Glucose-6-Phosphate Dehydrogenase Activity Assay Kit

Item No. 700300

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700273	G6PDH Assay Buffer (10X)	1 vial/5 ml
700274	G6PDH Substrate	2 vials
700275	G6PDH Cofactor	2 vials
700276	G6PDH Enzyme Mixture	2 vials
700277	G6PDH Fluorometric Detector	2 vials
700278	G6PDH Positive Control	2 vials/50 µl
700279	NADPH Standard	2 vials
400017	96-Well Solid Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Glucose-6-phosphate dehydrogenase (G6PDH) is a cytosolic enzyme that catalyzes the first step in the pentose phosphate pathway.[1] This pathway includes converting glucose to ribose-5-phosphate, a precursor to RNA, DNA, ATP, CoA, NAD, and FAD. The pathway also generates NADPH. Glutathione reductase uses NADPH to maintain the level of glutathione within the cell, thus protecting the cell from oxidative damage.[2] NADPH is also involved in fatty acid oxidation, lipid biosynthesis, and is the substrate for NADPH oxidase in activated macrophages and polymorphonuclear leukocytes to produce oxygen radicals which destroy pathogens. Most cells have alternate ways of generating intracellular NADPH such as the *de novo* pathway from amino acids. Since red blood cells do not contain mitochondria, the pentose phosphate pathway is their only source of NADPH; therefore, defense against oxidative damage is dependent on G6PDH. G6PDH deficiency becomes especially lethal in red blood cells, where any oxidative stress will result in hemolytic anemia.[3,4]

G6PDH deficiency, the most common enzyme deficiency worldwide, causes a spectrum of diseases including neonatal hyperbilirubinemia, acute hemolysis, and chronic hemolysis.[4-7] It is estimated that about 400 million people are affected by this deficiency. Fortunately, most people will remain clinically asymptomatic throughout their lives. The deficiency is an X-linked hereditary genetic defect caused by mutations in the G6PD gene, showing a typical X-linked distribution pattern with higher incidence in males than in females.[3,4] G6PDH deficiency is known to provide protection against malaria, particularly the deadliest form of malaria caused by *Plasmodium falciparum*.[4] Areas endemic to malaria usually have more individuals with the deficiency, possibly because of an evolutionary advantage.

G6PDH activity has been shown to be upregulated in rat and mouse models of obesity, hyperglycemia, and hyperinsulinemia.[7] G6PDH is an important mediator of insulin resistance and is strongly activated post-translationally in (pre)neoplastic lesions to produce NADPH.[7,8] The tumor suppressor p53, the most frequently mutated gene in human tumors, inhibits G6PDH by binding to the enzyme and preventing the formation of the active dimer.[9]

About This Assay

Cayman's Glucose-6-Phosphate Dehydrogenase Assay provides a fluorescence-based method for detecting G6PDH activity in a variety of samples including erythrocyte lysates, tissue homogenates, and cell culture samples. In the assay, G6PDH catalyzes the oxidation of glucose-6-phosphate to 6-phospho-D-gluconate, along with the concomitant reduction of $NADP^+$ to NADPH. NADPH reacts with the fluorometric detector to yield a highly fluorescent product which can be analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **G6PDH Assay Buffer (10X) - (Item No. 700273)**

   The vial contains 5 ml of 500 mM Tris-HCl, pH 7.4, containing 100 mM $MgCl_2$. Dilute the contents of the vial with 45 ml of HPLC-grade water. This final Assay Buffer (50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$) is used in the assay. The diluted Assay Buffer is stable for three months at 4°C.

2. **G6PDH Substrate - (Item No. 700274)**

   Each vial contains a lyophilized powder of glucose-6-phosphate. Reconstitute the contents of the vial with 600 µl of diluted Assay Buffer. This is sufficient Substrate to assay 60 wells. Prepare the additional vial as needed. The reconstituted Substrate is stable for one week at -20°C.

3. **G6PDH Cofactor - (Item No. 700275)**

   Each vial contains a lyophilized powder of $NADP^+$. Reconstitute the contents of the vial with 600 µl of diluted Assay Buffer. This is sufficient reagent to assay 60 wells. Prepare the additional vial as needed. The reconstituted Cofactor is stable for four hours at room temperature.

4. **G6PDH Enzyme Mixture - (Item No. 700276)**

   Each vial contains a lyophilized powder of enzymes. Reconstitute the contents of the vial with 600 µl of diluted Assay Buffer and put the vial on ice. This is enough Enzyme Mixture to assay 60 wells. Prepare the additional vial as needed. The reconstituted enzymes are stable for four hours at 4°C.

5. **G6PDH Fluorometric Detector - (Item No. 700277)**

   Each vial contains a lyophilized powder of fluorometric detector. Reconstitute the contents of the vial with 600 µl of diluted Assay Buffer. This is sufficient reagent to assay 60 wells. Prepare the additional vial as needed. The reconstituted mixture is stable for one week at -20°C.

6. **G6PDH Positive Control - (Item No. 700278)**

   Each vial contains 50 µl of glucose-6-phosphate dehydrogenase. Add 450 µl of diluted Assay Buffer to the vial and put the vial on ice. Further dilute 10 µl of enzyme with 1.99 ml of diluted Assay Buffer and store on ice. This enzyme will be assayed as the Positive Control. The diluted enzyme is stable for four hours at 4°C.

7. **NADPH Standard - (Item No. 700279)**

   Each vial contains a lyophilized powder of NADPH. Reconstitute the contents of the vial with 2 ml of diluted Assay Buffer to yield a 1 mM solution of NADPH. The reagent is ready to prepare the standard curve. The reconstituted Standard is stable for four hours at 4°C.

Sample Preparation

Erythrocyte Lysate

Typically, normal human erythrocyte G6PDH activity is in the range of 5-20 µmol/min/g hemoglobin.

1. Collect blood using an anticoagulant such as heparin or EDTA.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C. Remove the top plasma and buffy coat layers.
3. Dilute the erythrocytes 1:1 with phosphate buffered saline (pH 7.4) and place on ice (*i.e.*, 1 ml erythrocytes and 1 ml of buffer). *NOTE: This is a 1:2 dilution of the erythrocytes that will have to be included in the calculations.*
4. On ice, sonicate the erythrocytes with a few short bursts to break open the cells.
5. If not assaying the same day, freeze at -80°C. The sample will be stable for one month while stored at -80°C.
6. Further dilute the lysate 1:10-1:20 with Assay Buffer before assaying.

NOTE: Erythrocyte G6PDH activity can be standardized to hemoglobin content using Cayman's Hemoglobin Colorimetric Assay Kit (Item No. 700540).

Cell Lysate

1. Collect cells (~3 x 10^6 cells) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. On ice, sonicate the cell pellet in 1 ml of cold 1X phosphate buffered saline, pH 7.4.
3. Centrifuge at 10,000 x g for 10 minutes at 4°C. Remove the supernatant and store on ice.
4. If not assaying the same day, freeze at -80°C. The sample will be stable for one month while stored at -80°C.
5. The sample does not need to be diluted before assaying.

Tissue Homogenate

1. Prior to dissection, rinse tissue with a phosphate buffered saline solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 1X PBS, containing protease inhibitors of choice; see **Interferences** on page 19) per gram weight of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a NADPH Standard curve and the Positive Control in duplicate have to be assayed with the samples. We suggest that each sample be assayed at least in duplicate in the presence and absence of G6PDH substrate. A typical layout of standards, samples, and sample backgrounds to be measured in duplicate is given below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
B	B	B	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
C	C	C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
D	D	D	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
E	E	E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
F	F	F	B3	B3	B7	B7	B11	B11	B15	B15	B19	B11
G	G	G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
H	+	+	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-G = Standards
+ = G6PDH Positive Control
S1-S20 = Sample Wells
B1-B20 = Background Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 200 µl in all the wells.
- All reagents except the enzymes must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples at least in duplicate (triplicate preferred).
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Take seven clean glass test tubes or polystyrene tubes and mark them A-G. Add the amount of reconstituted NADPH (1 mM) and diluted Assay Buffer to each tube as described in Table 1, below. The diluted Standards are stable for four hours at room temperature.

Tube	NADPH (µl)	Assay Buffer (µl)	Final Concentration (µM)
A	0	500	0
B	50	450	100
C	100	400	200
D	200	300	400
E	300	200	600
F	400	100	800
G	500	0	1,000

Table 1. Preparation of NADPH standards

Performing the Assay

1. **Standard Wells -** add 150 µl of Assay Buffer, 10 µl of Cofactor, 10 µl Enzyme Mixture, and 10 µl of standard (tubes A-G) per well in the designated wells on the plate (see **Sample plate format**, Figure 1, page 11).
2. **G6PDH Positive Control Wells -** add 150 µl of Assay Buffer, 10 µl of Cofactor, 10 µl of Enzyme Mixture, and 10 µl of diluted Positive Control to at least two wells.
3. **Sample Wells -** add 150 µl of Assay Buffer, 10 µl of Cofactor, 10 µl of Enzyme Mixture, and 10 µl of sample to at least two wells.
4. **Sample Background Wells -** add 160 µl of Assay Buffer, 10 µl of Cofactor, 10 µl of Enzyme Mixture, and 10 µl of sample to at least two wells.

Well	Assay Buffer (µl)	Cofactor (µl)	Enzyme Mixture (µl)	Standard (µl)	Sample (µl)	Positive Control (µl)
Standard Wells	150	10	10	10	-	-
Positive Control Wells	150	10	10	-	-	10
Sample Wells	150	10	10	-	10	-
Sample Background Wells	160	10	10	-	10	-

5. Add 10 µl of Fluorometric Detector to all of the wells being used.
6. Initiate the reactions by adding 10 µl of G6PDH Substrate to all standard, Positive Control, and sample wells. DO NOT add to sample background wells.
7. Cover the plate with the plate cover and incubate the plate for 20 minutes at 37°C.
8. Remove the plate cover and read fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, Positive Control, sample, and sample background.
2. Subtract the fluorescence value of standard A from itself, all other standards, and the Positive Control. This is the corrected fluorescence (CF).
3. Plot the corrected fluorescence values (from step 2 above) of each standard as a function of the final concentration of NADPH from Table 1. See Figure 2, on page 18, for a typical standard curve.
4. Subtract the sample background fluorescence value from the sample value. This is the corrected sample fluorescence value (CSF).
5. Calculate the NADPH concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the corrected sample fluorescence value (CSF) for each sample.

$$\text{NADPH } (\mu M) = \left[\frac{\text{CSF - (y-intercept)}}{\text{Slope from NADPH curve (CF/}\mu M)}\right]$$

6. Calculate the G6PDH activity of the samples using the equation below. One unit is defined as the amount of enzyme that will catalyze the conversion of 1 nmol of glucose-6-phosphate into 6-phospho-D-gluconate and generates 1 nmol of NADPH per minute at 37°C.

$$\text{G6PDH (nmol/min/ml)} = \left[\frac{\text{NADPH } (\mu M)}{\text{20 minute}}\right] \text{ x } 2^{*} \text{ x Sample dilution}$$

*This is a dilution factor to correct for adding PBS to the erythrocytes. Do not use this dilution factor when determining cell lysate and tissue homogenate G6PDH values.

NOTE: G6PDH values from erythrocyte samples can be standardized using Cayman's Hemoglobin Colorimetric Assay Kit (Item No. 700540).

Performance Characteristics

Precision:

When a series of eight erythrocyte lysate mesurements were performed on the same day, the intra-assay coefficient of variation was 4.4%. When a series of eight erythrocyte lysate measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.3%.

Sensitivity:

Samples containing G6PDH activity between 5-50 nmol/min/ml can be assayed without further dilution or concentration.

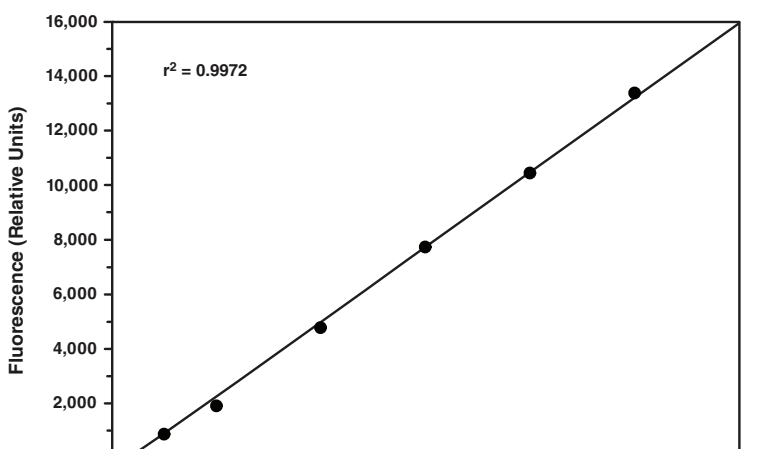

Figure 2. NADPH standard curve

RESOURCES

Interferences

The following reagents were tested in the assay for interference in the assay:

	Reagent	Will Interfere (Yes or No)
Buffers	Tris	No
	Borate	No
	HEPES	No
	Phosphate	No
	1X Phosphate Buffered Saline	No
Detergents	Polysorbate 20 (1%)	No
	Triton X-100 (1%)	No
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	BSA (0.1%)	No
Solvents	Ethanol (5%)	Yes
	Methanol (5%)	No
	Dimethylsulfoxide (5%)	Yes
Others	Glutathione (1 mM)	No
	Glycerol (10%)	No
	Dithiothreitol (1 mM)	No
	NaCl (150 mM)	No
	$CaCl_2$ (10 mM)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the sample wells	Sample was too dilute	Re-assay the sample using a lower dilution
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
The fluorescence of the sample wells were higher than the last standard	Sample was too concentrated	Re-assay the sample using a higher dilution

References

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2. Ursini, M.V., Parrella, A., Rosa, G., *et al.* Enhanced expression of glucose-6-phosphate dehydrogenase in human cells sustaining oxidative stress. *Biochem. J.* **323(3)**, 801-806 (1997).
3. Beutler, E. Glucose-6-phosphate dehydrogenase deficiency: A historical perspective. *Blood* **111(1)**, 16-24 (2008).
4. Cappellini, M.D. and Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. *Lancet* **371(9606)**, 64-74 (2008).
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6. Frank, J.E. Diagnosis and management of G6PD deficiency. *Am. Fam. Physician* **72(7)**, 1277-1282 (2005).
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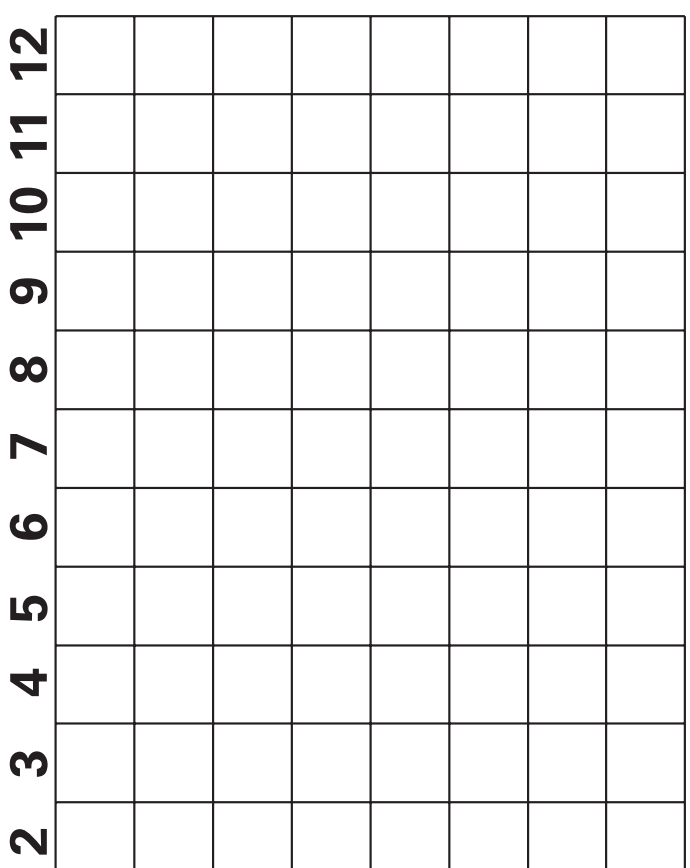
9. Jiang, P., Du, W., Wang, X., *et al.* p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. *Nat. Cell. Biol.* **13(3)**, 310-316 (2011).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.