Molecular Genetics and Metabolism 119 (2016) 168–173

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Regular Article

# Rapid detection of *G6PD* mutations by multicolor melting curve analysis

Zhongmin Xia [a,b], Ping Chen [c], Ning Tang [d], Tizhen Yan [d], Yuqiu Zhou [e], Qizhi Xiao [e], Qiuying Huang [a,*], Qingge Li [a,*]

[a] State Key Laboratory of Cellular Stress Biology, State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, Engineering Research Centre of Molecular Diagnostics, Ministry of Education, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China
[b] United Diagnostic and Research Center for Clinical Genetics, School of Public Health of Xiamen University & Xiamen Maternal and Child Health Hospital, Xiamen, Fujian 361102, China
[c] Hemoglobin Laboratory, the First Affiliated Hospital of Guangxi Medical University, Guangxi Key Laboratory of Thalassemia Research, Guangxi Zhuang Autonomous Region, Nanning 530021,China
[d] Department of Clinical Laboratory, Liuzhou Key Laboratory of Birth Defects Prevention and Control, Liuzhou Maternal and Child Health Hospital, Liuzhou, Guangxi 545001, China
[e] Zhuhai Institute of Medical Genetics & Department of Clinical Laboratory, Zhuhai Municipal Maternity and Child Healthcare Hospital, Zhuhai, Guangdong 519001, China

ARTICLE INFO

*Article history:*
Received 18 July 2016
Accepted 18 July 2016
Available online 20 July 2016

*Keywords:*
Glucose-6-phosphate dehydrogenase deficiency
Multicolor melting curve analysis
Newborn screening
Real-time PCR

ABSTRACT

The MeltPro G6PD assay is the first commercial genetic test for glucose-6-phosphate dehydrogenase (G6PD) deficiency. This multicolor melting curve analysis-based real-time PCR assay is designed to genotype 16 *G6PD* mutations prevalent in the Chinese population. We comprehensively evaluated both the analytical and clinical performances of this assay. All 16 mutations were accurately genotyped, and the standard deviation of the measured $T_m$ was <0.3 °C. The limit of detection was 1.0 ng/μL human genomic DNA. The assay could be run on four mainstream models of real-time PCR machines. The shortest running time (150 min) was obtained with LightCycler 480 II. A clinical study using 763 samples collected from three hospitals indicated that, of 433 samples with reduced G6PD activity, the MeltPro assay identified 423 samples as mutant, yielding a clinical sensitivity of 97.7% (423/433). Of the 117 male samples with normal G6PD activity, the MeltPro assay confirmed that 116 samples were wild type, yielding a clinical specificity of 99.1% (116/117). Moreover, the MeltPro assay demonstrated 100% concordance with DNA sequencing for all targeted mutations. We concluded that the MeltPro G6PD assay is useful as a diagnostic or screening tool for G6PD deficiency in clinical settings.



## 1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked hereditary defect caused by mutations in the *G6PD* gene. G6PD deficiency is one of the most prevalent human enzymopathies affecting over 400 million individuals worldwide, and particularly in those undeveloped and resource-limited countries. The clinical phenotype of G6PD deficiency varies significantly from asymptomatic to neonatal jaundice, kernicterus, or acute hemolytic anemia following the ingestion of certain drugs during some infections, and notably through eating fava beans (favism). This variability in clinical phenotypes has been attributed to diverse mutant types in the *G6PD* gene [1–3]. To date, >180 mutations have been reported worldwide, and each ethnic population presents a characteristic mutation spectrum [4]. For example, in the Chinese population, at least 21 different mutations have been associated with G6PD deficiency [4–7]. These mutations cause class II (severe) or class III (mild) deficiencies, in which anemia is not present in daily life, but hemolytic attack can occur upon ingestion of certain oxidative medicines or foods [8]. Therefore, screening for affected individuals is critical for prevention of the disease [9].

Biochemical assays based on G6PD-catalyzed production of nicotinamide adenine dinucleotide phosphate (NADPH) are widely used for newborn screening. Despite the success in identifying male patients, measurement of G6PD activity appears to be inadequate for the detection of heterozygous females due to lyonization (inactivation of one X chromosome) [5,10]. To overcome this limitation, many alternative molecular assays have been developed, including denaturing high-performance liquid chromatography (DHPLC) [11], amplification refractory mutation system (ARMS) [12], microarray-based assay [13] and reverse dot blot assay (RDB) [7,14]. Although each assay has unique advantages in terms of specificity and sensitivity, a common shortcoming of these methods is that they often involving multiple steps of post-PCR manipulations, which increase the technical complexity and the risk of amplicon contamination. High-resolution melting (HRM) [15,16] is a good choice to obviate the post-PCR complexity; nevertheless, the performance of the dye-based methods is compromised by an inability to precisely identify the mutation.

*Abbreviations:* G6PD, glucose-6-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; ARMS, amplification refractory mutation system; DHPLC, denaturing high-performance liquid chromatography; RDB, reverse dot blot assay; HRM, high-resolution melting; MMCA, multicolor melting curve analysis.

* Corresponding authors at: Engineering Research Center of Molecular Diagnostics, Ministry of Education, School of Life Sciences, Xiamen University, Xiamen 361005, Fujian, China.
*E-mail addresses:* hqying@xmu.edu.cn (Q. Huang), qgli@xmu.edu.cn (Q. Li).

http://dx.doi.org/10.1016/j.ymgme.2016.07.006
1096-7192/

The MeltPro G6PD (Zeesan, Xiamen, China) assay is a qualitative diagnostic assay developed based on multicolor melting curve analysis (MMCA) using dual-labeled, self-quenched probes [17–19]. This assay was designed to detect the genotypes of 16 mutations in the *G6PD* gene, which covers >95% of the Chinese *G6PD* mutations. The MeltPro assay is a closed-tube format performed on a real-time PCR platform, from which the mutation information is retrieved based on differences in melting temperature ($\Delta T_m$) compared to the wild-type. One distinct feature of this assay is its ease-of-use due to the omission of complex post-PCR manipulations. Moreover, the exact mutation type can be identified based on the predefined $T_m$ values and the detection channels.

In this study, we systematically evaluated the analytical and clinical performances of the MeltPro G6PD assay. For the analytical study, the accuracy of mutation detection, the limit of detection, the reproducibility, and the cross-platform compatibility were evaluated. For the clinical study, a multicenter validation study was performed using 763 clinical samples collected from three different hospitals in China. We examined both G6PD enzyme activity results and DNA sequencing results.

## 2. Materials and methods

### 2.1. Clinical samples

A total of 763 clinical, unrelated, peripheral blood samples (428 males and 335 females) were collected from Zhuhai Municipal Maternity and Child Healthcare Hospital (Zhuhai, Guangdong province), Liuzhou Municipal Maternity and Child Healthcare Hospital (Liuzhou, Guangxi Zhuang Autonomous Region), and the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi Zhuang Autonomous Region). The Research Ethics Committee of each hospital approved this study, and informed consent was obtained from individual patients. Genomic DNA (gDNA) was extracted from the blood samples using the Lab-Aid 820 nucleic acid extraction system (Zeesan Biotech, Xiamen, China) according to the manufacturer's instructions. The concentration of the gDNA was determined using a ND-1000 UV–VIS spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA).

The G6PD enzyme activity of the clinical samples was determined using a commercial G6PD detection kit (Zhongshan Bio-Tech, Zhongshan, China) according to the method suggested by the World Health Organization for the measurement of the G6PD/6PGD ratio [20].

### 2.2. PCR and melting curve analysis

The program for PCR amplification and melting curve analysis using a Bio-Rad CFX 96 real-time PCR machine (Bio-Rad, Hercules, CA) was as follows. For each sample, 5 μL of extracted gDNA was added to reaction mixtures A and B. Each reaction mixture contained 20 μL of PCR mix and 5 μL of DNA template. The reaction started with a contamination control procedure of 2 min at 50°C to prevent carryover of DNA amplicons by uracil-*N*-glycosylase. After a denaturation step at 95°C for 10 min, a touchdown program was performed with 10 cycles at 95°C for 15 s, 65°C (−1°C/cycle) for 15 s and 76°C for 20 s, followed by 50 cycles at 95°C for 15 s, 55°C for 15 s, and 76°C for 20 s. Melting curve analysis started with a denaturation step for 1 min at 95°C, a hybridization step for 3 min at 35°C and a stepwise temperature increase from 40°C to 85°C at 0.4°C/step with a 5 s stop at each step. Fluorescence from the FAM, HEX, ROX, and CY5 channels was recorded at the annealing step during the second set of 50 amplification cycles and at each step during the melting curve analysis. Melting curves were obtained by plotting the negative derivative of fluorescence with respect to temperature versus temperature ($-dF/dT$), and the $T_m$ values were obtained automatically from the melting curves through the CFX Manager 3.0 software.

### 2.3. Verification of the accuracy of mutation detection

First, we tested the accuracy of the MeltPro G6PD assay in mutation detection using a reference panel, which included 10 wild-type gDNA samples, 16 homozygous mutant samples, and 16 heterozygous mutant samples (see Table S1 in the Supplementary data). An assay was considered valid if it met the following eligible criteria: the variation of the $T_m$ values was within 1 °C for the wild-type samples and the $T_m$ shift value ($\Delta T_m$, which is calculated by the $T_m$ difference between the wild-type and mutant) was within the fluctuation ranges (see Table S2 in the Supplementary data) for the mutant sample at each detection channel.

### 2.4. Evaluation of reproducibility

Evaluation of reproducibility was performed using 10 ng/μL and 1 ng/μL wild-type gDNA samples within a period of 20 days. Each sample was detected in duplicate and twice within 1 day. The within-run and between-run variations were calculated according to the *Guideline for Evaluation of Analytical Performance of In Vitro Diagnostic Kits* [21].

### 2.5. Limit of detection study

One wild-type male gDNA, two hemizygous mutant gDNA (c.392G>T and c.1376G>T), and two heterozygous female gDNA (c.392G>T and c.1376G>T) were serially diluted 10-fold with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), yielding gDNA concentrations ranging from 100 ng/μL to 10 pg/μL. For each concentration, three replicates were analyzed. Water was used as a no-template control.

### 2.6. Cross-platform compatibility evaluation

Four models of real-time PCR machines, including Bio-Rad CFX 96, Rotor-Gene 6000 (Corbett Research, Mortlake, Australia), SLAN-96P (Zeesan, Xiamen, China), and Roche LightCycler 480 II (Roche, Rotkreuz, Switzerland) were used to evaluate cross-platform compatibility. A reference panel was used for accuracy validation of mutation detection. Identical PCR conditions were used for all instruments, except that the melting curve analysis program was adapted to be compatible with each machine. The filter combination of the Roche LightCycler 480 II was not compatible with the dyes used in the MeltPro G6PD assay, and an extra color compensation experiment was performed according to the manufacturer's instruction. The platform was considered compatible if the variation of the $T_m$ values was within 1°C for the wild-type samples and the $T_m$ shift value was within the fluctuation ranges (see Table S2 in the Supplementary data) for the mutant samples in each detection channel.

### 2.7. Clinical evaluation

A double blind test was performed using 763 clinical samples collected from three hospitals in southern China, representing the endemic region for G6PD deficiency in China. The samples were re-numbered by a technician who was solely in charge of the data collection and statistical analysis. The coded samples from each hospital were analyzed by a second technician of the corresponding laboratory. The results obtained from each laboratory were reported to a third individual, who checked the data and calculated the clinical sensitivity and specificity.

### 2.8. DNA sequencing

The following samples were subjected to DNA sequencing: 1) samples detected as positive for G6PD deficiency by MeltPro assay; 2) samples detected as negative for G6PD deficiency by MeltPro assay but positive by G6PD activity assay; and 3) eighty randomly chosen samples, including forty males and forty females detected as negative by both MeltPro assay and G6PD activity assay. Regions encompassing