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A Division of OXIS Health Products, Inc.

BIOXYTECH® G6PD¦6PGD-340™

Spectrophotometric Assay for Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21045

INTRODUCTION

The Analyte

Glutathione protects cells against reactive oxygen species (ROS) by a variety of mechanisms. One mechanism is the glutathione peroxidase (GPx) reaction in which hydrogen peroxide is reduced to water with the concomitant oxidation of glutathione (GSH) to the disulfide (GSSG) (Figure 1). Since the amount of GSH present in the cell is finite, GSSG must be recycled to the reduced (GSH) state to maintain protection against hydrogen peroxide. This is accomplished by glutathione reductase (GR), which utilizes electrons from the oxidation of NADPH to convert GSSG into GSH. In cells experiencing oxidative stress, a continual supply of reducing equivalents in the form of NADPH is required (1). NADPH is also used as a reductant in a wide range of anabolic processes, such as fatty acid biosynthesis. The pentose phosphate pathway (PPP) is a major provider of NADPH in a typical cell; in erythrocytes, it is the only source of NADPH. The oxidative branch of the PPP oxidizes glucose-6-phosphate (G6P) to ribulose-5-phosphate (Ru5P) and CO_2 with the formation of two molecules of NADPH per G6P (Figure 2). The two reactions of the PPP producing NADPH are catalyzed by the enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6-PGD).

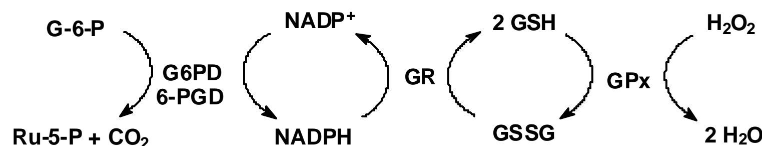


Figure 1. Relationship of the pentose phosphate pathway to glutathione metabolism.

G6PD deficiency is the most common enzyme disorder in humans with about 200 million people affected worldwide (2,3). The highest incidence of G6PD deficiency occurs in the tropics and coincides with regions where malaria is endemic: G6PD deficiency confers resistance to malaria. Heterozygotes for G6PD deficiency are often asymptomatic until subjected to an oxidative challenge. Then, the inability to synthesize adequate amounts of NADPH to support recycling of glutathione can result in severe hemolytic anemia. A variety of drugs, for example, the anti-malarial primiquine, can induce hemolytic anemia in G6PD-deficient individuals (3). Since G6PD catalyzes the first reaction of the PPP, a deficiency also reduces the production of NADPH by 6-PGD, even when that enzyme is present in normal amounts.

6-PGD deficiency in humans has been documented but is much less common than G6PD deficiency. Heterozygotes have only 2-5% of normal 6-PGD activity but have no clinical complications, suggesting that an individual with normal G6PD levels can produce adequate NADPH to meet the cell's requirements.

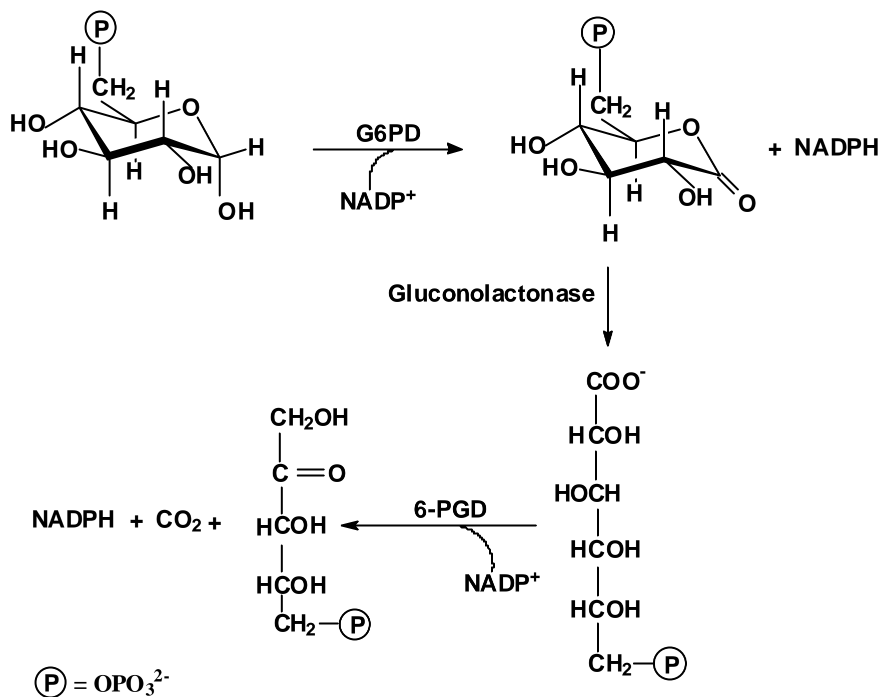


Figure 2. The oxidative branch of the pentose phosphate pathway.

Principle of the Assay

Assays for both enzymes measure the production of NADPH by the increase in absorbance at 340 nm due to the reduction of $NADP^+$ (Figure 2). This introduces a complication in measuring G6PD activity in a sample that contains both enzymes. The product of the G6PD reaction, 6-phosphogluconolactone, rapidly hydrolyzes, either spontaneously or enzymatically, to 6-phosphogluconic acid (6-PGA), the substrate for 6-PGD. As a result, initiation of the G6PD reaction with G6P gradually produces 6-PGA which enables 6-PGD to begin producing NADPH. The consequence is that the A_{340} vs. time plot is not linear but has increasing slope as the contribution from the 6-PGD reaction to total NADPH production increases. Secondly, the slope of the pseudo-linear region of the A_{340} vs. time plot will overestimate the G6PD activity since it will reflect additional production of NADPH by 6-PGD.

To avoid this problem, and to obtain accurate measurement of both G6PD and 6-PGD activity, the BIOXYTECH® G6PD¦6PGD-340™ assay employs the method of Glock and McLean (4,5). In this assay, two measurements are made: one with saturating concentrations of BOTH G6P and 6-PGA to measure the combined activity of both enzymes; and one with only 6-PGA to measure only 6-PGD activity. G6PD activity is then calculated as the difference in rate between the assays with both substrates and only 6-PGA.

In addition, procedures are given for measurement of G6PD activity using only G6P as substrate, as originally proposed by the World Health Organization (WHO) (5). This assay, referred to as the G6PD (WHO) assay, overestimates the G6PD activity in a sample which also contains 6-PGD activity. Finally, a procedure is given for determination of only 6-PGD activity, for researchers interested in that enzyme.

One unit of activity of either enzyme is defined as the amount of enzyme producing 1 µmol NADPH/min under the conditions of the assay.

REAGENTS

Materials Provided (Sufficient for 100 Tests)

- Assay Buffer — Tris-HCl, EDTA, $MgCl_2$, pH 8.0, 100 mL
- NADP Reagent — Lyophilized, 5 vials
- G6P Reagent — Lyophilized, 1 vial
- 6-PGA Reagent — Lyophilized, 1 vial
- Sample Diluent — EDTA, pH 7.0, 100 mL

Items Required But NOT Provided

- Spectrophotometer with thermostatted cell holder
- 37°C water bath
- Cuvettes with 1 cm optical path
- Pipettes, preferably adjustable, capable of accurately pipetting 5-1000 µL
- 2-Mercaptoethanol

Reagent Storage and Handling

Do not allow the reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 2-8°C. Subsequent to reconstitution, the NADP, G6P and 6-PGA reagents should be stored frozen at –20°C. Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

- NADP Reagent: Just prior to use, reconstitute one vial with 16 mL of Assay Buffer. Equilibrate the required volume to 37°C. Unused reagent should be stored frozen at –20°C.
- G6P Reagent: Just prior to use, reconstitute with 11 mL of deionized water. Unused reagent should be stored frozen at –20°C.
- 6-PGA Reagent: Just prior to use, reconstitute with 11 mL of deionized water. Unused reagent should be stored frozen at –20°C.
- Sample Diluent: Prepare just prior to use. Working in a fume hood, thoroughly mix 100 µL of 2-mercaptoethanol and 900 µL deionized water. Place 10 mL of Sample Diluent in a container that can be tightly capped. Add 5 µL of the diluted 2-mercaptoethanol, cap tightly and mix well. Store the required volume of working Sample Diluent on ice. Unused solution can be frozen at –20°C and is stable for several days.
- Substrate Mixtures: Prepare Substrate Mixtures for the assays to be done and the number of samples to be run, according to Table 1 below. Equilibrate at 37°C. Discard unused Substrate Mixtures at the end of the day.

Table 1
Preparation of Substrate Solutions (all volumes are in mL)

Method	Substrate	# of Samples					
		Glock and McLean			G6PD (WHO)		
		1	10	20	1	10	20
Combined Substrates	G6P	0.100	1.100	2.100	---	---	---
	6-PGA	0.100	1.100	2.100	---	---	---
6-PGA	6-PGA	0.100	1.100	2.100	---	---	---
	Deionized water	0.100	1.100	2.100	---	---	---
G6P (WHO)	G6P	---	---	---	0.100	1.100	2.100
	Deionized water	---	---	---	0.100	1.100	2.100
Sample Blank	Deionized water	0.200	2.200	4.200	0.200	2.200	4.200

Sample Preparation Guidelines

These procedures are intended as a starting point for sample preparation. The researcher will have to adjust them as necessary for their particular sample types.

Erythrocytes: Collect blood using heparin or EDTA as anticoagulant. Collect and wash erythrocytes. Lyse cells by adding 9 volumes of cold working Sample Diluent to 1 volume of packed cells (1/10). Suspend cells and rapidly freeze the sample. To assay, thaw in a water bath at 20-25°C. Dilute an aliquot of lysate 1/2 with working Sample Diluent to give a net 1/20 dilution of the packed red cells. Store on wet ice and assay as described below. Results may be normalized to hemoglobin for inter-sample comparison.

Solid Tissue: Homogenize ~200 mg of tissue in 1.0 mL of cold working Sample Diluent. Dilute an aliquot of the homogenate with working Sample Diluent and store on wet ice. Assay as described below. Results may be normalized to total protein for inter-sample comparison.

Note: Regardless of sample type, samples are diluted with working Sample Diluent to give 20-140 mU/mL of the enzyme being measured. Usually, the same sample dilution can be used for both the G6PD and 6-PGD assays. The appropriate degree of sample dilution must be determined experimentally.

Assay (in Cuvettes)

Below are given assay procedures for the Glock and McLean assay (for accurate determination of both G6PD and 6-PGD in a sample containing both activities), the 6-PGD assay (for accurate determination of only 6-PGD activity) and the G6PD (WHO) assay (overestimates G6PD in a sample which also has 6-PGD activity).

General

1. Add 750 µL of NADP solution to a small test tube and place in a 37°C water bath.
2. Add 50 µL of the diluted sample.
3. Add 200 µL of the selected substrate or deionized water (sample blank).
4. Vortex to mix and immediately transfer to a cuvette.
5. Record A_{340} vs. time for 5 minutes in a thermostatted cell holder maintained at 37°C.
6. For each run, determine the reaction rate (ΔA_{340}/min) over the linear portion of the absorbance vs. time plot.

Glock and McLean Assay (G6PD and 6-PGD)

1. Determine the reaction rate for G6PD using the Combined Substrate
2. Determine the reaction rate for 6-PDG using the 6-PGA Substrate.
3. Determine the reaction rate for sample blank using deionized water.

6-PGD Assay (Alone)

1. Determine the reaction rate for 6-PDG using the 6-PGA Substrate.
2. Determine the reaction rate for sample blank using deionized water.

G6PD Assay (WHO Method)

1. Determine the reaction rate for G6PD using the only G6P Substrate.
2. Determine the reaction rate for sample blank using deionized water.

Calculations

General

1. Determine the net reaction rate for the selected substrate by subtracting the sample blank rate from the measured rate for that substrate.
2. Convert each rate into mU/mL of activity in the original sample solution using the equation below:

$$\text{Activity (mU/mL)} = \frac{\text{Ä}A_{340}/\text{min}}{6.22 \text{x} 10^{-3}} \text{ x Dilution Factor}$$

   Where Dilution Factor is the total amount the sample was diluted.

Glock and McLean Assay (G6PD and 6-PGD)

1. Calculate the activity measured with the Combined Substrate.
2. Calculate the activity measured with the 6-PGA as substrate.
3. Calculate the G6PD activity of the sample by subtracting the 6-PGD activity from the activity determined using the Combined Substrates.

$$\text{G6PD (mU/mL)} = \text{Combined Substrates mU/mL} - \text{6-PGD mU/mL}$$

6-PGD Activity (Alone)

Calculate the activity measured with 6-PGA as substrate is the 6-PGD activity in mU/mL.

G6PD Activity (WHO Method)

Calculate the activity measured with the G6P as substrate.

Example

An erythrocyte lysate was prepared as described above (1/20 dilution of the packed red cell volume) and assayed in triplicate by the Glock and McLean Method and the G6PD (WHO) Method. The data are given in Table 2 below. The hemoglobin concentration of the diluted lysate was found to be 14.3 mg/mL.

Table 2
Enzyme Activity in Erythrocyte Lysate

Assay	Slope (ΔA_{340}/min)				
	Run 1	Run 2	Run 3	Mean	Net Rate
Blank	-0.0017	-0.0012	-0.0015	-0.0015	
Combined Substrates	0.0627	0.0627	0.0626	0.0627	0.0642
6-PGA Substrate	0.0298	0.0307	0.0304	0.0307	0.0322
G6PD (WHO) Substrate	0.0503	0.0498	0.0494	0.0498	0.0513

Glock and McLean Assay (G6PD and 6-PGD)

1. Calculate the mean of the three runs in each set. Note the sample blank slope is negative; this is typical for red cell lysates.
2. Subtract the sample blank slope from the mean slopes for the Combined and 6-PGA Assays to get the Net Rate for each (last column).
3. Calculate the Activity with the Combined Substrate and 6-PGA Substrate

   Combined Substrate

$$\text{Activity (mU/mL)} = \frac{0.0642}{6.22 \text{x} 10^{-3}} \text{ x } 20 = 206 \text{ mU/mL}$$

   Note: Since 50 µL of sample was used and the total reaction volume is 1000 µL, the dilution factor is 20.

6-PGA Substrate

$$\text{Activity (mU/mL)} = \frac{0.0322}{6.22\text{x}10^{-3}} \text{ x } 20 = 104 \text{ mU/mL}$$

4. Calculate the G6PD Activity

$$\text{G6PD (mU/mL)} = 206 \text{ mU/mL} - 104 \text{ mU/mL} = 102 \text{ mU/mL}$$

5. Express both activities per mg hemoglobin

$$\text{G6PD} = \frac{102 \text{ mU/mL}}{14.3 \text{ mg Hb/mL}} = 7.13 \text{ mU/mg Hb}$$

$$\text{6-PGD} = \frac{104 \text{ mU/mL}}{14.3 \text{ mg Hb/mL}} = 7.27 \text{ mU/mg Hb}$$

G6PD Assay (WHO Method)

1. Calculate the mean of the three runs. Note the sample blank slope is negative.
2. Subtract the sample blank slope from the mean slope for the G6PD (WHO) Assay to get the Net Rate (last column).
3. Calculate the G6PD (WHO) Activity.

$$\text{Activity (mU/mL)} = \frac{0.0513}{6.22\text{x}10^{-3}} \text{ x } 20 = 165 \text{ mU/mL}$$

4. Express the activity per mg hemoglobin

$$\text{G6PD(WHO)} = \frac{165 \text{ mU/mL}}{14.3 \text{ mg Hb/mL}} = 11.5 \text{ mU/mg Hb}$$

Note that in the example above, the G6PD activity determined with the WHO method (**11.5** mU/mg Hb) overestimated G6PD activity by 160% relative to the Glock and McLean method (**7.13** mU/mg Hb).

Alternative Microplate Assay Procedure

Reagent and sample preparation are identical to the cuvette method. The results will differ from the cuvette method for three reasons: 1) the pathlength will not be 1 cm, 2) the bandpass of the filter will be different from the monochromoter of the spectrophotometer, and 3) the temperature will not be 37°C.

These conditions will not prevent comparison of results from a series of samples to determine relative G6PD and 6-PGD activities. By running a given sample in both the cuvette and microplate methods, the researcher can determine a correction factor to convert microplate rates to activities in mU/mL in the standard cuvette assay.

A suggested protocol for the microplate assay follows.

1. Add 150 µL of NADP solution to the well
2. Add 10 µL of the diluted sample.
3. Add 40 µL of the selected substrate solution or deionized water (Blank).
4. Mix well.
5. Record A_{340} vs. time for 5 minutes.
6. For each run, determine the reaction rate (ΔA_{340}/min) over the linear portion of the absorbance vs. time plot.

PERFORMANCE CHARACTERISTICS

Precision

The precision of the assay was determined by assaying G6PD and 6-PGD controls, at three different concentrations, stored at –70°C. Each sample was assayed in duplicate on 10 different occasions over a two-week period (Table 3).

Table 3
Intra-assay and Inter-assay Precision

	G6PD			6-PGD		
mU/mL Enzyme in Cuvette	2.46	4.54	8.50	3.08	5.40	9.00
Mean (ΔA_{340}/min)	0.0153	0.0282	0.0529	0.0192	0.0336	0.0560
Intra-assay Precision (%CV)	2.07%	1.14%	2.45%	1.65%	1.38%	1.30%
Total Precision (%CV)	1.62%	1.05%	2.42%	1.84%	1.25%	1.42%

Sensitivity – Lower Limit of Detection (LLD)

The Lower Limit of Detection (LLD) is a measure of the lowest activity distinguishable from zero with confidence. Using purified G6P and 6-PGA as substrates, the ΔA_{340}/min for a Water Blank was determined and the mean and standard deviation of the slope was calculated. The LLD (~99.5% confidence) is the amount of enzyme giving a rate 3.29 times the standard deviation of the blank (Table 4).

Table 4
Lower Limit of Detection

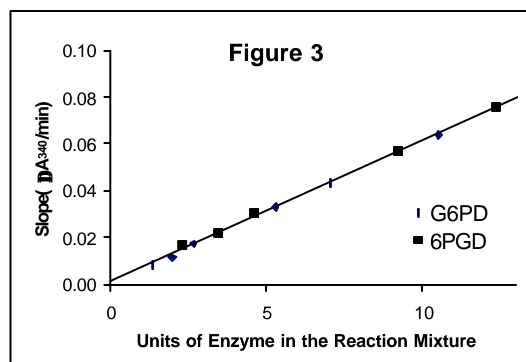
Parameter	G6PD	6-PGD
Number of Data Points	12	6
Mean ΔA_{340}/min	0.000025	0.0000067
Standard Deviation	0.000148	0.000170
LLD (mU enzyme/cuvette)	0.024	0.027

Assay Range

The theoretical range of the assay is from the LLD to the highest rate for which a linear slope is obtained. As a practical matter, the effective LLD is determined by the sensitivity of the spectrophotometer. A conservative estimate of the working range is 1-7 mU of enzyme/cuvette. This will provide an adequate slope at the lower end of the range to determine an accurate rate and a sufficiently long linear region of the A_{340} vs. time plot at the upper end of the range to give an accurate rate.

Linearity on Dilution

Purified G6PD and 6-PGD were diluted and assayed under standard conditions. Both assays measure the rate of NADPH production and both enzyme activity units are defined as the production of one micromole of NADPH per minute under the assay conditions. Therefore, the ΔA_{340}/min vs. mU of enzyme per cuvette should be the same for both. Figure 3 confirms this, with both enzymes demonstrating linearity on dilution between 1 – 12 mU/cuvette.



Recovery

Pure G6PD or 6PGD was added to working Sample Diluent and a red blood cell lysate. The recovery of added enzyme was the calculated by dividing the Net lysate activity by the Diluent activity as seen in Table 5.

Table 5
Recovery of Enzyme Added to Erythrocyte Lysate

Sample	G6PD mU/mL				6-PGD mU/mL		
	Measured		Added	% Recovery	Measured	Added	% Recovery
	Combined	G6PD					
Lysate	174	89	---	---	86	---	---
Spiked Diluent	87	87	---	---	157	---	---
Spiked Lysate	265	179	90	103	232	146	93

Specificity

The activity of purified yeast G6PD and 6-PGD was measured in the presence of various substances commonly used in enzyme isolation (Table 6). The percent activity was determined relative to a control with no additions. The concentration of each addition is given in the assay mixture.

Table 6
Interference by Selected Substances

Substance	Concentration in Reaction Mixture	G6PD Activity mU/mL	% of Control	6-PGD Activity mu/mL	% of Control
None	---	7.32	100.0	9.02	100.0
Urea	600 mM	7.28	99.6	6.80	75.4
Triton X-100	0.1%	7.27	99.3	8.75	97.0
Sucrose	6.0%	6.90	94.3	9.37	103.9
Na_2SO_4	100 mM	0.37	5.1	2.57	28.5
$(NH_4)_2SO_4$	100 mM	0.29	4.0	2.65	29.4
DTT	0.1%	7.25	99.1	9.15	101.4
Mercaptoethanol	0.1%	7.44	101.8	9.34	103.6
BSA	1 mg/mL	7.38	100.9	9.08	100.7

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol.

Thiols, Triton X-100, sucrose and BSA do not interfere in the assay of either enzyme. Both activities are severely affected by Na_2SO_4 and $(NH_4)_2SO_4$. Yeast G6PD is known to be strongly inhibited by sulfate ion. Urea causes a slight reduction in 6PGD activity but has no effect on G6PD.

Assay Temperature

Both G6PD and 6-PGD have significantly higher activity at 37°C compared to 25°C. For this reason, the assay temperature was chosen to be 37°C. For researchers who either do not have a thermostatted cell holder for their spectrophotometer, or who for other reasons wish to run the assay at 25°C, we provide information on conversion of activities measured at 25°C to 37°C values. Table 7 below gives the percent activity at 25°C relative to 37°C for G6PD and 6-PGD using the Glock and McLean assay procedure and for G6PD using the G6PD (WHO) procedure. The last column is the conversion factor for calculating activity at 37°C from 25°C data. Multiply the 25°C activity by the indicated number to convert to 37°C activity.

Table 7
Temperature Conversion 25°C to 37°C

Enzyme	Method	Activity at 25°C Relative to 37°C	Conversion Factor 25°C to 37°C
G6PD	Glock and McLean	62.8%	1.59
6-PGD	Glock and McLean	58.5%	1.71
G6PD	(WHO)	60.6%	1.65

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