Glucose-6-Phosphate Fluorometric Assay Kit

Item No. 700750

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, store the kit as supplied or remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
700751	Tris Assay Buffer 1	1 vial/5 ml	-20°C
700752	G6P Standard	2 vials/150 µg	-20°C
700753	G6PDH Assay Reagent	2 vials/100 µl	-20°C
700754	G6P Cofactor Mixture	2 vials/2 mg	-20°C
700755	G6P Fluorometric Detector	2 vials/30 µg	-20°C
700518	MPA Assay Reagent	1 vial/2 g	RT
700517	Potassium Carbonate Assay Reagent	1 vial/5 ml	-20°C
400017	96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

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.

It is recommended to take appropriate precautions when using the kit reagents (*i.e.*, lab coat, gloves, eye goggles, etc.) as some of them may be harmful. MPA (metaphosphoric acid) and potassium carbonate are corrosive and harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes.

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 plate reader 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

On entry into cells, glucose is converted by hexokinase (or glucokinase) into glucose-6-phosphate (G6P, D-glucose-6-phosphate, Robison ester). G6P has three principal intracellular fates.[1] It can: 1) enter glycolysis *via* phosphoglucose isomerase to provide cellular energy or carbon skeletons for biosynthesis; 2) be converted into glucose-1-phosphate by phosphoglucomutase, the first step in glycogen synthesis; 3) be metabolized by glucose-6-phosphate dehydrogenase (G6PDH) to NADPH, thereby entering the hexose monophosphate shunt to provide cells with reducing power and nucleic acid precursors.[2] 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, in which NADPH is used by glutathione reductase to maintain adequate GSH levels, is dependent on G6PDH. G6PDH deficiency becomes especially lethal in red blood cells, where any oxidative stress will result in hemolytic anemia.[4,5]

The major function of the liver is to maintain a near constant level of glucose in the blood. The liver contains the hydrolytic enzyme, glucose-6-phosphatase which cleaves the phosphoryl group from G6P to form free glucose and orthophosphate.[6] Glucose is then exported from the cell *via* glucose transporter membrane proteins.[6] This catalysis completes the final step in gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose levels. Glucose-6-phosphatase deficiency (glycogen storage disease type I or von Gierke's disease) is a group of inherited metabolic diseases, including types Ia and Ib, characterized by poor tolerance to fasting resulting in severe hypoglycemia, growth restardation, and hepatomegaly resulting from accumulation of glycogen and fat in the liver.[7]

About This Assay

Cayman's Glucose-6-Phosphate Fluorometric Assay provides a fluorescence-based method for detecting G6P in tissue homogenates and cell culture samples. In the assay, G6PDH catalyzes the oxidation of G6P 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. **Tris Assay Buffer 1 - (Item No. 700751)**

   The vial contains 5 ml of 500 mM Tris-HCl, pH 7.8, 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 7.8, containing 10 mM $MgCl_2$) is used in the assay. The diluted Assay Buffer is stable for three months at 4°C.

2. **G6P Standard - (Item No. 700752)**

   Each vial contains a lyophilized powder of glucose-6-phosphate. Reconstitute the contents of the vial with 1 ml of diluted Assay Buffer to yield a 500 µM stock. The 500 µM stock will be used to prepare the diluted standards (see page 13). The reconstituted mixture is stable for one week at -20°C.

3. **G6PDH Assay Reagent - (Item No. 700753)**

   Each vial contains 100 µl of glucose-6-phosphate dehydrogenase. Add 800 µl of diluted Assay Buffer to the vial, vortex, and put the vial on ice. This is enough enzyme to assay 85 wells. Prepare the additional vial as needed. The diluted enzyme is stable for four hours at 4°C.

4. **G6P Cofactor Mixture - (Item No. 700754)**

   Each vial contains a lyophilized powder of cofactors including $NADP^+$. Reconstitute the contents of the vial with 1.2 ml of diluted Assay Buffer and put the vial on ice. This is sufficient reagent to assay 60 wells. Prepare the additional vial as needed. The reconstituted cofactors are stable for four hours at 4°C.

5. **G6P Fluorometric Detector - (Item No. 700755)**

   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. **MPA Assay Reagent - (Item No. 700518)**

   The vial contains 2 g of metaphosphoric acid (MPA). To prepare 0.5 M MPA for deproteinating the samples, dissolve 1.6 g of MPA in 40 ml of HPLC-grade water. Store the diluted acid solution at room temperature. The diluted acid is stable for three months at room temperature.

7. **Potassium Carbonate Assay Reagent - (Item No. 700517)**

   The vial contains 5 ml of 5 M potassium carbonate. The reagent is ready to use as supplied.

Sample Preparation

Enzymes in the sample may consume G6P. We recommend deproteinating the sample upon collection and then storing at -80°C.

Cell Lysate

1. Collect cells (~10 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. Add 500 µl of diluted Assay Buffer to the cell pellet (or see **Interferences**, on page 19, for additional choices) and vortex.
3. To deproteinate, add 500 µl of 0.5 M MPA to the cells, vortex, and place on ice for five minutes.
4. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 10 µl of Potassium Carbonate to neutralize the acid.
5. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.
6. If not assaying the same day, freeze at -80°C. The deproteinated sample will be stable for one month while stored at -80°C.
7. Dilute the sample 1:2-1:4 with diluted Assay Buffer before assaying.

Tissue Homogenate

1. Prior to dissection, rinse tissue with a phosphate buffered saline (PBS) 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. To deproteinate, add 500 µl of 0.5 M MPA to 500 µl of tissue homogenate, vortex, and place on ice for five minutes.
4. Centrifuge at 10,000 x g for five minutes at 4°C to pellet the proteins. Remove the supernatant and add 10 µl of Potassium Carbonate to neutralize the acid.
5. Centrifuge at 10,000 x g for five minutes at 4°C to remove any additional debris. Remove the supernatant for assaying.
6. If not assaying the same day, freeze at -80°C. The deproteinated sample will be stable for one month while stored at -80°C.
7. Dilute the sample 1:5-1:10 with diluted Assay Buffer before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a G6P standard curve in duplicate has to be assayed with the samples. We suggest that each sample be assayed at least in duplicate in the presence and absence of G6PDH Assay Reagent. 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	B19
G	G	G	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20
H	H	H	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-H = Standards
S1-S20 = Sample Wells
B1-B20 = Sample Background 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 and cofactors 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 eight clean glass test tubes or polystyrene tubes and mark them A-H. Add the amount of G6P (500 µM) and diluted Assay Buffer to each tube as described in Table 1. The diluted Standards are stable for four hours at room temperature.

Tube	G6P (µl)	Assay Buffer (µl)	Final Concentration (µM)
A	0	500	0
B	5	495	5
C	10	490	10
D	25	475	25
E	50	450	50
F	100	400	100
G	150	350	150
H	200	300	200

Table 1. Preparation of G6P standards

Performing the Assay

1. **Standard Wells** - add 150 µl of Assay Buffer, 20 µl of Cofactor Mixture, and 10 µl of Standard (tubes A-H) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 1, page 11).
2. **Sample Wells** - add 150 µl of Assay Buffer, 20 µl of Cofactor Mixture, and 10 µl of sample to at least two wells.
3. **Sample Background Wells** - add 160 µl of Assay Buffer, 20 µl of Cofactor Mixture, and 10 µl of sample to at least two wells.
4. Add 10 µl of Fluorometric Detector to all of the wells being used.
5. Initiate the reactions by adding 10 µl of G6PDH Assay Reagent to all standard and sample wells. DO NOT add to sample background wells.
6. Cover the plate with the plate cover and incubate the plate for 15 minutes at 37°C.
7. Remove the plate cover and read fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

	Standard Wells (µl)	Sample Wells (µl)	Sample Background Wells (µl)
Assay Buffer	150	150	160
Cofactor Mixture	20	20	20
Standard	10	-	-
Sample	-	10	10
Fluorometric Detector	10	10	10
Initiate reactions			
G6PDH	10	10	-

Table 2. Pipetting summary

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, sample, and sample background.
2. Subtract the fluorescence value of standard A from itself and all other standards. 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 G6P from Table 1. See Figure 2, on page 16, 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 concentration of G6P in the samples using the equation below.

$$\text{G6P (µM)} = \left[\frac{\text{CSF - (y-intercept)}}{\text{Slope}}\right] \times 2^{*} \times \text{Sample dilution}$$

*This is a dilution factor to correct for diluting the samples during the deproteinating step.

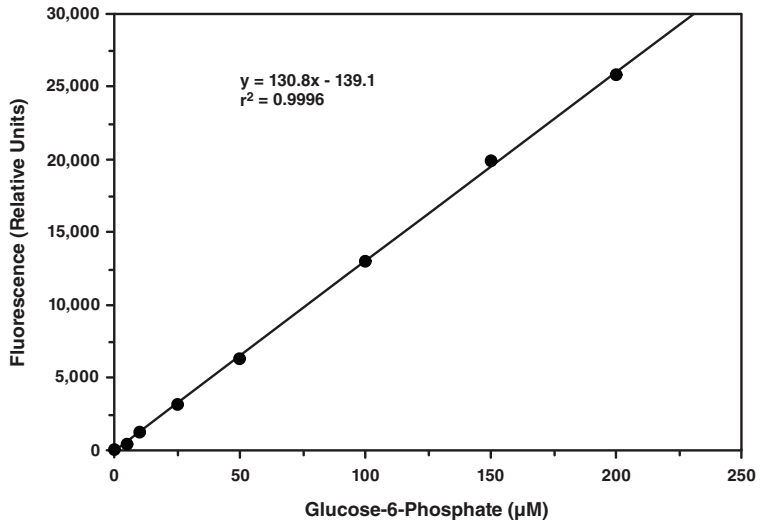


Figure 2. G6P standard curve

Performance Characteristics

Precision:

When a series of 16 deproteinated mouse liver homogenate measurements were performed on the same day, the intra-assay coefficient of variation was 2.5%. When a series of 16 deproteinated mouse liver homogenate measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.0%.

Sensitivity:

The limit of detection for the assay is 5 µM (±1 µM) G6P.

Assay Specificity:

To assess substrate specificity, the assay was performed with G6P replaced by structurally similar compounds such as glucose-1-phosphate (G1P), fructose-6-phosphate (F6P), ribose-5-phosphate (R5P), and glucose. G1P, R5P, and glucose were not utilized by G6PDH in this assay, whereas F6P had 2.4% conversion relative to G6P (see Figure 3).

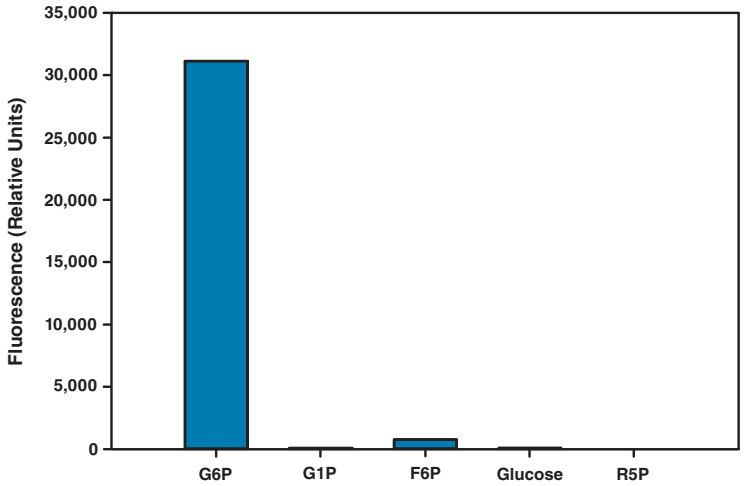


Figure 3. Assay specificity

Assay Recovery:

Deproteinated mouse liver homogenate was spiked with various concentrations of G6P. G6P was then determined for each spiked sample. The data in Figure 4 represents the amount of G6P added to the sample *versus* the measured amount of G6P. Based on the slope of the best fit line, the assay gives 99% recovery in this experiment.

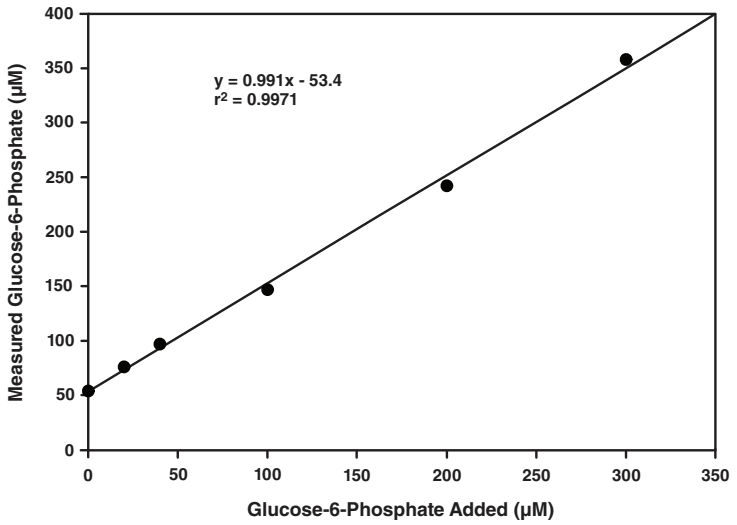


Figure 4. Assay recovery from mouse liver homogenate

Interferences

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

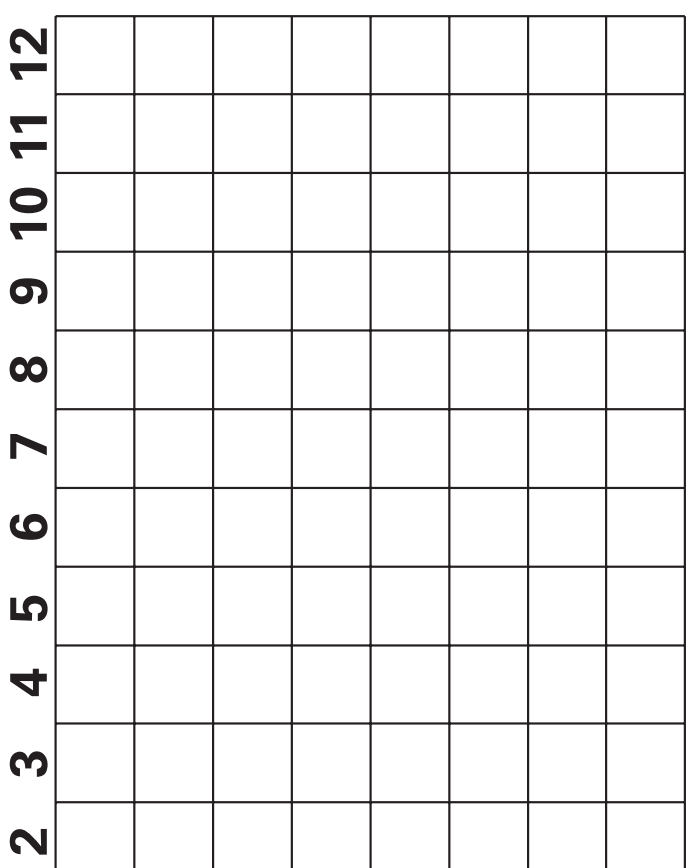
Reagent		Will Interfere (Yes or No)
Buffers	Tris	No
	Borate	No
	Phosphate	No
	1X Phosphate Buffered Saline	No
Detergents	Polysorbate 20 (0.1%)	Yes (9%)
	Triton X-100 (1%)	No
Protease Inhibitors/ Chelators/Enzymes	EDTA (1 mM)	No
	EGTA (1 mM)	No
	Trypsin (10 µg/ml)	No
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	BSA (0.1%)	No
Solvents	Ethanol (5%)	No
	Methanol (5%)	Yes (9%)
	Dimethylsulfoxide (5%)	Yes (16%)
Others	Dithiotreitol (1 mM)	Yes (16%)
	Fructose-6-Phosphate (1 mM)	Yes (19%)
	Glucose (1 mM)	No
	Glucose-1-Phosphate (1 mM)	No
	Glycerol (10%)	No
	NaCl (100 mM)	No
	Ribose-5-Phosphate (1 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

1. J.M. Berg, J.L. Tymoczko, L. Stryer. *in* Biochemistry. 5th Edition. W. H. Freeman. New York. (2002).
2. Gumaa, K.A. and McLean, P. The pentose phosphate pathway of glucose metabolism: Enzyme profiles and transient and steady-state content of intermediates of alternative pathways of glucose metabolism in Krebs ascites cells. *Biochem. J.* **115(5)**, 1009-1029 (1969).
3. 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).
4. Beutler, E. Glucose-6-phosphate dehydrogenase deficiency: A historical perspective. *Blood* **111(1)**, 16-24 (2008).
5. Cappellini, M.D. and Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. *Lancet* **371(9606)**, 64-74 (2008).
6. Foster, J.D. and Nordlie, R.C. The biochemistry and molecular biology of the glucose-6-phosphatase system. *Exp. Biol. Med.* **227**, 601-608 (2002)
7. Froissart, R., Piraud, M., Boudjemline, A.M., *et al.* Glucose-6-phosphatase deficiency. *Orphanet J. Rare Dis.* **6(27)**, 1-12 (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.