Published in final edited form as:

Anal Biochem. 2011 December 15; 419(2): 266–270. doi:10.1016/j.ab.2011.08.037.

An enzymatic colorimetric assay for glucose-6-phosphate

Aiping Zhu^a, Roberto Romero^{b,c}, and Howard R. Petty^{a,d}

^aDepartment of Ophthalmology and Visual Sciences, The University of Michigan Medical School, Ann Arbor, MI 48105

^bPerinatology Research Branch, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) of NIH, Bethesda, Maryland and Detroit, Michigan 48201

^cCenter of Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan; Hutzel Women's Hospital at the Detroit Medical Center, Detroit, Michigan, 3990 John R. Rd., 4 Brush South, Detroit, MI 48201

^dDepartment of Microbiology and Immunology, The University of Michigan Medical School, Ann Arbor, MI 48105

Abstract

A specific colorimetric assay for the determination of glucose-6-phosphate (G6P) was developed. This assay is based on the oxidation of G6P in the presence of glucose-6-phosphate dehydrogenase (G6PD) and NADP⁺; the NADPH thereby generated reduces the tetrazolium salt WST-1 (2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) to water-soluble yellow-colored formazan with 1-methoxy-5-methylphenazium methylsulfate (1-mPMS) as an electron carrier. The assay is optimized for reaction buffer pH, enzyme/dye concentration, and reaction time course. The limit of detection of the assay is 0.15 μ M (15 pmol/well). The usefulness of the assay is demonstrated by the accurate measurement of the G6P concentration in fetal bovine serum (FBS).

Keywords

WST-1; glucose 6-phosphate; colorimetric assay; glucose-6-phosphate dehydrogenase

1. Introduction

Hexokinase (EC 2.7.1.1) catalyzes the formation of glucose 6-phosphate (G6P) from glucose. G6P is the first substrate in glycolysis, the hexose monophosphate shunt, and in both the glycogen and starch biosynthesis pathways[1]. G6P concentrations may reflect ongoing biological processes such as catabolite de-repression, metabolic oscillations, and metabolic regulation in diabetes, to name a just few [2; 3; 4].

© 2011 Elsevier Inc. All rights reserved.

Address correspondence to: Dr. Howard Petty Department of Ophthalmology and Visual Sciences 1000 Wall Street The University of Michigan Medical School Ann Arbor, MI 48105 Phone: 734-647-0384/fax:734-936-3815/ hpetty@umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Several methods have been developed for the quantitative analysis of G6P concentrations in cells and tissues. These methods include chromatographic approaches [5; 6; 7; 8; 9], enzyme-based amperometric biosensors [10; 11; 12] and NADP⁺ coupled enzymatic assays [13; 14; 15]. As G6P has no chromophore, chromatographic methods require an expensive apparatus to separate and detect G6P. On the other hand, enzymatic methods are specific, rapid and convenient to perform. In these methods, G6P is oxidized to gluconolactone 6-phosphate by G6P dehydrogenase (G6PD) while NADP⁺ is reduced to NADPH. Hence, G6P can be determined by measuring NADPH absorbance at 339 nm or fluorescence at 470 nm [13; 14; 15]. However, due to its low extinction coefficient and weak fluorescence, the sensitivity of NADPH-coupled tools is not high [13]. Amperometric methods are based on enzyme reactions with electronic signal detection. As enzyme immobilization on the electrodes is required, long-term storage of functional enzyme-labeled electrodes is difficult.

Recently, we reported a sensitive enzymatic fluorimetric assay for the determination of intracellular G6P based upon the stoichiometric reduction of weakly-fluorescent resazurin to highly fluorescent resorufin [16]. We have found this fluorimetric assay to be particularly useful in the analysis of intracellular metabolites [17]. However, under certain conditions, a colorimetric methodology may be preferred. For example, colorimetric assays are not sensitive to autofluorescence background. Fluorescence methods are also more sensitive to impurities. We have found fluorimetric measurements are not sufficiently reliable to quantify metabolite levels in human serum. Although colorimetric assays typically exhibit moderate sensitivities, recently developed tools have improved the sensitivities of these assays. A new generation of sulfonated tetrazolium salts (WST-1 and WST-8) have been used in clinical and cellular assays [18; 19; 20; 21]. Ishiyama et al. [19] demonstrated that NADH reduced WST-1 to WST-1 formazan at pH>8.0. The absorbance change of WST-1 formazan is much more sensitive than the spectrophotometric measurement of NADH (or NADPH) at 339 nm.

In this paper, we report a WST-1 coupled enzymatic assay for measurement of G6P in a 96-well microplate using redox amplification to increase sensitivity [22]. The mechanism of the assay is shown in Scheme 1. G6P is converted to gluconate-6-phosphate by G6PD; the simultaneously generated NADPH participates in a cycling reaction that employs tetrazolium salt WST-1 to produce water-soluble yellow-colored formazan with 1-methoxy-5-methylphenazinium methylsulfate (1-mPMS) as an electron carrier. The concentration of WST-1 formazan is measured by the change in absorbance at 440 nm, which is proportional to the G6P concentration. Colorimetric read-out is convenient, and the dynamic range is much higher than the fluorimetric method [16]. We also tested the assay's performance by measuring G6P concentrations in fetal bovine serum samples.

2. Experimental

2.1 Materials

WST-1 was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). G6P, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PD), 1-methoxy-5-methylphenazinium (1-mPMS), and nicotinamide adenine dinucleotide phosphate (NADP⁺) were obtained from Sigma-Aldrich Co. (St. Louis, MO).

2.2 Preparation of FBS samples

Heat-inactivated FBS samples were deproteinized by addition of an equal volume of ice-cold 0.5 M HClO₄. After incubation for 5 min on ice, the mixture was centrifuged at 10000g for 5 min to remove proteins and then the supernatant was collected. Two hundred µl of

supernatant was neutralized with 10 μ l of 2.5 M K_2CO_3 at 4°C. Using an Integrated SpeedVac System (model ISS110; Thermo Savant, Holbrook, NY), samples were simultaneously degassed and centrifuged while set to a low drying rate for 5 min. The clear supernatant was pooled for the G6P assay, as described below.

2.3 Microplate assay procedure

A total assay volume of 100 μ l was used for each reaction. The assay solution was freshly prepared by mixing the following: 250 μ l of 20 mM $MgCl_2$, 250 μ l of 0.5 mM $NADP^+$, 250 μ l of 10 mM WST-1, 250 μ l of 1-mPMS, 250 μ l of 4U/ml G6PD, and 2.25 mL of 50 mM Tris buffer (pH 8.5). A total assay solution volume of 3.5 ml is sufficient for ~50 assays.

The assay procedure was as follows: a 30 μ l volume of serially diluted G6P standards were pipetted into individual wells of a 96-well plate (Costar 3626, Corning, Corning, NY) followed by the addition of 70 μ l of the assay solution. These mixtures were incubated for 30 minutes at room temperature in the dark. The absorbance at 440 nm was measured with a plate reader (Molecular Devices, Sunnyvale, CA). Background absorbance was corrected by subtracting the value of the no G6P control from all sample readings. For FBS samples, we used 0.2 M Tris buffer (pH 8.5) for the assay to stabilize the reaction pH. G6P standards were treated in the same way as FBS to prepare the calibration curves.

2.4 Statistical analyses

Unless otherwise noted, all experiments were performed in triplicate and the results are presented as mean $\pm$ standard deviation.

The repeatability (within-run) and intermediate precision of the assay were determined by the percent coefficient of variation (%C.V.) as shown in equation (1) and (2) [23]:

$$\text{Repeatability (within-run) (\%C.V.)} = 100 \times s_w / \text{mean} = 100 \times (MS_w)^{0.5} / \text{mean} \quad (\text{Eq. 1})$$

$$\text{Intermediate precision (\%C.V.)} = 100 \times (s_w^2 + s_b^2)^{0.5} / \text{mean} \quad (\text{Eq. 2})$$

Where $s_b^2 = (MS_b - MS_w)/n$, MS_b and MS_w are within-run and between-run ANOVA mean square errors; s_w and s_b are within-run and between-run variance components, respectively. The mean square variances were calculated with Excel analysis ToolPak.

3. Results and discussion

3.1 Optimization of the assay

The G6P assay is expected to exhibit sensitivity to the concentrations of WST-1, the electron mediator, $NADP^+$, and G6PD, as well as the reaction pH and time. Therefore, experiments were conducted to optimize the assay conditions. We performed the G6P assay with G6P standards at final concentrations of 10 μ M and 100 μ M. As our results at these two concentrations gave identical trends, we only show typical curves of G6P at 10 μ M in Fig. 1. The buffer pH is one of the most important parameters of the assay. Color change is a general reaction characteristic of sulfonated tetrazolium salts such as WST-1. The sensitivity of WST-1 is increased at high pH (pH>8.0). The recommended pH range for G6PD is 7.0-8.5 with maximal activity at 7.8. Therefore, the influence of pH on the G6P assay was examined in the pH range from 7.0 to 8.5 at fixed chemical concentrations and reaction time. As shown in Fig. 1A, the optimum pH range for the assay is 7.8-8.5. The maximal

response was observed at pH 8.5. Unless mentioned otherwise, we performed G6P assays in Tris buffer at pH 8.5. The assay was also tested in TEA buffer at pH 8.5; the results were indistinguishable using these two buffers (data not shown).

Previous studies have documented the moderate extinction coefficient of WST-1 formazan (3.7×10^4 , pH 8.0 at 433 nm) [19] and that the yield of formazan is ~80% at pH 8.5. Hence, high concentrations of WST-1 are required to obtain optimal sensitivity. As shown in Fig. 1B, the absorbance increases with WST-1 concentration, until the concentration of WST-1 reaches 0.5 mM. We employed 0.5 mM WST-1 for the assay, since at that concentration the assay is both economical and highly sensitive. It is worth mentioning that WST-8 (another highly water soluble disulfonated tetrazolium) can replace WST-1 in the assay. WST-8 is less pH dependent and more sensitive to NADH than WST-1 [19]. Unfortunately, WST-8 is only available in kit form from Dojindo Molecular Technologies.

The measured absorbance was not influenced by the concentration of the electron mediator 1-mPMS in the range of 2.5 to 25 μ M (Fig. 1C). A final concentration of 10 μ M of 1-mPMS provided satisfactory signals (Fig. 1C). It should be pointed out that diaphorase (0.2 U/ml) can replace 1-mPMS as an electron carrier in the assay. 1-mPMS shows red color at concentrations as low as 10 μ M, while diaphorase is a colorless enzyme, suggesting that the latter has lower background absorbance. However, the signal to noise ratios are similar in both assay configurations.

Unlike previous assays requiring the use of stoichiometric quantities of NADP^+ (up to 200 μ M) [14], the assay reported herein employs a recycling reaction wherein NADPH formed in one step is recycled back to NADP^+ during the formation of WST-1 formazan (Scheme 1), which then participates in another round oxidation-reduction reactions. Thus, one molecule of G6PD and NADP^+ are coupled to produce many molecules of WST-1 formazan. We did not observe significant differences in absorbance during our standard conditions using NADP^+ concentrations from 5 to 150 μ M in test wells (Fig. 1D). Hence, we performed the assays with NADP^+ at 25 μ M.

G6PD is another important assay component. In a previous paper [16], we demonstrated that the G6PD from *Leuconostoc mesenteroides* expressed more activity than that isolated from baker's yeast. Taking this fact and sample handling and the storage stability into consideration, we used G6PD from *Leuconostoc mesenteroides* (Sigma Cat #: G8404) throughout the assays. The measured absorbance is not sensitive to G6PD concentration when G6PD level is greater than 0.05 U/ml (Fig. 1E). We adopted a concentration of 0.2 U/ml G6PD to ensure good sensitivity and a robust upper limit of quantitation (ULOQ).

Using the standardized conditions, sample absorbance was also measured as a function of time. As shown in Fig. 1F, the absorbance is independent of time at times of 15-60 min. Unless otherwise noted, reactions were incubated for 30 min at room temperature in all assays.

3.2 Validation of the assay

Under the optimized conditions described above, we evaluated the selectivity of the assay by measuring reaction product in the presence of the control substrate G6P at a final concentration of 100 μ M and that of other sugar phosphates (fructose-6-phosphate, glucose-1-phosphate, ribulose-5P, and ribose-5P at 100 μ M) as well as that of glucose at a high concentration (100 mM). These experimental compounds were chosen because they are the most likely components of samples to interfere with the assay. Typical results are shown in Fig. 2. As seen in Fig. 2A, the assay is highly specific for G6P. Other sugar phosphates do not respond, except ribulose-5P, whose interference is minimal (Fig. 2A).

As shown in Fig. 2B, the plot of absorbance versus G6P concentration shows a linear response from 0–300 μM . However, at G6P concentrations higher than 200 μM , the plate reader becomes a limiting factor (maximum reliable absorbance unit of 2, which corresponds to 200 μM in the G6P assay). From 1.0 to 200 μM , the data points can be fitted to a line with a slope of $0.0107 (\mu\text{M})^{-1}$ and a correlation coefficient of 0.998 (Fig. 2C). Therefore, we operationally set the upper limit of quantitation (ULOQ) of the assay at 200 μM (20 nmol/well).

We have calculated several performance criteria of the assay. The Z' factor is a widely accepted criteria used to assess the quality of a bioassay. The means and standard deviations were measured from 12 separate wells containing buffer alone and, separately, 12 wells containing 200 μM G6P. The Z' factor for the assay was calculated as 0.94 by using the equation introduced by Zhang and coworkers [24]. This indicates that this is an excellent assay for the measurement of G6P. The limit of detection (LOD) and lower limit of quantitation (LLOQ) were calculated as: three times and ten times of the standard deviation of the reagent blank samples [22]. The LOD and LLOQ of this assay were calculated to be 0.15 μM (15 pmol/well) and 0.5 μM (50 pmol/well) ($n=12$), respectively.

Each set of quality control samples (0.5 μM , 1.0 μM , 100 μM , and 200 μM) was tested on six sequential days with four replicates per sample. Data were examined by a one-way analysis of variance (ANOVA) to calculate the repeatability and intermediate precision and accuracy of the assay [23]. The accuracy of assay was expressed as the percent relative error (% RE). $\% \text{RE} = 100\% \times (\text{mean value} - \text{nominal value}) / \text{nominal value}$. The statistical findings are summarized in Table 1. At the LLOQ concentration (0.5 μM), both within-run and between-run precisions are smaller than the statistical acceptance criteria (20%).

3.3 Application of the assay

We applied this assay in the measurement of G6P concentrations in fetal bovine serum (FBS) samples. We used 0.2 M Tris buffer (pH 8.5) instead of 50 mM buffer to ensure pH stability for these measurements. The calibration curve in FBS (Fig. 3) was determined by treating G6P standard solutions with the same procedure as FBS samples. It should be noted that the concentrations of G6P in Fig. 3 are original concentrations of standard G6P solutions not final concentrations of each well as shown in Fig. 2.

3.3.1 Spike-recovery studies—To estimate the influence of sample preparation on G6P measurements in FBS samples, we spiked known amounts of G6P (0, 15 μM , 30 μM and 60 μM) into the deproteinized FBS. The calculated recovery values are shown in Table 2. The results in Table 2 indicate that G6P was satisfactorily recovered in FBS samples.

3.3.2 FBS sample measurement—To further test the precision of the assay, we measured G6P concentrations in FBS samples over four sequential days using six replicates for each sample. The results and statistics are shown in Table 3. We freshly prepared FBS samples each day, while using the same assay reagents (stored at 4°C) to test their stability. The day-to-day results did not show measureable differences. Hence, the reagents used in the assay are stable and the within-run and between-run precisions of the assay are excellent. Furthermore, the G6P concentration in heat-inactivated FBS is $6.2 \pm 0.5 \mu\text{M}$, which is very close the values that we measured with a conventional enzymatic assay ($5.9 \pm 0.3 \mu\text{M}$) [14].

4. Conclusions

A practical and specific colorimetric assay for measuring G6P concentrations in biological samples was developed. This assay used G6PD coupled to a cyclic NADP⁺-NADPH reaction and the tetrazolium WST-1. In comparison with other reported methods for G6P

quantitation (Table 4), the new assay is of very high sensitivity and has a very large linear range. Another advantage of the assay was that the color changes are easily observed by the naked eye. The assay is compatible with high throughput approaches and will be useful in studies of small clinical samples. Finally, the G6P assay's sensitivity is compatible with numerous biological settings including blood, cells, tissues and certain disease states [e.g., 25, 26].

Acknowledgments

This work was supported, in part, by the Division of Intramural Research of the Eunice Kennedy Schriver National Institute of Child Health and Human Development, NIH, DHHS, contract number N01-HD-2-3342 and subcontract WSU04055.

References

- [1]. Berg, JM.; Tymoczko, JL.; Stryer, L. Biochemistry. W.H. Freeman; New York: 2007.
- [2]. Aiston S, Andersen B, Agius L. Glucose 6-phosphate regulates hepatic glycogenolysis through inactivation of phosphorylase. *Diabetes*. 2003; 52:1333–1339. [PubMed: 12765941]
- [3]. Nilsson T, Schultz V, Berggren PO, Corkey BE, Tornheim K. Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca^{2+} in glucose-stimulated pancreatic beta-cells. *Biochem J*. 1996; 314:91–94. [PubMed: 8660314]
- [4]. Quain DE, Haslam JM. Changes in glucose-6-phosphate and storage carbohydrates during catabolite derepression in *Saccharomyces cerevisiae*. *J Gen Microbiol*. 1979; 113:195–198.
- [5]. Buchholz A, Takors R, Wandrey C. Quantification of intracellular metabolites in *Escherichia coli* K12 using liquid chromatographic-electrospray ionization tandem mass spectrometric techniques. *Anal Biochem*. 2001; 295:129–137. [PubMed: 11488613]
- [6]. van Dam JC, Eman MR, Frank J, Lange HC, van Dedem GWK, Heijnen SJ. Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection. *Anal Chim Acta*. 2002; 460:209–218.
- [7]. Sekiguchi Y, Mitsuhashi N, Kokaji T, Miyakoda H, Mimura T. Development of a comprehensive analytical method for phosphate metabolites in plants by ion chromatography coupled with tandem mass spectrometry. *J Chromatogr A*. 2005; 1085:131–136. [PubMed: 16106859]
- [8]. Antonio C, Larson T, Gilday A, Graham I, Bergstrom E, Thomas-Oates J. Quantification of sugars and sugar phosphates in *Arabidopsis thaliana* tissues using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr A*. 2007; 1172:170–8. [PubMed: 17961583]
- [9]. Antonio C, Larson T, Gilday A, Graham I, Bergstrom E, Thomas-Oates J. Hydrophilic interaction chromatography/electrospray mass spectrometry analysis of carbohydrate-related metabolites from *Arabidopsis thaliana* leaf tissue. *Rapid Commun Mass Spectrom*. 2008; 22:1399–407. [PubMed: 18384194]
- [10]. Cui Y, Barford JP, Renneberg R. Development of a glucose-6-phosphate biosensor based on coimmobilized p-hydroxybenzoate hydroxylase and glucose-6-phosphate dehydrogenase. *Biosens Bioelectron*. 2007; 22:2754–8. [PubMed: 17137780]
- [11]. Tzang, C. Hung; Yuan, R.; Yang, M. Voltammetric biosensors for the determination of formate and glucose-6-phosphate based on the measurement of dehydrogenase-generated NADH and NADPH. *Biosens Bioelectron*. 2001; 16:211–9. [PubMed: 11340000]
- [12]. Bassi AS, Tang D, Bergougnou MA. Mediated, amperometric biosensor for glucose-6-phosphate monitoring based on entrapped glucose-6-phosphate dehydrogenase, Mg^{2+} ions, tetracyanoquinodimethane, and nicotinamide adenine dinucleotide phosphate in carbon paste. *Anal Biochem*. 1999; 268:223–8. [PubMed: 10075811]
- [13]. Kirkman HN, Gaetani GF. Regulation of glucose-6-phosphate-dehydrogenase in human-erythrocytes. *J Biol Chem*. 1986; 261:4033–4038. [PubMed: 3081513]

- [14]. Bergmeyer, HU.; Bergmeyer, J.; Grassl, M. *Methods of Enzymatic Analysis*. Verlag Chemie; Weinheim: Deerfield Beach, Fla.: 1983.
- [15]. Gibon Y, Vigeolas H, Tiessen A, Geigenberger P, Stitt M. Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. *Plant J*. 2002; 30:221–235. [PubMed: 12000458]
- [16]. Zhu A, Romero R, Petty HR. An enzymatic fluorimetric assay for glucose-6-phosphate: application in an in vitro Warburg-like effect. *Anal Biochem*. 2009; 388:97–101. [PubMed: 19454216]
- [17]. Xu Y, Madsen-Bouterse SA, Romero R, Hassan S, Mittal P, Elflin M, Zhu A, Petty HR. Leukocyte pyruvate kinase expression is reduced in normal human pregnancy but not in pre-eclampsia. *Am J Reprod Immunol*. 2010; 64:137–51. [PubMed: 20560913]
- [18]. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He PG. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. *Chem PharmBull*. 1993; 41:1118–1122.
- [19]. Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta*. 1997; 44:1299–1305. [PubMed: 18966866]
- [20]. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev*. 2005; 11:127–52. [PubMed: 16216776]
- [21]. Tsukatani T, Suenaga H, Higuchi T, Akao T, Ishiyama M, Ezoe K, Matsumoto K. Colorimetric cell proliferation assay for microorganisms in microtiter plate using water-soluble tetrazolium salts. *J Microbiol Methods*. 2008; 75:109–116. [PubMed: 18586343]
- [22]. Self CH. Enzyme amplification--a general method applied to provide an immunoassisted assay for placental alkaline phosphatase. *J Immunol Methods*. 1985; 76:389–93. [PubMed: 3973395]
- [23]. Karnes HT, Shiu G, Shah VP. Validation of bioanalytical methods. *Pharm Res*. 1991; 8:421–6. [PubMed: 1871036]
- [24]. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen*. 1999; 4:67–73. [PubMed: 10838414]
- [25]. Fujii S, Beutler E. High glucose concentrations partially release hexokinase from inhibition by glucose 6-phosphate. *Proc Natl Acad Sci USA*. 1985; 82:1552–4. [PubMed: 3856279]
- [26]. Heath H, Kang SS, Philippou D. Glucose, glucose-6-phosphate, lactate and pyruvate content of the retina, blood and liver of streptozotocin-diabetic rats fed sucrose- or starch-rich diets. *Diabetologia*. 1975; 11:57–62. [PubMed: 124673]

Fig. 1. Optimization of G6P assay. The G6P concentration was 10 μ M. A: pH effect on the reaction; B: dye effect on the assay; C: Electron carrier effect on the assay; D: NADP⁺ effect on the assay; E: effect of G6PD concentration on absorbance; F: reaction time course of the assay. Unless otherwise noted, each point in the figure is a mean of three replicates; error bars represent the standard deviation. In some panels, error bars are invisible because of the symbols are larger than the error bars.

Fig. 2. Validation of the assay. A: The G6P assay was tested using a panel of compounds (G6P, F6P, G1P, ribulose-5P and ribose-5P at 100 μ M and glucose at 100 mM); B: the relationship of absorbance with G6P concentration; C: linear range of the assay (from 1.0 to 200 μ M).

Fig. 3.

A typical calibration curve for G6P measurement in FBS samples. Each datum point is a mean of triplicate measurements). $y = 0.001169 x$, $n = 8$.

Scheme1.

Mechanism of the chemical reactions for detection of G6P.

Table 1

The precision and accuracy of the assay for measurement of G6P (six experiments with six replicates per experiment).

prepared concentration	measured concentration	Accuracy	Precision (%C.V.)	
(μ M)	(μ M)	(% RE)	within-run	between-run
0.5	0.53	6.1	13.2	15.9
1.0	0.94	−6.0	8.2	9.5
100	96	−4.5	1.5	1.6
200	206	2.7	1.6	2.1

Table 2

Spike-recovery of G6P in FBS.

Spiked G6P * (μM)	Found (μM)						Expected G6P** (μM)	Recovery (%)
	1st	2nd	3rd	4th	5th	6th		
0	3.2	3.2	3.1	3.3	3.1	3.3		
15	16.2	16.5	16.5	18.4	16.4	16.2	18.2	91.8 ± 4.7
30	29.6	28.5	30.4	32.2	30.6	30.6	33.2	91.4 ± 3.7
60	57.0	56.8	58.3	62.6	58.0	56.7	63.2	92.2 ± 3.6

* 10 μl of unknown amounts of G6P standard solution and 490 μl of FBS (deproteinized with same volume of 0.5 M HClO₄) and neutralized with 25 μl of 2.5 M K₂CO₃.

** Expected value = spiked concentration + average G6P concentration of 6 runs the calculated average of G6P concentration in the deproteinized FBS is 3.2 μM .

Table 3

Results for heat-inactivated FBS sample measurements and statistics.

Batch Run	Replicate results						Ancillary Statistics			
	1st	2nd	3rd	4th	5th	6th	Mean	SD	%C.V.	
1	6.8	6.3	7.2	6.7	6.0	5.8	6.5	0.5	7.2	$MS_w = 0.1435$
2	5.7	6.3	6.2	6.4	5.7	6.3	6.1	0.3	5.5	$MS_b = 0.8544$
3	5.3	5.6	6.0	5.6	5.7	5.9	5.7	0.3	4.4	$s_w^2 = 0.1435$
4	6.9	6.3	6.7	6.0	6.2	6.8	6.5	0.4	5.7	$s_b^2 = 0.1422$
Intra-assay (within-run) statistics							6.2	0.4	6.1	
Inter-assay (between-run) statistics							6.2	0.5	8.6	

Table 4

Comparison of the proposed assay with some other reported methods for G6P detection.

Method	Linear range (μM)	%C.V.	LOD (μM)	Ref.
Amperometric	2.0 -1000	4.6	1.2	10
Spectrophotometric	3.4-180	1.8	3.0	14
Fluorimetric	0.3-10	3.3	0.1	16
LC-MS	5.0 -100	–	1.5	8
WST-1 + enzymatic	0.5-200	6.1	0.15	This paper