Laboratory Diagnosis of G6PD Deficiency. A British Society for Haematology Guideline

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Methodology

This guideline was compiled according to the BSH process at: http://b-s-h.org.uk/guidelines/proposing-and-writing-a-new-bsh-guideline/. The Grading of Recommendations Assessment, Development and Evaluation (GRADE) nomenclature was used to evaluate levels of evidence and to assess the strength of recommendations. The GRADE criteria can be found at http://www.gradeworkinggroup.org.

Literature review details

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Literature review details

In addition to the Authors' comprehensive databases, a specific literature review was conducted on 18th April 2017 on the following databases: MEDLINE (OVID), EMBASE (OVID), CENTRAL (The Cochrane library) and Web of Science (SCI-Expanded, CPCI-S) using the search terms: G6PD activity in heterozygous beta thalassaemia, G6PD activity in reticulocytes, Glock and McLean assay procedure,

Leukocyte G6PD activity, Molecular characterization of G6PD variants, `Correction' for the presence of young red cells and reticulocytes. The search covered the period back to 1950. Exclusions included articles not in English, non-human papers and those without abstracts. This yielded 9443 publications which, with exclusions and duplications, resulted in 236 articles which were additionally reviewed.

Review of the manuscript

Review of the manuscript was performed by the British Society for Haematology (BSH) Guidelines Committee General Haematology, the BSH Guidelines Committee and the General Haem Task Force sounding board of BSH. It was also on the members section of the BSH website for comment.

Introduction

This guideline is an update of the first G6PD guideline [The Assessment of Glucose-6-Phosphate Dehydrogenase Deficiency; prepared by the General Haematology Task Force, 1991]. Data from recent External Quality Assessment (EQA) exercises show that there is continued variation in both the results obtained and laboratory practice and this may be sufficient to affect clinical outcome. The guideline is for use by staff working in diagnostic laboratories and is intended to promote the harmonisation of analytical methods through sharing best practice in the diagnosis of G6PD deficiency.

Background

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme expressed in all tissue cells where it catalyses the first step in the pentose phosphate pathway. In the red blood cell, this is the sole pathway for the production of NADPH, which is required to maintain glutathione in the reduced state (GSH). Failure of this process

impairs the ability of the red cell to deal with oxidative stress, which may lead to haemolytic episodes and anaemia that can be severe and in some cases fatal.

G6PD deficiency shows marked genetic heterogeneity. Minucci and colleagues described some 186 mutations (Minucci et al. 2012) since when an additional 31 mutations have been reported (Gomez-Manzo et al. 2016). However, of the estimated 400 million people worldwide that have G6PD deficiency, a few polymorphic mutations account for the vast majority (Vulliamy et al. 1988). The most common G6PD variants are found in people who originated from the Mediterranean countries (the Mediterranean type), parts of Africa (the African type; G6PD A-) and parts of India and South East Asia (Beutler 1971, Grimes 1980, Wintrobe 1981, Dacie 1985).

DNA sequence analysis has shown that the vast majority of mutations arise from single amino acid substitutions, mostly leading to a decrease in enzyme stability or to reduced catalytic efficiency. The degrees of enzymatic dysfunction detected in variants have been found to be in accordance with the severity of the clinical manifestations (Vulliamy et al. 1998, Gómez-Manzo et al. 2017). A list of G6PD variants can be found at http://www.bioinf.org.uk/g6pd/db.

The gene encoding G6PD is located near the telomeric region of the distal arm of the X chromosome (band Xq28) in a region that includes the genes for haemophilia A, dyskeratosis congenita and colour blindness. The G6PD gene consists of 13 exons and spans 18.5 Kb, with a GC-rich promoter region (Persico et al. 1986). Being X-linked, males can be either hemizygous normal or hemizygous deficient, whereas females may be either homozygous normal, homozygous deficient or heterozygous. A heterozygous female will be a mosaic for cells expressing the wild type enzyme

and cells expressing a deficient variant. The variable proportion of normal and deficient red cells, as a consequence of random X-chromosome inactivation (Lyonisation), renders diagnosis in some female heterozygotes difficult. A fraction of red cells in heterozygotes (on average, 50%) is as enzyme-deficient as in hemizygous males and therefore susceptible to haemolysis. Such individuals are usually less severely affected than homozygous females or hemizygous males. The severity of haemolysis and its potential clinical complications are roughly proportional to the fraction of deficient red cells. As a result of the random nature of X-chromosome inactivation during embryogenesis, individual females can manifest skewing in favour of either the normal or deficient G6PD allele. The latter, if extreme, will result in most red cells being G6PD deficient and therefore susceptible to haemolysis.

The great majority of G6PD-deficient individuals have no clinical manifestations in the steady state and the condition remains undetected until they are exposed to an exogenous haemolytic trigger such as bacterial or viral infections, ingestion of fava beans (favism) or drugs. However, some G6PD-deficient individuals suffer from a chronic non-spherocytic haemolytic anaemia (CNSHA), often requiring blood transfusion, in some cases on a long-term basis. These are due to the so-called Class 1 variants, in which G6PD activity is less than 10% of normal (Table 1; adapted from (Luzzatto et al. 2016).

Table 1. Classification of G6PD variants

Class	Residual G6PD Activity (% of normal)[a]	Clinical Manifestations
I[b]	<10[c]	CNSHA[d] (NNJ, acute exacerbations)
II	<10[c]	None in the steady state
III	10-60	None in the steady state
IV	100	None
V	>100	None

CNSHA, chronic non-spherocytic haemolytic anaemia

NNJ, neonatal jaundice

[a] Levels of residual G6PD activity in hemizygous males.

[b] The definition of class I variants is not biochemical but clinical (i.e., class I variants cause CNSHA).

[c] The range of G6PD activity is similar in class I and class II variants, which may seem strange because the clinical phenotype is significantly different. It must be considered, however, that (1) in CNSHA, there is always reticulocytosis, which increases G6PD levels, and (2) in some class I variants, the residual G6PD activity may be similar to a class II or even a class III variant, but the enzyme kinetics may be unfavourable.

[d] When haemolysis is not compensated, chronic anaemia is present and blood transfusions may be necessary at times or even at regular intervals.

G6PD deficiency is never complete, as this is not compatible with survival, but enzyme activity may be so low as to be undetectable in red blood cells by standard methods. As the red cells age in the circulation there is a gradual reduction in G6PD activity and the mean value in young red cells has been shown to exceed that of old red cells by a factor of 8.5 (Bonsignore et al. 1964).

One of the most clinically significant complication of G6PD deficiency is neonatal jaundice (NNJ), which peaks 2 to 3 days after birth (Luzzatto 2010). Although highly variable in severity, without effective treatment it can lead to bilirubin encephalopathy (kernicterus) and permanent neurological damage or death. Haemolysis does not seem to contribute as much as impaired bilirubin conjugation and clearance by the liver (Kaplan et al. 1996). G6PD-deficient newborn babies who also inherit a mutation of the uridine-diphosphate-glucuronosyltransferase 1 (*UGT1A1*) gene promoter, responsible for Gilbert syndrome, are particularly at risk for neonatal

jaundice (Kaplan et al. 1997). Neonatal screening for G6PD deficiency is routinely performed in many countries, mainly on dried blood spots, though not in the UK at the time of publication.

Both favism and G6PD-related NNJ are often regarded as being primarily disorders of males: there are many more hemizygous deficient males than homozygous deficient females. However, both favism and NNJ are well documented in females, including heterozygotes (Meloni et al. 1983, Meloni et al. 1992, Luzzatto 1993). The offending chemicals present in fava beans (broad beans), particularly in the fresh green beans, are divicine and isouramil, powerful oxidising agents which result respectively from hydrolysis of the alkaloid glucosides vicine and convicine. While intrauterine haemolysis in G6PD-deficient fetuses exposed passively to fava beans appears rare, neonatal favism has been reported (Mentzer and Collier 1975, Corchia et al. 1995). Postnatal haemolysis through breast milk intake after maternal ingestion of fava beans is a recognised cause of NNJ (Yeruchimovich et al. 2002, Al-Azzam et al. 2009). Although placental transfer of the culpable agents in fava beans has not been demonstrated, other glucosides are known to be transferred.

Accidental ingestion of mothballs containing naphthalene was one of the more frequent causes of acute haemolysis in G6PD deficiency in the UK (Santucci and Shah 2000), but is less common now as naphthalene-containing products have been banned in the EU since 2008.

Bacterial and viral infections are known triggers of acute haemolytic anaemia in G6PD-deficient individuals although the mechanism of haemolysis is not well defined. Reactive oxygen species (ROS) have been implicated in the pathogenesis of many infections and these are known to cause oxidative damage. Severe

haemolysis has been attributed to hepatitis viruses A and B, cytomegalovirus, pneumonia and typhoid fever (Cappellini and Fiorelli 2008). After severe trauma, G6PD-deficient individuals may be at higher risk of sepsis and once infected may have a more severe clinical course (Spolarics et al. 2001).

Some antimalarial drugs cause significant oxidative stress to the red cell and therefore it is important to test patients for G6PD deficiency before starting antimalarial therapy with these specific drugs. However, careful consideration for delaying treatment is required in what may be a life-threatening malaria. Although many drugs have been claimed to cause haemolysis in G6PD deficiency, in only a few of these is there a well-documented causal relationship (Table 2; from (Luzzatto and Seneca 2014) and the British National Formulary).

Table 2. Drugs that can trigger haemolysis in G6PD-deficient subjects

Category of drug	**Predictable haemolysis**	**Possible haemolysis**
Antimalarials	Dapsone	Chloroquine
	Primaquine	Quinine
	Pamaquin[a]	
	Tafenoquine	
	Methylene blue	
Analgesics/Antipyretic	Phenazopyridine	Aspirin (high doses)[b]
		Paracetamol (Acetaminophen)
Antibacterials	Cotrimoxazole	Sulfasalazine
	Sulfadiazine	
	Quinolones[c]	
	Nitrofurantoin	
Other	Rasburicase	Chloramphenicol
	Toluidine blue	Isoniazid
	Niridazole[a]	Ascorbic acid

	Pegloticase	Glibenclamide
		Vitamin K (Menadione)
		Isosorbide
		Dinitrate

[a] not on UK market

[b] acceptable up to a dose of at least 1 g daily in most G6PD-deficient individuals

[c] including ciprofloxacin, moxifloxacin, nalidixic acid, norfloxacin and ofloxacin

G6PD deficiency should also be considered in other clinical situations as described in Table 3. Although it has been claimed that haemolysis is usually not as severe with the African variant, drug-induced acute haemolytic anaemia in G6PD A- subjects can be life-threatening, therefore in clinical terms, the A- type of G6PD deficiency cannot be regarded as benign.

The severity and course of a haemolytic episode depend both on the G6PD variant and the type and duration of oxidative stress. In addition, the age of the individual and any coexisting disease conditions are factors. Little is understood about how G6PD deficiency interacts with other genetic traits that affect red cell structure or function. In populations where G6PD deficiency is prevalent, haemoglobin S and thalassaemia coexist at a significant frequency. Some studies have suggested that co-inheritance of sickle cell anaemia and G6PD deficiency is associated with more severe anaemia and greater risk of cerebrovascular disease, although this has not been found consistently (Rees et al. 2009). G6PD testing in sickle cell disease and thalassaemic disorders is nevertheless recommended to avoid exposure of G6PD-deficient individuals to oxidant drugs or other agents that may exacerbate anaemia in the context of chronic haemolysis. The impact of transfusing blood from G6PD-deficient donors in high prevalence regions should be considered, especially when transfusing children.

Table 3. Indications for G6PD testing

Prior to treatment with certain antimalarial or other 'oxidant' drugs (see Table 2)
Haemolytic anaemia in the newborn (non-immune)
Prolonged or severe neonatal jaundice
Haemolysis associated with 'oxidant' drugs (see Table 2) or infection
Favism
Red cell morphology suggestive of oxidant damage or positive Heinz body stain
Congenital non-spherocytic haemolytic anaemia in males or females
Haemoglobinuria
Sickle cell disease*
Thalassaemic disorders
Family history of G6PD deficiency or favism
Patients likely to need rasburicase, such as those with leukaemia, lymphoma or other malignancies
Acute haemolysis following haemopoietic stem cell transplantation if donor is G6PD deficient or status unknown

*Sickle cell anaemia and compound heterozygous states

Specimen collection and storage

For quantitative assays and for the fluorescence screening test, blood anticoagulated with ethylenediamine tetra-acetic acid (EDTA), heparin or acid-citrate-dextrose (ACD) solution can be stored for up to 3 weeks at 4°C or up to 5 days at room temperature with less than 10% loss of G6PD activity (Beutler 1984). Samples containing variants may be less stable than samples containing the normal enzyme. However a fresh sample (less than 24 hours old) is required for dye decolourisation tests and heparin should not be used as an anticoagulant as it may affect the decolourisation time. The cytochemical test must be carried out on the day of blood collection if anticoagulated with EDTA or heparin, or within 1 week if ACD is used. Since G6PD in haemolysates is unstable at room temperature, 4°C or - 20°C, haemolysates should not be stored.

Recommendations: sample collection and storage

- **Whole blood samples should not be stored for more than 5 days if anticoagulated with EDTA or not for more than 3 weeks if anticoagulated with ACD (one week for cytochemical test) (GRADE 1C).**

Principles of measurement of G6PD activity

G6PD promotes the conversion of its specific substrate glucose-6-phosphate (G6P) to 6-phosphoglucono-δ-lactone (6PGL) with a simultaneous reduction of the coenzyme NADP to NADPH. The 6PGL produced undergoes hydrolysis either spontaneously or via lactonase to 6-phosphogluconate (6PG), and is the substrate for the next enzyme in the metabolic pathway, 6-phosphogluconate dehydrogenase (6PGD), which is also present in the red cells, and which also reduces an additional amount of NADP to NADPH. Since NADPH is produced by both reactions, the only way to measure true G6PD activity is by carrying out assays with two different reaction mixtures: one containing an excess of both G6P and 6PG and the other containing only 6PG. The difference in activity between the first (G6PD + 6PGD) and the second (6PGD) assay gives the true G6PD activity (Glock and McLean 1953): Although this assay can be regarded as more accurate for certain research purposes, it is not necessary for diagnostic purposes. Indeed, for G6PD deficiency it tends to introduce an error greater than the one it is meant to correct for. The WHO (World Health Organization 1967) method which measures the overall reaction is satisfactory and is simpler to perform. Commercial kits simulate the Glock & McLean method by incorporating maleimide in their reagent to inhibit the 6PGD activity. All tests for measuring G6PD activity depend on detecting the rate of reduction of NADP to NADPH and are based on one of the following properties of NADPH.

1 Absorption of light at 340 nm.

2 Fluorescence produced by long wavelength UV light (approximately 340 nm).

3 Ability to decolourise or lead to the precipitation of certain dyes.

In recent years, affordable, qualitative 'point of care' (POC) lateral flow tests have become available. These tests generally require capillary blood from finger-prick, and can be performed and interpreted by health workers at the bedside or in the field in <30 minutes. These cassette-based enzyme chromatographic devices are based on the reduction of colourless nitroblue tetrazolium dye to dark coloured formazan. However, to address the problem of misclassification of females with intermediate G6PD activities, more sophisticated *quantitative* POC devices have been developed that accurately measure G6PD activity normalised for haemoglobin concentration by reflectometry/spectrosocopy (Bancone et al. 2018, Pal et al. 2019).

Cytochemical staining to demonstrate intracellular G6PD activity presents an alternative way to assess G6PD deficiency, and because it assays intact red blood cells, it can identify heterozygous females. Staining allows visualization (by microscopy) or enumeration (by flow cytometry) of the two distinct red cell populations resulting from the G6PD-normal and G6PD-deficient erythrocytes.

Recommendation: principles of measurement of G6PD activity

- **Check the absorbance of the spectrophotometer for NADPH at the bandwidth (slot width) used and use the value obtained in your calculations. The theoretical molar extinction (of 6.22) is only obtained with narrow (≤4 nm) bandwidth instruments that are regularly serviced (GRADE 2C).**

Laboratory Diagnosis

If a male patient is suspected of having G6PD deficiency on clinical grounds, either the fluorescent spot or the dye decolourisation screening test are acceptable first-line tests. The complications of a raised WBC, raised retics, low Hb (and the inability to reliably identify female carriers) must be understood. If the screening test is abnormal or equivocal, the quantitative assay should be undertaken to confirm, or exclude, the diagnosis unless the patient is known to the laboratory. It is best to proceed directly to quantitative assay for female patients, who may be heterozygous and thereby possibly misclassified by any screening test. It is up to the individual laboratory to use either the one substrate (WHO) assay or the Glock and McLean (Glock and McLean 1953) technique.

If a woman has an intermediate or equivocal result in the quantitative assay then the cytochemical test should be undertaken. If there is a clinical or genetic reason to suspect that a woman is heterozygous for G6PD deficiency then the cytochemical test should be undertaken even if the quantitative assay is normal because the cytochemical test may be the only way to detect a deficiency in some cases (other than by DNA analysis). After a haemolytic episode all tests may give normal or equivocal results in G6PD deficiency; therefore, it is recommended that a patient is retested following a haemolytic episode of unknown cause to ensure that G6PD deficiency is not missed. In this clinical scenario, the WHO quantitative technique is satisfactory but suffers the disadvantage that the activity of 6-phosphogluconate dehydrogenase is not measured and therefore this test cannot be used to assess the effect of young red cells and reticulocytes. After a haemolytic episode or if the reticulocyte count is raised, the Glock and McLean technique is therefore more

informative with respect to interpretation. Alternatively, comparison with the activity of either PK or HK assayed in parallel often proves helpful.

If G6PD deficiency is confirmed, the implications should be explained to the patient (or parent) who may also be given a 'card' or information leaflet containing the relevant information. A suggested format for such a card is given in the Appendices.

A description of various laboratory tests which may be used for the diagnosis of G6PD deficiency together with useful practical points can be found in the Appendices. Laboratories undertaking these screening tests and assays should participate in External Quality Assessment Schemes.

Reagent costs for these procedures are relatively inexpensive and readily obtainable from reliable biochemical supply manufacturers. However, the complexity of the tests requires experienced laboratory staff and some costly equipment (e.g. spectrophotometer). Commercially available kits for screening/assay are more straightforward to perform and generally provide a cost-neutral alternative to the procedures described below.

Recommendations: Laboratory diagnosis

- **Do not rely on screening tests for female patients; measure G6PD activity by quantitative spectrophotometric assay directly (GRADE 1C).**
- **The quantitative assay must be carried out if the screening test is abnormal or borderline (GRADE 1C).**
- **Re-assay following a haemolytic episode of unknown cause to ensure the diagnosis of G6PD deficiency is not missed (GRADE 1C).**

Data from External Quality Assessment (EQA) G6PD surveys show that there is continued variation in both the results obtained and laboratory practice of participants. As this may be sufficient to affect clinical outcomes, several recommendations are given with particular reference to the quantitative assay.

- **Since the G6PD reaction is temperature dependent, an accurate cuvette temperature is very important (GRADE 1C):**

  (a) Where possible, measure the temperature inside the reaction cuvette using a certificated thermometer/thermistor. Use a thermostatically controlled, recirculating water bath or a Peltier heating component attached to the spectrophotometer or use an automated analyser for the assay.

  (b) Run assays at either 30ºC or 37ºC. In laboratories without air-conditioning, a temperature of 37ºC may be easier to control than 30ºC unless a cooling unit is available (and these are expensive).

  (c) Allow the assay mixture or reagents to equilibrate at the assay temperature before starting measurements.

  (d) Report results at the temperature assayed, rather than use a 'correction factor' to adjust the numeric results to those that might have been achieved at a different temperature.

- **Run controls with every batch of samples: a normal and deficient sample obtained through a commercial company is preferable to an in house control (GRADE 1C).**

- **White cells contain a significant amount of G6PD, and ideally they should be removed prior to assay, and especially if the count is above the lab's reference interval upper limit: Consider removal of white cells with a cellulose/'real' cotton wool column before assay (see Beutler, 1984) (GRADE 1C).**

- **Perform assays in duplicate if assays are undertaken only infrequently (GRADE 1C). On normal samples, the results of these duplicates should be within 0.5 iu/g of haemoglobin of each other (GRADE 1C).**

- **Establish a laboratory reference range by in-house testing; referring to a kit manufacturer's leaflet (if using a commercial kit) or literature search only as a guide (GRADE 1C).**

- **Check that the assay absorbance increases in a linear fashion (it may take a minute or two for this to be achieved) and measure the absorbance over 10 minutes at 20 second intervals (for non-kit methods) (GRADE 1C).**

- **Measuring the haemoglobin concentration of the haemolysate is just as important as measuring the enzyme activity, as both measurements will affect the final result to a similar extent. Similarly with well mixed whole blood where a kit indicates such use (GRADE 1C).**

Normal values

The normal range for G6PD activity should be determined in each laboratory whether using an "in-house" (ICSH) procedure or a commercial kit. It is not acceptable to report the kit manufacturer's reference range, which is too broad and

will provide a false-normal result on many heterozygotes. If the ICSH method is used, values should not differ widely from the published values. Results are expressed in international units (iu), which are the µmoles of substrate converted per minute. For adults, these values are 8.7 ± 1.7 iu/g haemoglobin at 30°C; 12.1 ± 2.09 iu/g haemoglobin at 37°C (without correction for 6-PGD activity) and 8.34 ± 1.59 iu/g haemoglobin at 37°C (corrected for 6-PGD activity).

Newborns and infants have enzyme activities that deviate appreciably from the adult value (Konrad et al. 1972). In one study, the newborn mean activity was about 150% of the adult mean (Oski 1969). G6PD activity has been found to be higher in premature infants born between 29 and 32 weeks gestation than in term neonates (Mesner et al. 2004).

The Laboratory Report

The report should conform to the requirements of the international standard (currently) “ISO 15189:2012 – Medical laboratories – Requirements for quality and competence”. Screening test results should be reported either as “normal”, “deficient” or “equivocal – please repeat”, and the results of quantitative assays should include the enzyme activity, the unit of measurement (iu/g haemoglobin or 10^{12} rbc) or whether the result is expressed as a ratio. In addition, the assay temperature and the reference interval should be stated, together with the reticulocyte count. An interpretation of the result and recommendation(s) for action that reflect the clinical question being asked should be included.

Factors affecting clinical interpretation

With some G6PD variants, including the African type, young red cells and particularly reticulocytes have much higher G6PD activity than mature red cells. For this reason

tests carried out during, or soon after, a haemolytic episode may result in normal or even raised enzyme levels producing a false-normal result. In heterozygous females, the impact of selective haemolysis of G6PD-deficient red cells may compound the effect of reticulocytosis and the enzyme activity of residual non-deficient cells can mask the diagnosis of G6PD deficiency. Where G6PD deficiency is suspected on clinical grounds and no alternative explanation for haemolysis is forthcoming a G6PD assay should be repeated at least 2-3 months after resolution of the haemolytic episode. In this situation, examination of a blood film for typical features of oxidant damage is very important and is usually spectacular – almost diagnostic on its own – only if done very promptly, within 2 days from the onset of the haemolytic episode. If clinically indicated, to avoid delay it is possible to apply a 'correction' for the presence of young red cells and reticulocytes by measuring G6PD activity in the heaviest (oldest) red cells after microhaematocrit centrifugation (Herz et al. 1970). Alternatively, since the activity of several other red cell enzymes is similarly affected by the red cell age, comparison of their activity to that of G6PD can be undertaken. This approach also takes into account the effect of older cells lost by haemolysis. Hexokinase (HK), pyruvate kinase (PK) and 6-phosphogluconate dehydrogenase may be used for this purpose. 6PGD is the most convenient since it is assayed as part of the Glock and McLean procedure (see Appendix), which forms the basis of the International Council for Standardization in Haematology (ICSH) recommended method for G6PD assay (ICSH 1977). If HK, PK or 6PGD activity is raised in the presence of a G6PD level at the lower end of the ‘reference’ range it is likely G6PD activity would be subnormal in the absence of haemolysis and with a ‘normal’ age distribution of red cells. Improved identification of heterozygotes by utilizing the G6PD/6PGD ratio has been reported (Minucci et al. 2009).

Samples with a very low MCH (<25 pg), as seen for example in thalassaemia, frequently give G6PD activity levels *above* the reference range, so values falling within range (especially at the lower end) should be viewed with caution and confirmatory molecular testing considered if clinically indicated. Sanna and colleagues (Sanna, et al. 1980) found this was a particular issue when G6PD levels are expressed as activity per g of haemoglobin and to lesser extent as activity per number of red cells x 10^9.

Sex chromosome aneuploidy is a rare cause of uncertainty in the interpretation of G6PD activity. Male individuals with Klinefelter syndrome (XXY) may have intermediate levels similar to those of heterozygous females. Conversely in Turner syndrome (XO), G6PD activity may be as low as that seen in hemizygous deficient males or in women with extreme Lyonisation.

All screening tests are useful to differentiate between normal and grossly deficient samples, but none of these techniques can reliably detect G6PD deficiency in heterozygous women. Equivocal results are difficult to interpret. It is advisable that the activity of these as well as of all deficient samples be confirmed by quantitative assay wherever possible.

Anaemic samples and samples with a high leucocyte count can give misleading results in both screening tests. These problems can be avoided if the buffy coat is removed and packed red cells are used instead of whole blood.

Recommendations: clinical interpretation

- **The final G6PD activity should be interpreted in light of the reticulocyte count measured on the same sample (GRADE 1C).**

- **The MCH (pg) of the test sample should be taken into account, as very low values (as seen in thalassaemia and iron deficiency) will overestimate the G6PD level where results are expressed in units per g haemoglobin (GRADE 1D).**

Point of Care Tests

G6PD “point of care” (POC) is an area of rapid development with a number of different commercial products on the market and in development. Undoubtedly these will be invaluable both for mandated neonatal screening programs and in the use of 8-aminoquinolines for the elimination of *Plasmodium vivax* malaria especially in “outreach” areas. However, they need not be limited to out of lab or field testing as they have potential to be used as a laboratory screening test, especially when a rapid result is required such as for anti-malarial treatment or Rasburicase administration. It is essential that any kit is evaluated for its fitness for purpose before use, especially with reference to storage temperatures, impact of environmental conditions and whether or not the kit incorporates a reference or control line where appropriate.

Molecular diagnosis

Molecular analysis should be considered in cases where a precise diagnosis is required for clinical reasons, e.g. to confirm the condition in a recently transfused patient, a heterozygous female or where a deficiency may be masked by a reticulocytosis. It should be remembered that molecular analysis may not detect all variants and that it does not reflect the functional activity of the enzyme in all conditions, which will be affected by the reticulocyte concentration and the erythrocyte lifespan.

The majority of G6PD mutations are point mutations and to date, 217 have been identified worldwide in both the coding DNA, introns and the 5' and 3' untranslated regions. There are common mutations in certain geographical areas such as the Mediterranean mutation and the G6PD A- in Africa and so one potential approach is to use a targeted direct mutation analysis method such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Poggi et al. 1990) aimed at these particular mutations. If this method produces a negative result or if there are no clear common mutations or if geographical origin is unclear then direct Sanger sequencing (Sanger and Coulson 1975, Minucci et al. 2008) can be performed, which will also detect less common and novel mutations. Given the improvements in efficiency and cost of Sanger sequencing and the considerable heterogeneity of the mutations encountered in G6PD deficiency, proceeding directly to Sanger sequencing has become a more widely used approach. A positive result from this sequence analysis can be regarded as a stand-alone diagnostic result. However, if a mutation is not found, then only a quantitative assay can establish G6PD deficiency. The recent advances in next generation sequencing have also made it possible for many genes related to red cell disorders to be sequenced simultaneously. This means that in cases of unexplained haemolysis, all potential genes related to red cell enzyme deficiency can be looked at in a single molecular assay rather than several molecular and biochemical tests.

It should be noted that in heterozygous females, molecular analysis is likely to be the only method of definitive identification of carrier status.

Recommendations: Molecular diagnosis

- **Molecular analysis can be undertaken if results of initial diagnostic procedures are equivocal or borderline, especially in (heterozygous) woman and male individuals with Klinefelter syndrome (XXY) who may have intermediate levels similar to those of heterozygous females (GRADE 1D).**

Quality assurance

Quality assurance is the process by which the laboratory demonstrates that the diagnostic results it produces are reliable in terms of accuracy and precision. In addition to the inclusion of internal quality control materials with each batch, a key element in quality assurance is participation in an accredited external quality assessment (EQA) programme, where available. EQA for G6PD qualitative and quantitative tests is offered by the major national and international EQA organisations and the laboratory should understand the type of programme provided and how it should be used in the laboratory to gain maximum benefit from participation (James et al. 2014). EQA is not available for the cytochemical assay and a laboratory undertaking this test should consider some other means of interlaboratory comparison, e.g. sample exchange. The frequency of EQA distributions, the inclusion of specimens that test the laboratory at clinical decision-making activities and the regular review of EQA results are particularly important. Any out-of-consensus EQA result should be fully investigated.

Recommendations: Quality assurance

- **Laboratories undertaking these screening tests and assays should participate in an accredited External Quality Assessment Schemes (GRADE 1C).**

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Declaration of Interests

None of the authors had conflicts of interest to declare. All authors have made a declaration of interests to the BSH and Task Force Chairs which may be viewed on request.

Review Process

Members of the writing group will inform the writing group Chair if any new evidence becomes available that would alter the strength of the recommendations made in this document or render it obsolete. The document will be reviewed regularly by the relevant Task Force and the literature search will be re-run every three years to search systematically for any new evidence that may have been missed. The document will be archived and removed from the BSH current guidelines website if it becomes obsolete. If new recommendations are made an addendum will be published on the BSH guidelines website (http://www.b-s-h.org.uk/guidelines).

Disclaimer

While the advice and information in this guidance is believed to be true and accurate at the time of going to press, neither the authors, the BSH nor the publishers accept any legal responsibility for the content of this guidance.

Audit Tool

Template obtained from BSH guidelines website (http://www.b-s-h.org.uk/guidelines).

Appendix 1

Fig. 1. Example of card template to be carried by a patient with G6PD deficiency

Appendix 2

Fluorescent Spot Test

Reagent kits for both the fluorescent spot test (e.g. Trinity Biotech Qualitative G6PD FST Kit; catalogue number 203-A) and the dye decolourisation test (Trinity Biotech G-6-PDH Deficiency Screen Kit; catalogue number 400k) can be obtained commercially.* Such kits have the advantage of being “CE” ("Conformité Européene") marked and meet the requirements of the IVD (In Vitro Diagnostic

* Trinity Biotech, (Trinity Biotech, Bray, Co Wicklow, Ireland).

Device) directive (98/79/EC), ensuring they are “fit for purpose”. The new In Vitro Diagnostic Device Regulation (EU) 2017/746, published in the Official Journal of the European Union on 5 May 2017, strengthens the approval system for *in vitro* diagnostics.

A procedure based on the recommended method (ICSH 1979) is given below. Blood is mixed with an appropriate reaction mixture containing a detergent-like compound to lyse the red cells. After a standard time the mixture is 'spotted' onto filter paper, dried and inspected under long wavelength UV light. The appearance and brightness of the fluorescence due to NADPH gives a measure of the activity of G6PD.

REAGENTS

β-NADP	7.5 mmol/l	2 ml
Glucose-6-phosphate	l0 mmol/l	4ml
Saponin	10 g/l	4ml
Tris-HCI buffer†	0.75 mol/l, pH 7.8	6 ml
Oxidized glutathione (GSSG)	8 mmol/l	2 ml
Distilled water		2 ml
TOTAL		20ml

†It is essential to use a pH electrode which is suitable for Tris buffer.

Mix the reagents in the volumes stated to make a total volume of 20 ml and then dispense this reaction mixture in 0.2 ml aliquots (*e.g*. in microfuge tubes) and store frozen at - 20°C. This mixture is stable for up to I year at this temperature.

BLOOD SAMPLES

Blood may be anticoagulated with EDTA (any sodium or potassium salt), heparin or ACD and dried blood spots collected onto filter paper can also be used. Samples

give reliable results even after storage for up to 5 days at 25°C or for up to 21 days at 4°C.

Method

Thaw an aliquot of the reaction mixture and allow it to come to room temperature. Mix 20 µl of whole anticoagulated blood with 0.2 ml of reaction mixture. Spot one drop (20 µl) of this mixture onto non-fluorescent filter paper such as Whatman No. 1, as soon as it has been mixed, and again at intervals of 5 and I0 minutes from the mixing time. Examine the spots under long wavelength UV light as soon as they have thoroughly dried. Samples with a very high haemoglobin concentration (Hb) (*e.g*. cord bloods) should be diluted to match the Hb of the normal control (using plasma from the test sample), because “quenching” of the fluorescence has been noted with such samples.

Note. The presence of GSSG in the reaction mixture increases the sensitivity of the method. This is because the GSSG allows the small amounts of NADPH which may be formed by residual G6PD in mildly deficient samples to be reoxidised by glutathione reductase, another enzyme present in the red cell haemolysate.

Interpretation

At the beginning of the incubation no fluorescence should be visible and the samples from people with normal G6PD activity will fluoresce after 10 minutes incubation. G6PD deficiency is indicated by delayed or absent fluorescence.

Dye decolourisation screening test

A freshly prepared haemolysate is added to the reaction mixture containing a coloured dye at an appropriate concentration. The rate of decolourisation of the dye

by the NADPH produced gives a measure of the activity of G6PD. In the Motulsky Test (Motulsky 1959) the dye is brilliant cresyl blue, while in the Trinity colorimetric test (Procedure No. 400k), 2, 6-dichlorophenol indophenol is used. Unfortunately only certain batches of brilliant cresyl blue are suitable for the dye decolourisation test and so it is important that all new batches are tested for suitability.

Motulsky *et al.* (1959)

REAGENTS

Stored reagents are stable for up to one year at the temperatures given.

β -NADP	0.7 mmol/l. Freeze 1 ml aliquots at - 20°C
Glucose-6-phosphate	30 mmol/l. Freeze 1 ml aliquots at - 20°C
Liquid paraffin	Store at room temperature
Buffer-dye mixture	0.7 mol/l Tris-HCl, pH 8.5, containing 320 mg/l brilliant cresyl blue. Freeze 4.5 ml aliquots at - 20°C. It is essential to use a pH electrode that is suitable for Tris.
Working mixture	Thaw one aliquot of NADP, G6P and the buffer-dye mixture and mix together.

BLOOD SAMPLE

Blood should be anticoagulated with EDT A and used within 24 hours of collection. Make a haemolysate by adding 20 μl of whole blood to 1 ml of water, mix well and use within 6 hours. Always include a control sample with normal G6PD activity and if possible a control sample with reduced G6PD activity.

Method

Add 0.65 ml of the working mixture to the haemolysate, mix and cover with a layer of liquid paraffin. Place in a waterbath at 37°C and record the time for decolourisation to occur.

Interpretation

Normal range	35-60 minutes
G6PD deficiency	1.5-24 hours

Note. In very anaemic subjects (Hct less than 0. 25) adjust the Hct to 0.4-0.5 before making the haemolysate.

Cytochemical assay

The technique described by Fairbanks and Lampe (Fairbanks and Lampe 1968) is satisfactory and is summarised below, although various attempts have since been made to improve the reliability of this procedure for detecting heterozygotes, such as “mild” fixation of the red cells and accelerating the reaction with an exogenous electron carrier, 1-methoxyphenazine methosulphate (Van Noorden and Vogels, 1985; Gurbuz et al. 2005). The procedure is based on the fact that haemoglobin can reduce certain dyes to form a granular precipitate while methaemoglobin (MetHb) cannot do this. The test involves a preliminary incubation of red cells with nitrite to change all the haemoglobin to methaemoglobin. This is followed by a second incubation with a reaction mixture containing the dye. The presence of G6PD in the red cells leads to the formation of NADPH which reduces methaemoglobin to haemoglobin and hence precipitation of the dye to form granules. The presence of these granules in the red cells therefore indicates G6PD activity. Counting the granules in the red cells can give a semi-quantitative assessment of G6PD. This method may sometimes be the only way to identify G6PD deficiency in some heterozygous females. However in some women the effects of Lyonisation of the X-chromosome are such that even this test may fail to detect the heterozygous state and in such cases the only way to diagnose the condition is by family studies or by DNA analysis.

Fairbanks and Lampe (1968)

REAGENTS:

Sodium chloride	0.15 mol/l
Sodium chloride	0.1 mol/l
Sodium nitrite	0.18 mol/l
MTT*	5 mg/ml
Incubation mixture:	Mix the following reagents so that the mixture will have the final concentration stated and divide into 1 ml aliquots which are stable for 3 weeks at 4°C or for at least I year at - 20°C Glucose (28 mmol/l), Phosphate buffer (50 mmol/ l, pH 7.0) Sodium chloride (58 mmol/l), and Nile blue sulphate (11 mg/l)

*MTT = 3-(4,5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide.

BLOOD SAMPLES

It is best to use blood anticoagulated in ACD (4 ml of blood in 1 ml of ACD) which can be used for up to 1 week after collection. Heparinized and EDTA blood samples can also be used, but must be used on the day of venesection to avoid excessive red cell crenation (affecting membrane permeability), or alternatively, transferred into ACD on the day of collection.

Procedure

1 Centrifuge sample, remove the supernatant plasma and add 0.5 ml of packed red cells to a centrifuge tube containing 9 ml 0.15 mol/l sodium chloride and 0.5 ml of 0.18 mol/l sodium nitrite. Incubate undisturbed at 37°C for 20 minutes.

2 Centrifuge at 4°C for 15 minutes at 550 g. Remove and discard the supernatant without disturbing the buffy coat.

3 Wash the red cells three times with 9 ml of cold 0.15 mol/l sodium chloride at 4°C as in step 2 above. After each wash remove the buffy coat taking care to remove as few red cells as possible.

4 Mix the packed red cells and transfer 50 μl to a tube containing l.0 ml of the incubation mixture. Incubate undisturbed at 37°C for 30 minutes.

5 Add 0.2 ml of MTT and resuspend the red cells by gentle agitation. Continue the incubation at 37°C for a further 60 minutes.

6 Resuspend the red cells thoroughly and mix one drop of this suspension with one drop of 0.1 mol/l sodium chloride on a microscope slide and cover with a coverslip.

7 Within 30 minutes of making the preparation examine 500 red cells with a x 100 oil immersion objective and score their granularity as follows:

No granules	0
1-3 granules	I +
4-6 granules	2 +
More than 7 granules	3 +

Interpretation

In subjects with normal G6PD activity most of the red cells will contain some granules and more than 30% will score 3 +. In deficient hemizygous males less than 20% of the red cells will contain any granules and often only the youngest red cells (reticulocytes) will contain any granules. Mosaicism in heterozygous females should be easy to recognize since one population will have normal granules and the other population will have few or no granules. The results are often easier to interpret if they are plotted as a bar graph.

More recently, a cytofluorometric method has been developed (Shah et al. 2012) which utilises flow cytometry to analyse the G6PD activity in individual red cells, and a new software tool (Kalnoky et al. 2014) has been designed to improve precision in measurements generated by different flow cytometers and to give better quantitative interpretation of cytochemical staining results.

Quantitative assay

The introductory chapters of Beutler's practical manual (Beutler 1984) contain very useful details concerning the preparation, stability and storage of reagents and calibration of spectrophotometers as well as details of the assay technique (unfortunately no longer in print).

In the quantitative method, a recording spectrophotometer is helpful but the assay can be undertaken with any spectrophotometer which can measure absorbance at 340 nm and in which the temperature of the cuvette can be maintained within 1-2°C at 30°C or 37°C. Since the activity of G6PD is calculated from the change in absorption at 340 nm due to the reduction of NADP to NADPH, it is important to know the millimolar extinction coefficient of NADPH in the spectrophotometer being used for the assay. In practice the millimolar extinction coefficient of 6.22 can be used (the absorption of a millimolar solution in a 1 cm cuvette at 340 nm) if the bandwidth of the spectrophotometer is 4 nm or less. If the bandwidth is broader than this it may be necessary to calibrate the spectrophotometer using an accurately prepared solution of NADPH since the extinction coefficient will fall as the bandwidth gets broader. In any case a normal range for a given laboratory (and spectrophotometer) should be established and used for comparison.

Like all enzyme reactions, the chemical reactions associated with G6PD are affected by temperature: a higher temperature will lead to an increase in the reaction rate and will therefore appear to give both an increase in activity and an apparent loss of precision if assessed by the standard deviation, but no change in precision if assessed by the coefficient of variation. For the same reason the apparent sensitivity of the tests will increase with temperature. Since the effect of temperature can be significant, it is essential that the reaction is undertaken at a known constant temperature and that a 'normal range' is used which has been established at that temperature. Formulae have been drawn up to indicate the effect of temperature on reaction rate (Beutler, 1984) but these should not be relied on in a clinical situation. In practice it is much easier to control the temperature of the cuvette if the temperature chosen is significantly above ambient temperature, because most temperature controllers only have heaters and rely on the ambient temperature for cooling.

It is important to remember that the normal range for G6PD activity by the WHO “one-substrate” (“one-stage”) method is different from (and higher than) that obtained using the Glock and McLean method (see Appendix).

G6PD quantitative “one-substrate” assay

BLOOD SAMPLES

Blood samples may be anticoagulated with EDTA, heparin or ACD and give reliable results after storage at 5 days at 25°C or 21 days at 4°C.

Haemolysate

Filtration of whole, anticoagulated blood through a mixed cellulose column to remove white cells and platelets is preferable but if this is not practical, centrifugation of the blood and removal of the plasma and buffy coat is satisfactory.

Wash the red cells twice in cold 0.15 mol/l sodium chloride and then resuspend the red cells in an equal volume of cold 0.15 mol/l sodium chloride. Add 0.2 ml of the cell suspension to 1.8 ml of the haemolysing reagent consisting of 2.7 mmol/l EDTA, pH 7.0, and 0.7 mmol/l 2-mercaptoethanol (100 mg of EDTA disodium salt and 5 µl of 2-mercaptoethanol in 100 ml of water; adjust the pH to 7.0 with HCl or NaOH).

Place the suspension on ice for 10 minutes to ensure haemolysis and mix before use. If lysis appears incomplete, freeze and thaw by placing the tube containing the haemolysate in an ice-alcohol mixture until it is frozen and then thaw by placing the tube in a beaker of water at room temperature. Next measure the haemoglobin concentration. This haemolysate should be kept cold (preferably in ice) and should be used within 2 hours. Removal of the stroma is not necessary.

The haemolysate should be prepared freshly for each batch of enzyme assays. Most enzymes in haemolysates are stable for 8 hours at 0°C, but it is best to carry out assays immediately. G6PD is one of the least stable enzymes in this haemolysate, and its assay should be conducted within 1 or 2 hours of the lysate being prepared. The storing of frozen cells or haemolysates is not recommended; it is preferable to store whole blood in ACD.

Procedure

The assay is carried out at constant temperature (37°C or 30°C), the cuvettes containing the first three reagents and water plus haemolysate being incubated for 10 min before starting the reaction by adding the substrate, as shown in Table 3. The

change in absorbance following the addition of the substrate is measured over the first 10 to 15 minutes of the reaction. The value of the blank is subtracted from the test reaction, either automatically or by calculation. Commercial assay kits are also available.*

The reaction involving G6PD can be written as follows:

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

Table 3 Glucose-6-phosphate dehydrogenase assay

Reagents	Assay (µl)	Blank (µl)
0.1M Tris-HCl/0.5mMEDTA buffer, pH8.0	100	100
$MgCl_2$, 100 mmol/l	100	100
NADP, 2 mmol/l	100	100
1:20 Haemolysate	20	20
Water	580	680
Start reaction by adding: G6P, 6 mmol/l	100	–

EDTA, ethylenediaminetetra-acetic acid; NADP, Nicotinamide adenine dinucleotide phosphate; G6P, glucose-6-phosphate.

Calculation of enzyme activity

The reaction rate, and therefore the rate of increase in absorbance at 340 nm, usually increases for the first few minutes, then becomes linear and finally slows down as the substrate is used up.

The linear part of the curve over a 10 minute period is used for the calculation. Draw a straight line through the recorded points then read off the increase in absorbance and divide by 10 to obtain the increase in one minute (ΔA).

The activity of the enzyme in the haemolysate is calculated from the initial rate of change of NADPH accumulation:

* Trinity Biotech, (Trinity Biotech, Bray, Co Wicklow, Ireland); Pointe Scientific (Pointe Scientific, Inc. 5449 Research Drive, Canton, MI 48188).

Enzyme activity in iu/g haemoglobin

$$= \frac{\text{change in absorbance at 340 nm/minute}}{6.22} \times \text{dilution factor} \times \frac{100}{Hb}$$

where 6.22 is the mmol extinction coefficient of NADPH at 340 nm. Results are expressed per 10^{12} red cells, per ml red cells, or per g haemoglobin by reference to the respective values obtained with the washed red cell suspension. However, the ICSH recommendation is to express values per g haemoglobin, so the haemoglobin concentration must be measured on the haemolysate directly.

Normal G6PD is very stable and with most variants venous blood may be stored in ACD for up to 3 weeks at 4°C without loss of activity.

Some enzyme-deficient variants lose activity more rapidly, and this will cause deficiency to appear more severe than it is. Therefore, for diagnostic purposes, a delay in assaying well-conserved samples should not be a deterrent.

G6PD quantitative assay incorporating Glock and McLean procedure

See Beutler (1984)

REAGENTS

These may be stored at the temperatures given for up to l year.

β-NADP	2 mmol/l. Freeze 2 ml aliquots at - 20°C
Glucose-6-phosphate	6 mmol/l. Freeze I ml aliquots at - 20°C
6-phosphogluconate	6 mmol/l. Freeze I ml aliquots at - 20°C
Magnesium chloride	0.1 mol/l Store at 4°C
Sodium chloride	0.15 mol/l Store at 4°C
Tris-HCI, I mol/l, EDTA 5 mmol/l,	Store at 4 °C
pH 8.0	It is essential to use a pH electrode which is suitable for Tris

Haemolysing reagent	Mix 0.05 ml of 2-mercaptoethanol and 10 ml of neutralised 10% (0.27 mol/l) EDTA and make up to I litre with water

BLOOD SAMPLES

Blood samples may be anticoagulated with EDTA, heparin or ACD and give reliable results after storage at 5 days at 25°C or 21 days at 4°C.

Haemolysate

Filtration of whole, anticoagulated blood through a mixed cellulose column to remove white cells and platelets is preferable (Beutler 1984), but if this is not practical centrifugation of the blood and removal of the plasma and buffy coat is satisfactory. Wash the red cells twice in cold 0.15 mol/l sodium chloride and then resuspend the red cells in an equal volume of cold 0.15 mol/l sodium chloride. Add 0.2 ml of the cell suspension to 1.8 ml of the haemolysing reagent. Freeze and thaw the haemolysate by placing the tube containing the haemolysate in an ice-alcohol mixture until it is frozen and then thaw by placing the tube in a beaker of water at room temperature. Next measure the haemoglobin concentration. This haemolysate should be kept cold (preferably in ice) and should be used within 2 hours. Removal of the stroma is not necessary.

Procedure

The reagents shown in Table 4 are added to cuvettes with a critical volume of less than l.0 ml. If cuvettes with a critical volume of less than 3 ml are used the volumes of all the reagents must be multiplied by three.

Mix, incubate at constant temperature (37° or 30°C) and record the absorbance at 340 nm for 15 minutes. The recorder should have a full scale expansion of 1.0 A.

Table 4

Cuvette	1	2	3	4
Tris-HCl, EDTA buffer	100*	100	100	100
$MgCl_2$	100	100	100	100
NADP	100	100	100	100
Water	680	580	580	480
Haemolysate	20	20	20	20
Mix and incubate at constant temperature for 10 minutes				
G-6-P	–	100	–	100
6-PGA	–	–	100	100

**µl.*

The reaction rate, and therefore the rate of increase in absorbance at 340 nm usually increases for the first few minutes, then becomes linear and finally slows down as the substrates are used up.

Use the linear part of the curve for the calculation. Draw a straight line through the recorded points then read off the increase in absorbance over a 10 minute period and divide by 10 to obtain the increase in one minute (∆A).

Calculation

Enzyme activity in iu/g haemoglobin

$$= \frac{\text{change in absorbance at 340 nm/minute}}{6.22} \times \text{dilution factor} \times \frac{100}{Hb}$$

Where 'Hb' = Hb concentration of the haemolysate in g/dl. In the method given above this can be simplified to:

$$= \frac{\text{G6PD (WHO method)}}{\text{Hb concentration of haemolysate (g/dl)}} \quad \frac{\Delta A \text{ (cuvette 2-cuvette 1)} \times 804}{\text{Hb concentration of haemolysate (g/dl)}} \text{ iu/g haemoglobin}$$

G6PD (Glock & McLean method)

$$= \frac{\Delta A \text{ (cuvette 4-cuvette 3)} \times 804}{\text{Hb concentration of haemolysate (g/dl)}} \text{ iu/g haemoglobin}$$

$$\text{6PGD} = \frac{\Delta A \text{ (cuvette 3-cuvette 1)} \times 804}{\text{Hb concentration of haemolysate (g/dl)}} \text{ iu/g haemoglobin}$$

Where ΔA = change in absorbance in 1 minute.

Normal values (mean ± 2SD at 37°C)

G6PD (WHO method)	12.1 ± 4.2 iu/g haemoglobin
G6PD (Glock & McLean method)	8.3 ± 3.2
6PGD	8.8 ± 1.7

It is important for each laboratory to establish its own normal range and to include a normal control sample with each assay.

Interpretation

The Glock and McLean method (cuvettes 4-3) is usually a more accurate expression of the true G6PD activity than the WHO method (cuvette 2-1). However, at very low enzyme activities this involves subtracting a large, experimentally determined value (6PGD, cuvette 3), from a slightly larger one (G6PD + 6PGD, cuvette 4). In this situation the WHO method (cuvette 2-1) is preferred.

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