POINTE SCIENTIFIC, INC.
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Glucose-6-Phosphate Dehydrogenase Reagent Set

Intended Use
For the quantitative, kinetic determination of glucose-6-phosphate dehydrogenase (G6PD) in blood at 340 nm. For *in vitro* diagnostic use only.

Clinical Significance[1]
G6PD assays are most commonly performed to determine deficiency of G6PD, which is widely prevalent throughout the world. It has been determined that G6PD deficiency in red cells is the basis for certain drug-induced hemolytic anemias. This type of susceptibility to drug-induced hemolysis is often called "primaquine sensitivity" because studies which led to its characterization were made during investigations of the hemolytic properties of this antimalarial compound.

Summary
Glucose-6-phosphate dehydrogenase (G6PD, D-glucose-6-phosphate: oxidoreductase, EC 1.1.1.49) catalyzes the first step in the pentose phosphate shunt, oxidizing glucose-6-phosphate (G-6-P) to 6-phosphogluconate (6-PG) and reducing NADP to NADPH. This procedure is a modification of the spectrophotometric methods of Kornberg and Horecker[2] and of Lohr and Waller[3], involving the following reaction:

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

Nictotinamide adenine dinucleotide phosphate (NADP) is reduced by G6PD in the presence of G-6-P. The rate of formation of NADPH is proportional to the G6PD activity and is measured spectrophotometrically as in increase in absorbance at 340nm. Production of a second molar equivalent of NADPH by erythrocyte 6-phosphogluconate dehydrogenase (6-PGDH) according to the reaction:

$$\text{6-PG} + \text{NADP}^+ \xrightarrow{\text{6PGD}} \text{Ribulose-5-Phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

is prevented by use of maleimide, an inhibitor of 6-PGDH.

Reagents
G6PD R1 Reagent: Reconstituted reagent will contain NADP, 1.5 mM, and maleimide, 12 mM. Also contains buffer, stabilizer and lysing agent.
G6PD R2 Reagent: Glucose-6-Phosphate, 1.05 mM, buffer and magnesium salt. Sodium azide added as preservative.

Precautions
1. These reagents are for *in vitro* diagnostic use only.
2. Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state and federal laws.
3. R1 Reagent is HARMFUL. May cause sensitization by inhalation and skin contact. Wear suitable protective clothing.
4. R2 Reagent contains sodium azide which may react with lead and copper plumbing to form highly explosive metal azides. Avoid azide accumulation.

Reagent Preparation
1. R1 reagent is prepared by reconstituting with the volume of deionized water indicated on vial label or application sheet. Swirl gently and invert several times to dissolve contents. Wait 2-3 minutes and mix again. **NOTE**: For manual use, see reagent preparation instructions listed in "MANUAL PROCEDURE" section.
2. R2 reagent is supplied ready to use.

Storage and Stability
1. When unopened R1 reagent vials and the R2 reagent are stored at 2-8°C. They are stable until the expiration date on the labels.
2. Reconstituted R1 reagent is stable for 8 hours at room temperature (18-26°C) or 5 days refrigerated (2-8°C).

Optional Reagents
G6PD controls: Lyophilized controls with G6PD in a stabilized human red cell hemolysate base. Pointe Scientific catalog number G7583-CTL.
G6PD Lyse reagent: Triton X-100, 0.2%. For use with discrete analyzer applications. Pointe Scientific catalog number G7583-LYS.

Specimen Collection and Storage
1. It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2.
2. Whole blood collected in EDTA, heparin or acid-citrate-dextrose (ACD) is satisfactory.[4-8]
3. Red cell G6PD is stable in whole blood for one week refrigerated (2-8°C), but is unstable in red cell hemolysates.[9]
4. Freezing of blood is not recommended.[4]
5. Since activity is reported in terms of grams hemoglobin or the number of red blood cells, the hemoglobin concentration or red cell count must be determined prior to performing the G6PD assay. The integrity of erythrocytes collected in ACD is preserved even after prolonged storage so that obtaining accurate red cell counts usually poses no problem.[6] However, red cell counts on specimens collected in heparin become unreliable after about 2 days.[6] Thus, for heparinized samples, results are best reported in terms of hemoglobin concentration.

Interfering Substances
1. Copper completely inhibits G6PD at a concentration of 100 umol/L, and sulfate ions (0.005 mol/L) decrease observed levels of G6PD activity.[10]
2. Certain drugs and other substances are known to influence circulating levels of G6PD.[11]
3. Reticulocytes have higher G6PD levels than mature red cells. It is recommended that assays **not** be performed after a severe hemolytic crisis, since G6PD levels may appear falsely elevated. Under those conditions, detection of deficiency may require family studies. Testing may be performed after the level of mature red cells has to returned to normal.
4. Under normal circumstances, activity contributed by leukocytes, platelets and serum is relatively small. However, in cases of extreme anemia, grossly elevated white counts or, very low levels of red cell G6PD activity, the contribution to the total made under these conditions may be significant. See "Use of Buffy-Coat-Free Samples" section.

Automated Analyzer Applications
Application procedures are available for various automated instruments. Please contact Pointe Scientific Technical Service Department (1-800-445-9853) for more information.

Materials Provided
See "reagents" section

Materials Required But Not Provided
1. Spectrophometer capable of measuring at 340 nm with temperature controlled cuvette compartment (water bath or incubator may be used instead)

2. Pipeting devices for delivery of volumes required for the assay
3. Cuvettes with optical properties suitable for use at 340 nm
4. Equipment and reagents for determining hemoglobin concentration or performing a red cell count. Pointe Scientific offers catalog number H7504 for the determination of hemoglobin.

Manual Procedure

Prepare working R1 reagent by adding lyse as the diluent instead of DH2O. Add volume stated on the R1 vial. This reagent can now be used as stated below. **NOTE**: Do not use DH2O to reconstitute the R1 vial for manual procedure.

The temperature of the reaction must be maintained at 37°C or some other constant temperature (see "Temperature Correction" section).

1. Prepare reaction mixture:
   a. To a labeled cuvet, add 1.0 ml R1 Reagent.
   b. Add 0.01 ml blood and mix thoroughly to completely suspend erythrocytes. Let stand at room temperature (18-26°C) for 5-10 minutes.
   c. Add 2.0 ml R2 Reagent and mix gently by inverting several times. Proceed to step 2.
2. Place cuvette in constant temperature cuvette compartment or water bath and incubate for approximately 5 minutes.
3. Read and record absorbance (A1) of TEST at 340 nm vs water. (If using a water bath or incubator, return cuvette to it.)
4. Exactly 5 minutes later, read and record absorbance (A2).
5. To determine G6PD activity, refer to "Calculations" section.

Calibration

The procedure is standardized on the basis of the millimolar absorptivity of NADPH, which is 6.22 at 340 nm. Measurement of the rate of increase in absorbance (ΔA) at 340 nm serves to quantitate enzymatic activity.

Quality Control

Reliability of test results should be monitored by use of control materials with known values within each run. Pointe Scientific glucose-6-phosphatase dehydrogenase controls are suitable for this purpose. (Catalog number G7583-CTL) It is recommended that each laboratory establish its own frequency of control determination.

Calculations

$$\Delta A \text{ per min} = \frac{A2 - A1}{5}$$

G6PD activity can be expressed as either U/g hemoglobin (Hb) or as U/10^{12} erythrocytes (RBC).

$$\text{G6PD (U/g Hb)} = \Delta A \text{ per min} \times \frac{100 \times 3.01}{0.01 \times 6.22 \times \text{Hb (g/dl)}} \times \text{TCF}$$

$$= \Delta A \text{ per min} \times \frac{4839}{\text{Hb (g/dl)}} \times \text{TCF}$$

Where: 100 = Factor to convert activity to 100 ml
3.01 = Total reaction volume (ml)
0.01 = Sample volume (ml)
6.22 = Millimolar absorptivity of NADPH at 340 nm
Hb (g/dl) = Hemoglobin concentration for each specimen
TCF = Temperature Correction Factor (1 at 37°C)

or

$$\text{G6PD (U/}10^{12}\text{RBC)} = \frac{\Delta A \text{ per min} \times 3.01 \times 10^{12} \times \text{TCF}}{0.01 \times 6.22 \times (N \times 10^6) \times 1000}$$

Where: 3.01 = Total reaction volume (ml)
10^{12} = Factor for expressing activity in 10^{12} cells
0.01 = Sample volume (ml)
6.22 = Millimolar absorptivity of NADPH at 340 nm
$N \times 10^6$ = Red cell count (red cells/mm^3) determined for each specimen
1000 = Conversion of red cell count from mm^3 to ml
TCF = Temperature correction factor (1 at 37°C)

This equation reduces to:

$$\text{G6PD (U/}10^{12}\text{ RBC)} = \Delta A \text{ per min} \times \frac{48{,}390}{N} \times \text{TCF}$$

Where: N = red cell count divided by 10^6
*TCF= temperature correction factor (1 at 37°C)

* Additional temperature correction factors are available upon request.

Example:
Assay of a specimen which had a red cell count of $4.6 \times 10^6/mm^3$ and a hemoglobin concentration 15.2 g/dl resulted in a ΔA per min at 37°C of 0.028.

$$\text{G6PD (U/g Hb)} = 0.028 \times \frac{4839}{15.2} = 8.9$$

$$\text{G6PD (U/}10^{12}\text{ RBC)} = 0.028 \times \frac{48{,}390}{4.6} = 295$$

NOTE: If ΔA per min is greater than 0.060, repeat determination using 5 µl blood and multiply results by 2.

Use of Buffy-Coat-Free Sample

Under normal circumstances G6PD activity contributed by leukocytes, platelets and serum is relatively small. However, as reported by Echler[12] and others[13], more accurate measurement of red cell G6PD activity, especially in the presence of anemia and/or leukocytosis, can be achieved by using buffy coat-free blood samples for assay. Thus, in case of a borderline value obtained with whole blood, it may be warranted to repeat the assay on a buffy coat-free sample.

Temperature Correction

When temperature is 37°C, no temperature correction factor (TCF) is required in the calculations. If assay is performed at another temperature a TCF must be used.[14]

Cuvette Temperature	TCF
25°C	1.98
30°C	1.37

Unit Definition

One International Unit (U) is that amount of G6PD activity that will convert 1 micromole of substrate per minute under the conditions specified in this insert.

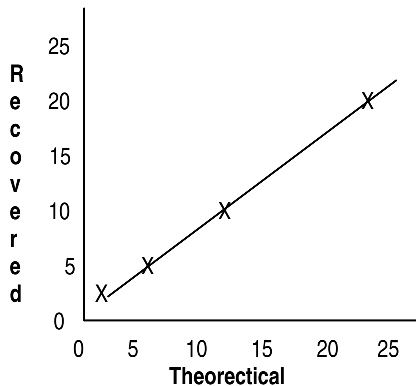
Expected Values[1]

A recommended reference range for G6PD measured at 37°C is:
12.1 ± 2.09 U/g Hb
351 ± 60.6 U/10^{12} RBC

Values for newborns may range somewhat higher. It is highly recommended that each laboratory establish its own expected range.

Performance Characteristics

Assay Range: The maximum G6PD activity which may be measured by this procedure is approximately 21.0 U/g Hb or 609 U/10^{12} RBC.



Observed Data	Theorectical Result	Precent Recovery
2.78 U/g Hb	2.78 U/g Hb	100.0%
5.29 U/g Hb	5.56 U/g Hb	95.1%
10.80 U/g Hb	11.12 U/g Hb	97.1%
20.69 U/g Hb	22.24 U/g Hb	93.0%

Precision: Precision studies were performed on a Roche Cobas Mira following the guidelines contained in NCCLS document EP5-T2.[15] The data is presented in units that an automated analyzer will produce for G6PD activity (U/L). It is highly recommended that precision of the assay be verified on each analyzer before use.

Within Day (n=20)

Mean	S.D.	C.V.
257	23.7	9.2%
658	18.3	2.8%
1939	48.0	2.5%

Day to Day (n=20)

Mean	S.D.	C.V.
269	30.8	11.4%
700	28.7	4.1%
2014	43.0	2.1%

Sensitivity: Assuming the limit of sensitivity to represent a change in absorbance at 340nm of 0.001 per minute, a G6PD activity of 0.4 U/g Hb or 11 U/10^{12} RBC may be detected using this procedure (assuming a hemoglobin concentration of 12.0 g/dL and a red cell count of 4.5 x 10^6/mm^3).

Specificity: The oxidation of glucose-6-phosphate by G6PD is specific. Any non-specific formation of NADPH due to oxidation of other substrates by endogenous enzymes occurs during the preincubation period. 6-Phosphogluconate dehydrogenase is completely inhibited by maleimide in the reagent system.

Correlation: A comparison study between the Pointe Scientific method and that of Sigma Diagnostics yielded a linear regression equation with y = 0.97x + 0.07 and a correlation coefficient of 0.994.

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Rev. 12/03 P803-G7583-01