



# Higher Cut-off Value Improves Identification of Heterozygous Glucose-6-Phosphate Dehydrogenase-Deficient Female Neonates

Kittipong Sakunthai<sup>1</sup>, Noppmats Khemtonglang<sup>2</sup>, Pakaphan Kiatchoosakun<sup>3</sup>,  
Kriengkrai Kitcharoen<sup>2</sup>, Suttiphan Kitcharoen<sup>2,\*</sup>

<sup>1</sup>Medical Science Program, Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand

<sup>2</sup>Department of Medical Technology, Faculty of Associated Medical Sciences,

Khon Kaen University, Khon Kaen 40002, Thailand

<sup>3</sup>Department of Pediatrics, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

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## ABSTRACT

Female neonates heterozygous for glucose- 6- phosphate dehydrogenase (G-6-PD) deficiency exhibit variations in enzyme activity due to X chromosome inactivation, and may not be recognized by a fluorescent screening test or a quantitative enzyme activity assay. Given the known association of G-6-PD deficiency with risk of the severe outcome of neonatal hyperbilirubinemia, an accurate identification of heterozygous G-6-PD-deficient neonates is needed. The objective of this study was to measure red blood cell G-6-PD activity in female neonates with identified G-6-PD alleles for the determination of a cut-off value for heterozygous G-6-PD-deficient neonates. Residual EDTA blood samples from 334 female neonates were evaluated for G-6-PD deficiency status using a fluorescent spot test (FST), measurement of G-6-PD activity, and detection by multiplex allele-specific-PCR of seven G-6-PD mutations common to northeastern Thailand. A receiver operator characteristic (ROC) curve analysis was employed to determine a G-6-PD activity cut-off value for heterozygous G-6-PD deficiency. FST classified 4.8% and 7.2% of neonates as complete and partial deficiency, respectively. Comparison of G-6-PD activities clearly allowed discrimination between G-6-PD-normal and -deficient neonates, but enzyme activity of G-6-PD-deficient heterozygotes ranged from 0 to 22.7 U/g Hb, covering the activities of deficient and normal groups. ROC curve analysis indicated a G-6-PD activity cut-off of 15.4 U/g Hb for identification of G-6-PD-deficient heterozygotes. Prevalence of G-6-PD deficiency determined by this cut-off value was comparable to the prevalence of G-6-PD mutations, 37.4% and 35.6%, respectively. The established cut-off value improves identification of heterozygous G-6-PD-deficient female neonates, who, together with those with frank deficiency, are at risk of developing severe neonatal hyperbilirubinemia.

**Keywords:** G-6-PD variant; Multiplex allele-specific-PCR; Neonatal hyperbilirubinemia; Receiver operator characteristic curve analysis

## 1. Introduction

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency affects more than 330 million people worldwide [1]. It is the major cause of acute hemolytic anemia and neonatal hyperbilirubinemia [2-5]. G-6-PD is coded by the *G-6-PD* gene located on the long arm of the X chromosome (Xq28). G-6-PD deficiency in males is hemizygous while in females it can be homozygous or heterozygous. Red blood cells of hemizygotes and homozygotes contain very low G-6-PD activity, and thus these red blood cells are readily detected by either a qualitative or quantitative G-6-PD activity assay [5-6]. However, red blood cells of heterozygotes contain a wide variety of G-6-PD activity, ranging from a normal level to one comparable to homozygous state, owing to random inactivation of X chromosomes during embryogenic development [6-7]. Consequently, many heterozygous G-6-PD deficient females could be identified as having normal enzyme level by either qualitative or quantitative G-6-PD activity assays [3, 8], particularly when a low cut-off threshold is used [9-13].

G-6-PD-deficient newborns are at risk of developing severe neonatal hyperbilirubinemia, which in some populations can lead to severe neurological damage (kernicterus) and even death [14-15]. The WHO recommends that neonatal screening for G-6-PD deficiency should be performed for populations in which G-6-PD deficiency prevalence is greater than 3-5% in males [16]. In Thailand, the prevalence of G-6-PD deficiency in males is >10% [1, 17], but neonatal screening for G-6-PD deficiency is not fully implemented [18]; however, a G-6-PD deficiency test is required for all neonates who develop jaundice. In theory, heterozygous G-6-PD-deficient female neonates should be detected as early as possible and treated as cases of

homozygous G-6-PD deficiency to prevent serious complications arising through synergistic interaction with metabolic defects in bilirubin metabolism [2-3, 6, 14]. In order to accurately identify G-6-PD-deficient heterozygotes, genotyping *G-6-PD* alleles must be done [6, 8]. However, a quantitative G-6-PD activity assay should be able to determine the overlap of high heterozygous G-6-PD-deficient and low normal enzyme levels if an appropriate cut-off value for heterozygous G-6-PD-deficiency can be established for a target population [12-13]. A G-6-PD activity cut-off value is best determined using a receiver operating characteristic (ROC) curve generated from data of both affected and unaffected individuals in the population of interest [19].

This study sought to determine an appropriate G-6-PD activity cut-off value using ROC curve analysis for heterozygous G-6-PD-deficient female neonates by identifying genotypes of G-6-PD deficiency variants and their correlation with G-6-PD activity.

## 2. Materials and Methods

### 2.1 Samples

Residual EDTA blood samples of female full-term or near full-term ( $\geq 35$  weeks of gestation) neonates were used. The samples were from hyperbilirubinemic neonates whose blood samples were sent to the Diagnostic Microscopy Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, for G-6-PD deficiency screening by fluorescent spot test (FST) [16]. G-6-PD status (normal, partially deficient, and deficient) was preliminarily classified using the FST. Hyperbilirubinemia is defined according to the American Academy of Pediatrics 2004, using a nomogram based on hour-specific serum bilirubin values [20].

The study was approved by the Institutional Review Board of Khon Kaen University Ethics Committee in Human Research (HE591151) and no prior written consent from parents or legal guardians of neonates was required.

## 2.2 G-6-PD activity assay

G-6-PD activity was quantified using a commercial kit (Trinity Biotech Plc., Bray, Ireland). In brief, a 15  $\mu$ L aliquot of whole blood was added to 135  $\mu$ L of lysing reagent and G-6-PD activity in lysate was determined at 37 °C from the rate of increase in  $A_{340nm}$  of NADPH. G-6-PD activity and hemoglobin (Hb) concentration of lysate were simultaneously measured in an automated analyzer (Synchron CX-4; Beckman Coulter Inc., Brea, CA, USA). Reliability of the results was monitored concurrently using normal and intermediate G-6-PD activity controls (Trinity Biotech Plc., Bray, Ireland). An assay is considered valid if control values are within the manufacturer's range.

## 2.3 Identification of G-6-PD mutant alleles

Genomic DNA was extracted from blood leukocytes using a DNAzol isolation kit (Invitrogen, Carlsbad, CA, USA). The concentration of DNA in each sample was quantified using a GE NanoVue spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK) and adjusted to 10 ng/ $\mu$ L. Seven G-6-PD variants commonly present in Southeast Asian populations [21-26], namely, G-6-PD Canton (c.1376G>T, Arg459Leu), Chinese-4 (c.392G>T, Gly131Val), Chinese-5 (c.1024C>T, Leu342Phe), Kaiping (c.1388G>A, Arg463His), Mahidol (c.487G>A, Gly163Ser), Union (c.1360C>T, Asp454Cys), and Viangchan (c.871G>A, Val291Met) were identified employing multiplex allele-specific (MAS)-PCR developed in our laboratory. All mutant alleles were confirmed using restriction fragment length polymorphism (RFLP)-PCR [17, 22, 27] or by direct DNA sequencing,

which also provided information on zygosity of the mutant allele.

## 2.4 Statistical analysis

Data were analyzed with Minitab version 17 software (Minitab Inc., State College, PA, USA). Descriptive statistics were used to describe general characteristics of the samples and are reported as mean  $\pm$  standard deviation (SD). Prevalence of G-6-PD deficiency was calculated as percent total samples ( $n = 334$ ) and G-6-PD variant allele frequency as percent total G-6-PD alleles ( $n = 668$ ). ROC curve analysis was performed using MedCalc version 15.8 software (MedCalc Software bv, Ostend, Belgium). Genotyping of G-6-PD variants was set as the gold standard method for determining the optimal G-6-PD activity cut-off value. The MedCalc software calculated sensitivity =  $[a/(a + c)] \times 100$ , and specificity =  $[d/(b + d)] \times 100$ , where  $a$  = true positive,  $b$  = false positive,  $c$  = false negative, and  $d$  = true negative, for different cut-off points and displayed the ROC curve together with the optimal cut-off threshold, area under the curve (AUC), sensitivity, and specificity.

## 3. Results

### 3.1 Genotypic classification of G-6-PD deficiency

A birth weight (mean  $\pm$  SD) of 2,971  $\pm$  568 g and a gestational age of 38  $\pm$  2 weeks of female neonates ( $n = 334$ ) were within normal range [20]. Peak total serum bilirubin (14  $\pm$  3 mg/dL) was indicative of jaundice (normal range 5 - 6 mg/dL) [28].

Genotyping of G-6-PD variants by MAS-PCR and subsequent confirmation by RFLP-PCR or DNA sequencing revealed that 106 (31.7%) and 13 (3.9%) cases of the 334 cases were heterozygous and homozygous respectively for one of the seven G-6-PD variants investigated, including one G-6-PD Chatham (c.1003 G>A, Ala335Thr) heterozygote (by DNA sequencing) and a compound G-6-PD Chinese-5/Kaiping heterozygote. The G-6-

PD Viangchan allele was the most frequent (13%), followed by Canton and Chinese-4 (1.6%), Kaiping and Union (1.0%), and Chinese-5 and Mahidol (0.6%) (Table 1).

**Table 1.** Frequency of mutant *G-6-PD* alleles carried by female neonates (n = 334) in northeastern Thailand.

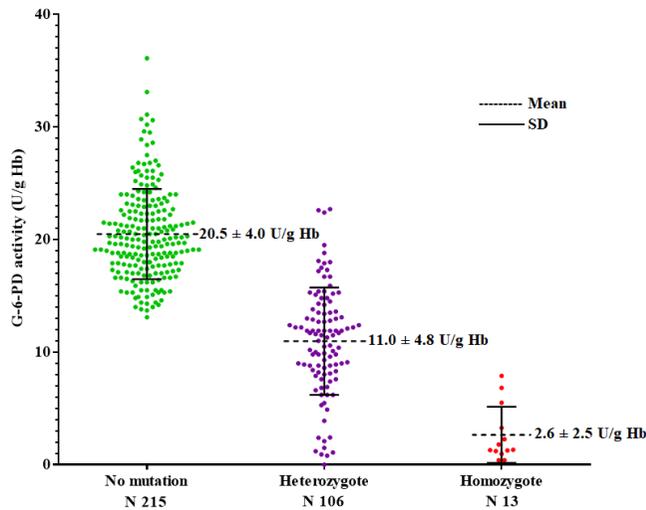
Mutant <i>G-6-PD</i> allele	Heterozygote	Homozygote	Total number of mutant alleles	Mutant allele frequency (%) (n = 668)
Viangchan (c.871G>A)	71	8	87	13.0
Chinese-4 (c.392G>T)	9	1	11	1.6
Canton (c.1376G>T)	7	2	11	1.6
Kaiping (c.1388G>A)	6	1*	7	1.0
Union (c.1360C>T)	7	0	7	1.0
Mahidol (c.487G>A)	4	0	4	0.6
Chinese-5 (c.1024C>T)	1	2*	4	0.6
Chatham (c.1003 G>A)	1	0	1	0.1
Total	106	13	132	19.8

\*Compound heterozygote of Chinese-5/Kaiping

**3.2 Quantification of G-6-PD activity and determination of cut-off value for heterozygous G-6-PD deficiency**

Red blood cell G-6-PD activity was measured at 37 °C using the Synchron CX-4 analyzer. The distribution of G-6-PD activity

showed, as expected, a clear difference in distribution of G-6-PD activity in G-6-PD-normal (13.1 - 36.1 U/g Hb) and -deficient (0.4 - 7.9 U/g Hb) neonates (Fig. 1).



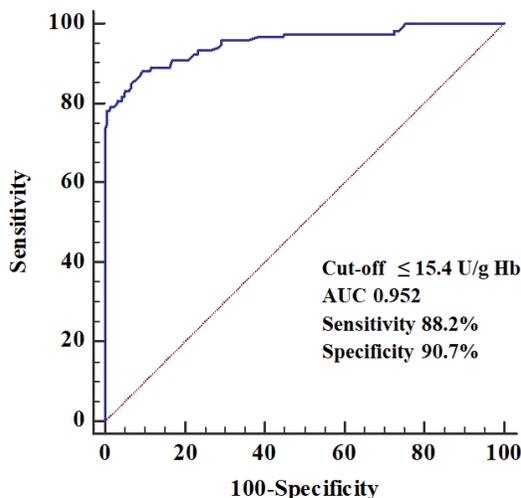
**Fig. 1.** Red blood cell G-6-PD activity of female neonates with hyperbilirubinemia in northeastern Thailand. G-6-PD activity was measured at 37 °C using the Synchron CX-4 analyzer and genotyping of the *G-6-PD* allele was performed by multiplex allele-specific-PCR, targeting seven *G-6-PD* variants common to the region.

Due to the overlap of enzyme activity among G-6-PD-normal and heterozygous G-6-PD-deficient neonates (0-22.7 U/gHb), employment of ROC curve analysis allowed the determination of a G-6-PD activity cut-

off value for heterozygous G-6-PD-deficient neonates of 15.4 U/g Hb (Fig. 2), which yielded a sensitivity of 88.2% ([ 105/( 105+ 14) ]x100) and a specificity of 90.7% ([195/(20+195)]x100) with excellent

accuracy, and an AUC of 0.952 [19]. According to this cut-off value, 125 (37.4%) cases were classified as G-6-PD-deficient, including 20 (6.0%) cases that carried no

mutation, whereas FST classified only 16 (4.8%) and 24 (7.2%) neonates as G-6-PD-deficient and partially G-6-PD-deficient, respectively (Table 2).



**Fig. 2.** ROC curve determination of G-6-PD activity cut-off value for differentiation between G-6-PD-deficient and G-6-PD-normal female neonates ( $n = 334$ ) in northeastern Thailand. ROC curve analysis was carried out using MedCalc version 15.8 software (MedCalc Software bv, Ostend, Belgium). AUC, area under the curve.

**Table 2.** Comparison of phenotypic classification of G-6-PD status according to G-6-PD activity assay and fluorescent spot test (FST) with genotypic classification.

Phenotype	Genotype, n (%)			Total
	No mutation*	Heterozygote	Homozygote	
<b>G-6-PD activity</b>				
Deficiency ( $\leq 15.4$ U/g Hb)	20 (6.0)	92 (27.5)	13 (3.9)	125 (37.4)
Normal ( $>15.4$ U/g Hb)	195 (58.4)	14 (4.2)	0	209 (62.6)
<b>Total</b>	<b>215 (64.4)</b>	<b>106 (31.7)</b>	<b>13 (3.9)</b>	<b>334 (100)</b>
<b>Fluorescent spot test</b>				
Deficiency	0	11 (3.3)	5 (1.5)	16 (4.8)
Partial deficiency	0	16 (4.8)	8 (2.4)	24 (7.2)
Normal	215 (64.4)	79 (23.6)	0	295 (88.3)
<b>Total</b>	<b>215 (64.4)</b>	<b>106 (31.7)</b>	<b>13 (3.9)</b>	<b>334 (100)</b>

\* No seven mutations analyzed in this study; G-6-PD Canton, Chinese-4, Chinese-5, Kaiping, Mahidol, Union, and Viangchan.

#### 4. Discussion

G-6-PD deficiency is the most common enzyme deficiency in the world. The global prevalence of G-6-PD deficiency has been estimated to be 4.9% [1]. This inherited enzyme deficiency is recognized as an important risk factor for neonatal hyperbilirubinemia [2-3]. Accurate screening for G-6-PD deficiency in newborns is very important in providing

proper medical management to prevent bilirubin-induced neurological dysfunction. Heterozygous female neonates are also at risk of increased hemolysis, hyperbilirubinemia, and even fatal bilirubin encephalopathy; hence it is prudent to attempt to identify these individuals [14, 29-30]. Routine screening for G-6-PD deficiency in neonates relies on FST [16], which in the present study, produced an

under reporting of the numbers of complete and partial G-6-PD deficiency among female neonates when compared with classifications based on genotyping. However, the prevalence of G-6-PD deficiency (12%) is comparable to those of previous reports which classified G-6-PD deficiency based on enzyme activity with a low cut-off value. Based on G-6-PD activity assays with the cut-off value  $<1.5$  U/g Hb, the prevalence of G-6-PD deficiency is 10.1% among female neonates with hyperbilirubinemia in central Thailand [17] and 15% among female newborns in northern Thailand [24]. Our report confirms that FST as well as quantitative G-6-PD activity assay using a low cut-off value underestimates the prevalence of G-6-PD deficiency in females.

In Thailand, based on quantitative G-6-PD activity assays, the prevalence of G-6-PD deficiency among neonates with hyperbilirubinemia has been reported at 22.1% in males and 10.1% in females, [17]. However, the prevalence of G-6-PD deficiency based on genotyping of mutant alleles has not been established among female neonates. According to the Hardy-Weinberg rule, the calculated frequency of G-6-PD-deficient homozygous females is 4.9% and that of heterozygotes is 34.4% [5]. In the present study, based on genotyping for seven common G-6-PD variants in all samples, the frequencies of homozygotes (3.9%) and heterozygotes (31.7%) agree with the Hardy-Weinberg rule. Moreover, the relative prevalence of each of the seven G-6-PD variants examined, of which G-6-PD Viangchan is the most prevalent, corresponds to previous reports from central [17], northeastern [22], and southern Thailand [23].

Various studies have been concerned with cut-offs and how to maximize the identification of female heterozygotes and suggested that higher cut-offs allow detection of more female heterozygotes [9-13,31]. We employed an ROC curve analysis to determine a G-6-PD activity cut-off value

for classification of heterozygous G-6-PD-deficient female neonates. Our cut-off value is higher than those of previous reports in China (2.55 to 2.8 U/g Hb) [9-13], Greece ( $<6.4$  U/g Hb) [9], Israel ( $<9.5$  U/g Hb [10], Saudi Arabia ( $\leq 6.6$  U/g Hb) [11], and the USA ( $<8.95$  U/g Hb) [31]. This might, firstly, be due to a difference in recruitment of sample donors: previous studies employed FST or an activity assay to identify intermediate deficiency (i.e. heterozygous G-6-PD deficiency) and then identified G-6-PD variants only in the selected samples, whereas the present study identified variant G-6-PD heterozygotes by genotyping all samples. Secondly, in each study, G-6-PD activity was measured using different assay kits either at 30 °C or 37 °C, resulting in a difference in G-6-PD levels. We performed the G-6-PD activity assay using Trinity Biotech G-6-PDH Quantitative kit in a Synchron CX-4 analyzer at 37 °C, using the standard reaction temperature of the automated chemistry analyzer [31], rather than at 30°C for conventional spectrophotometry assays [32-34]. Thirdly, G-6-PD activity is age dependent, with higher levels in neonates than in children and adults [35-37]. Our samples were from neonates whose ages ranged from 0 to 28 days; thus, their G-6-PD activities should be higher than other age groups. Finally, different populations may have different G-6-PD variants and thus, levels of enzyme activity. Therefore, a universal cut-off value may not be suitable for all populations. The specific cut-off value for G-6-PD activity assays should be defined in each population.

Based on this higher cut-off value, the prevalence of G-6-PD deficiency in female neonates is becoming closer to the prevalence determined by genotyping of G-6-PD mutations. However, 20 cases (6%) were classified as G-6-PD deficient but no mutation could be detected. These cases might carry G-6-PD variants that were not investigated in this study and need to be verified by DNA sequencing. In clinical

practice, female neonates with borderline G-6-PD activity should receive further testing, either by performing a family study or genotyping for G-6-PD-deficient variants [31], especially in countries in which the prevalence of G-6-PD deficiency is high, including Thailand. Heterozygous females should be warned as early in life as possible and treated as if they were completely G-6-PD deficient since they also are at risk, for developing severe hyperbilirubinemia during the neonatal period, and experiencing hemolytic episodes throughout their lifetime [6]. Moreover, genetic counselling should be advised to the patients and their families [6].

In summary, the present study confirms that heterozygous G-6-PD-deficient female neonates (identified by genotyping) present levels of red blood cell G-6-PD activity that overlap with those of G-6-PD-normal and -deficient (homozygous/compound heterozygous) individuals. A G-6-PD activity cut-off value for heterozygous G-6-PD deficiency was arrived at by using receiver operator characteristics curve analysis, the use of which should expedite identification of such neonates as reliance on fluorescent spot test produces false-negatives.

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