Atlas Medical CE

G6PD Quantitative Kit

IVD *For In Vitro Diagnostic Use Only*

Store at 2°C to 8°C

Intended Use

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

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). This coenzyme is required as the 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 is ultimately and heavily depends on the presence of G-6-PD. The red cells are exquisitely sensitive for oxidative damage and lack of 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 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, 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.

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

1. Drug induced hemolysis: certain anti-malarials, sulfonamides, sulfones and other drugs or chemicals are associated with significant hemolysis in subjects.
2. Infection induced hemolysis: numerous bacterial, viral and rickettsial infections have precipitated hemolysis, but the most important are infectious hepatitis, pneumonia and typhoid fever.
3. Favism: sudden onset of acute hemolytic anemia within 24 to 48 hours of ingesting fava beans.
4. Neonatal jaundice: jaundice usually appears by 1 to 4 days of age.
5. Chronic nonspherocytic hemolytic anemia .

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.

$$\text{Glucose-6-P} + NADP^+ \xleftrightarrow{\text{G-6-PD}} \text{Gluconate-6-P} + NADPH + H^+$$

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 Normal Control with known G-6-PD activity (kinetic mode). Alternatively, the reduced tetrazolium salt can be measured in endpoint mode using two measurements one at time = 0 and a second one at time = 12-15 minutes later. In this case the total ΔOD / sample should be used for calculations.

Hemoglobin (Hb) Normalization (patented): A concomitant measurement of Hemoglobin is performed at 405nm and the results are rated against a Control with known enzymatic activity and hemoglobin content.

Kit Contents

1. **2X 8 ml ELUTION BUFFER**: Liquid. Contains 0.15% (w/v) sodium azide as preservative.
2. **6 X 2 ml REAGENT VIALS**: Lyophilized. *CAUTION: Very toxic to humans.*
3. **2 X 1 ml COLOR REAGENT BOOSTER:** liquid.
4. **2 X 8 ml COLOR REAGENT:** bright yellow liquid. *CAUTION: Keep away from strong light sources.*
5. **Filter Paper**.*(Optional)*

Reagents / Materials required but not provided

- U -bottomed well micro titration plates (elution plates).
- Water for injection (de-ionized water).
- Flat-bottom micro titration plates (assay plates).
- Single or multichannel automatic pipettes to deliver volumes in the range of 5 to 75µl with an accuracy of +/- 1.5% over this range.
- Microtitration plate reader capable of reading absorbencies at 550nm in kinetic reading mode and 405nm in endpoint 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.
- G-6-PDH controls.

Stability of the Chemicals

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

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

Precautions

1. *For in-vitro diagnostic use only. For professional use only.*
2. Don't use the kit if damaged or leaking and discard the contents immediately.
3. Do not mix reagents of different lots. Do not use expired reagents.
4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. 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 24-29°C. The color reagent is the exception to this rule. Do not warm it before use. The Color Reagent in its reduced form [Blue] will stain glass or plastic after prolonged contact. It is advisable to discard such material after the test is over, especially when the microplate or other equipment is to be reused.
6. Disposal of these reagents should be accompanied by copious flushing with water to avoid accumulation of explosive salts in plumbing systems.
7. Color Reagent should be protected from direct exposure to light sources.
8. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.

Sample Collection

6. **DRIED BLOOD SPOTS (DBS):** 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.
7. **WHOLE BLOOD SPECIMEN:** Whole blood specimen from Venous, Cord or finger prick is suitable for the assay. No interferences with anticoagulants. Do not freeze blood. It is recommended to store blood specimen in the refrigerator and use them within 3-4 days after collection.

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 24-29°C.

- **Reagent Mixture (RM):**

Reconstitute 1 vial of REAGENT with 2 ml of deionized water. Each vial has sufficient reagent for 25 tests.

- **Color Reagent Mixture (CRM):**

Color Reagent Mixture is prepared by mixing 10 parts Color Reagent with 1 part Color Booster. Take the quantity from the two reagents you need for one day run (assuming 100 µl /sample) and place reagents back in the refrigerator. Avoid the formation of foam. Color Reagent Mixture should be discarded if not used within 8 hours.

- **Elution buffer** is ready to use.

ASSAY PROCEDURE

1. Punch Blood spots 4.7 (3/16") inch diameter (or 2x3.2 mm (1/8") and place it in U bottom micro titer plate. Alternatively, you can use 5 micro-liters of whole blood (with anticoagulant). Use position A1, A2 for the Normal Standard, use position A3, A4 for Deficient (Optional). 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 U-bottomed microtiter plate on a plate shaker for 30 minutes at room temperature (10 minutes for whole blood samples). This step can be performed in an incubator at temperature.
4. During the elution, reconstitute and prepare Reagent Mixture. The reagents must be warmed up slowly to 30 or 37ºC prior to use if this is the temperature the assay is going to be performed at.
5. Add **75 µl of the Reagent Mixture** to the corresponding wells of a new F-bottomed microtiter plate (Assay Plate).
6. Transfer **15 µl of the eluant** from each U-well to the corresponding well in the Assay Plate and mix thoroughly.
7. Add **100 µl of the prepared Color Reagent Mixture** (CRM) to each well.
8. Read the plate in a plate reader at **550 nm (500-570 nm)** for 12-15 minutes with 60 seconds intervals (kinetic mode). Alternatively an endpoint mode can be used, taking two measurements one at time = 0 and a second one at time = 12-15 minutes later. The latter protocol is encouraged if your microplate reader is not equipped with kinetic software. If an incubator is used, it is advised to place the microplate in during measurements.
9. After the final reading at 550 nm is taken, read the plate at wavelength 405 nm (to get the Hb content of each sample) read the plate (containing the same mixture) once again.

Calculation of Results

Use the following formula to express your results directly into U / g Hb (Unit per gram of Hemoglobin):

$$\frac{(\delta OD sample 550nm / \delta OD contorl 550nm)}{OD\ sample 405nm / OD control 405nm} \times \text{Control value} = \text{Sample Value (Activity in U/g Hb)}$$

Where:

- δOD sample550nm = Final OD sample550nm at 12-15 min - Initial OD sample550nm at 0 min
- δOD control550nm = Final OD control550nm at 12-15 min - Initial OD control550nm at 0 min
- δOD sample550nm is the change in optical density for the sample, measured at a particular wavelength (550 nm).
- δOD control550nm is the change in optical density of a control measured at a particular wavelength (550 nm).
- OD sample405nm is the optical density for the sample measured once at a particular wavelength (405 nm).
- OD control405nm is the optical density of the control measured once at that particular wavelength (405 nm).

Quality Control

Each assay must include the Normal Control and optionally the Deficient Control. The assay is valid if there is a distinct color development in the wells corresponding to Normal Control within 10-15 min after the addition of Color Reagent Mixture (CRM). The initial yellowish red color is turned into dark purple. When a Deficient Control is used the G6PD activities must be within the limits stated on the Control label.

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.
- Do not perform the assay at temperatures over 37ºC 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.

Interpretation of the results - Expected Values

Each sample is classified according to the obtained result as follows:

- Totally Deficient : Residual Activity Range U/g Hb < 2.5
- Partially Deficient : Residual Activity Range U/g Hb 2.6 – 6.5
- Normal : Residual Activity Range U/g Hb 6.6 – 17.0

It is highly recommended to repeat the examination of all the classified Deficient neonates after a period of 6 months.

Following a statistical analysis performed in association with the Greek National Screening Center involving more than 5000 neonates, the following frequency distribution was found, resulting in the classification of neonates as follows:

	Residual Activity Range U/g Hb	Average Activity Range U/g Hb	Frequency (%)
Totally Deficient	0-2.1	0.67	3.1
Partially Deficient	2.2-6.4	4.11	2.4
Normal	6.5-17	11.07	94.5

It is highly recommended that each laboratory establishes its own expected range and cut off points for classification since mutations prevailing in one region may be absent in another and vice versa. Briefly, a large population of neonates (> 5000) is tested and the mean value for that population is calculated. Following that, the lower 20% of the values is discarded and the mean for the remaining population (normal samples) is recalculated, which is the average activity for healthy newborns for this population. The cut off points are then set at 20% and 60% of that value to discriminate deficient and partially deficient samples. The larger the population used to extract the mean the more accurate the cut off points will be.

Performance Characteristics

- **Accuracy**

Kit was compared against the other kit and showed an excellent correlation of the results obtained (R-squared > 0.99).The range of the measured G6PD activities was 0.5 – 13.8 U/g Hb.

- **Reproducibility**

Twenty replicate assays of a normal and a deficient sample in two separate runs were performed on an Awareness Technology ChemWell and yielded standard deviations of 0.37 and 0.088 U/g Hb respectively and coefficient of variation of 2.75% and 2.80% respectively (intra assay variation). Mean values were 13.81 and 3.16 U/g Hb respectively. Inter assay variation was 2.7% (13.81 U/g Hb) and 2.8% (3.16 U/g Hb)

- **Sensitivity**

Assuming the limit of sensitivity to represent a change in absorbance of 1 mOD/min (0.001/min) of G6PDH activity at 550nm a G6PDH activity of 0.9 U/g Hb may be detected using this procedure. Using kinetic mode and four decimal displays, the detection limit becomes 0.3 U/g Hb.

- **Specificity**

The oxidation of glucose-6-phosphate by G6PDH is specific. Any nonspecific formation of NADPH due to oxidation of other substrates due to endogenous enzymes occurs during the elution time while the other substrates are exhausted. Specificity is 99% (0.99).

- **Interfering Substances**

The effects of bilirubin, triglyceride and protein, at concentrations which mimic severely icteric, lipemic, and abnormal protein specimens, were determined by spiking whole blood and preparing dried blood spot specimens.

Substance	Amount (mg/dL)	G6PD (U/gHb)	Recovery (%)
Unconjugated Bilirubin	0	17.77	-
	40	16.15	90.87
Conjugated Bilirubin	0	17.77	-
	40	17.18	96.66
Triglycerides (Liposyn II)	0	18.32	-
	1000	17.49	95.46
gG	0	18.20	-
	2500	17.73	97.43

The effect of these substances on the assay's results is marginal (2.57 – 9.13%) and will never lead to the misclassification of a positive (Deficient) sample.

References

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REF	Catalogue Number		Temperature limit
IVD	In Vitro diagnostic medical device		Caution
Σ	Contains sufficient for <n> tests and Relative size		Consult instructions for use (IFU)
LOT	Batch code		Manufacturer
	Fragile, handle with care		Use-by date
	Manufacturer fax number		Do not use if package is damaged
	Manufacturer telephone number		Date of Manufacture
	Keep away from sunlight		Keep dry