prior to use if this is the temperature the assay is going to be performed at.
5. Transfer **15µl** of the eluant from each well to the corresponding well of a new flat bottom microtiter plate. If out of range values are obtained in step 9 below, the transfer volume can be reduced to 10 microliters.
6. Add **75 µl** of the pre-warmed REAGENT to each well and **mix well**.
7. Add **80 µl** the Color Reagent Mixture prepared by mixing 1 part of Color Reagent Booster with 10 parts of Color Reagent.
8. Read the plate in a plate reader at **550 nm** (500-570 nm) for 12-15 minutes with 60 seconds intervals. The reader must be fitted with a temperature-controlled incubator. Alternatively, the NADPH produced can be measured in endpoint mode using two measurements one at zero time and a second one after 12-15 minutes. During the measurements the, microplate should remain in the incubator. This protocol is strongly encouraged if your microplate reader is not equipped with a kinetic software or a heating plate. In this case the total OD / sample should be used for calculations. See the attached "Benchtop" instructions for the formulas.
9. After the readings are completed, change the program of the reader to an ENDPOINT MODE select wavelength = 405 nm and read the plate (containing the same mixture) once again. See section 10 **"OBTAINING RESULTS DIRECTLY EXPRESSED IN U/g Hb"**

10. Calculation of Results

The optical densities for the 12-15 measurements per sample are plotted against time (in minutes) from which the increase in OD (slopes) for each well can be calculated in OD/min.

These slopes are evaluated against the slope for a Standard with known G-6-PD activity (mean of the slopes of the duplicates). The value for the Standard is divided by slope for the Standard to obtain a FACTOR. This FACTOR is used to multiply the slopes obtained for the unknowns:

$$\text{Factor} = \frac{\text{value Control}}{\text{slope Control}}$$

$$\text{Slope unknown} \times \text{factor} = \text{U G-6-PD activity}$$

Example: mean slope Normal Control = 0.015829 OD/min.

Value Control = 17.0 U/mgHb

Factor = 17.0 / 0.015829 = 1074

Slope sample = 0.00845 G-6-PD activity = 9.075 U

OBTAINING RESULTS DIRECTLY EXPRESSED IN U / g Hb. (37, 38, 39)

The following formula expresses your results directly into U / g Hb (Greek Patent # 1003227; International Patent Pending)

$$\frac{(\delta\text{Odsample}_{340\text{nm}}/\text{min})/(\delta\text{ODcontrol}_{340\text{nm}}/\text{min})}{\text{Odsample}_{405\text{nm}}/\text{ODcontrol}_{405\text{nm}}} \times \text{Control Value} = \text{Sample Value (Activity in U/g Hb)}$$

wherein $\delta\text{OD}_{\text{sample}}$ is the change in optical density (per minute) for the sample, 340nm is the wavelength at which optical density is measured under kinetic mode, $\delta\text{OD}_{\text{control}}$ is the change in optical density (per minute) of a control measured under kinetic mode conditions at a particular wavelength (340nm), $\text{OD}_{\text{sample}}$ is the optical density for the sample measured once at a particular wavelength (405nm), and $\text{OD}_{\text{control}}$ is the optical density of the control measured once at that particular wavelength (405nm), 405nm is the wavelength at which a reading of Hemoglobin contained can be taken, the control value being determined by the change in optical density (per minute) of the control measured under kinetic mode at 340nm divided by the optical density of the control at 405nm i.e.

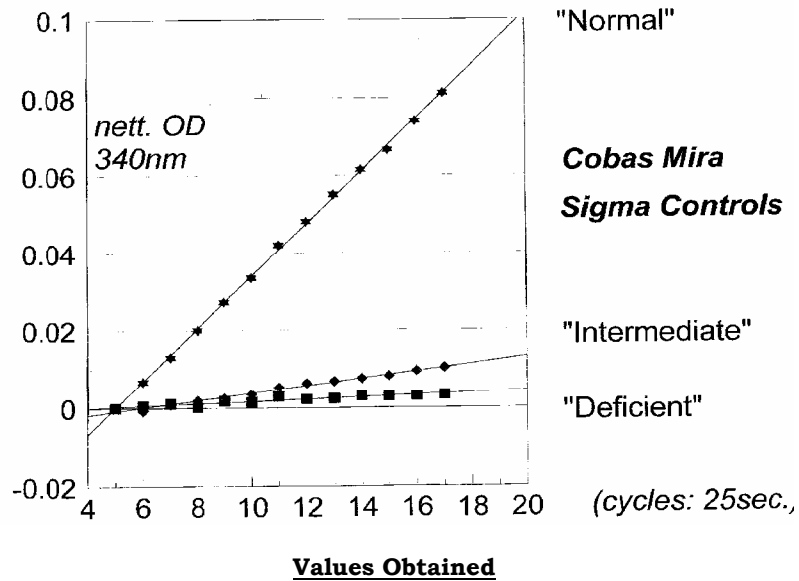
$\delta\text{OD}_{\text{control}_{340\text{nm}}}/\text{min}/\text{OD}_{\text{control}_{405\text{nm}}}$.

11. Quality Assurance

This kit has been thoroughly checked before shipment. Moreover all kits are checked at regular intervals during their shelf life to ensure compliance to the specifications set. The reproducibility of the control values should be within defined limits of laboratory acceptability. Commonly used measures of variability are discussed by Westgard, J.O., et al.³⁶ If the precision of the assay does not correlate with this standard and repetition excludes errors in technique, check the following areas:

- a. Pipetting and timing devices
- b. Instrument calibration
- c. Expiration dates on reagent labels and prepared working solutions
- d. Storage conditions
- e. Temperature control devices
- f. Wrong use (or no use) of the Hemoglobin normalization procedure.

12. Expected Values



Cycle No.	Deficient *	Intermediate *	Normal *
5	0	0	0
6	0.0060	-0.0008	0.0067
7	0.0011	0.0009	0.0130
8	0.0001	0.0019	0.0200
9	0.0019	0.0025	0.0273
10	0.0015	0.0035	0.0335
11	0.0029	0.0052	0.0420
12	0.0022	0.0062	0.0481
13	0.0024	0.0068	0.0551
14	0.0030	0.0075	0.0615
15	0.0029	0.0081	0.0667
16	0.0030	0.0094	0.0740
17	0.0033	0.0102	0.0812

• Values are expressed in ΔOD_s

13. Limitations of Use

Low results are not the sole diagnostic tool of G-6-PD deficiency but indicate the need for further study of the newborn from which a presumptive screen positive sample was received.

Important note: Do not perform the assay at temperatures over 37°C as this may inactivate the enzyme and lead to erroneous results.

In case of bubble formation in the well a FALSE NEGATIVE result may occur, caused by diffusion of the light passing vertically through the well leading to falsely high absorption of light. As a consequence, a G-6-PD Deficient specimen could be **misclassified** as NORMAL.

In some forms of G-6-PDH deficiency, young erythrocytes manifest normal enzyme activity. Blood from patients who have just experienced a hemolytic crisis must first be treated by the procedure described by Herz et al (37) to separate the older erythrocytes from the prevailing population of young ones. Use 0,005 ml of the suspension so obtained for the assay.

If the patient has received a blood transfusion, this test is clinically significant only after 30 days have elapsed, because the donor's erythrocytes generally manifest a normal G-6-PDH activity and can thus bias the result before the expiration of this time.

15. References

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