SCREENING AND MOLECULAR CHARACTERISATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY AMONG ELIGIBLE BLOOD DONORS

By

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ABSTRACT

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LEE SU XIAN

Glucose-6-Phosphate Dehydrogenase (G6PD) enzyme deficiency is a common worldwide and it is an X-linked inherited disorder that affects the males predominantly. It is a cytoplasmic enzyme which is involved in the first reaction of the pentose phosphate pathway to maintain the reduced form of gluthathione (GSH) from the oxidized disulphide form (GSSG) for detoxification of reactive oxygen species (ROS). Besides, GSH also protects erythrocytes from oxidative stress which could be induced by either ingestion of drugs or fava beans and infection. Without GSH, haemoglobin will denature and lead to formation of Heinz bodies. As G6PD deficiency individuals are asymptomatic until haemolytic crisis occurs, thus many G6PD deficiency individuals are unaware and attend for routine blood donation. The objective of this study was to screen and characterise G6PD variants at molecular level among eligible blood donors. In this study, sample recruitment was conducted at a blood donation campaign and 3 ml of blood samples were collected with informed consent. All the samples were screened using qualitative (fluorescent spot test) and quantitative method, OSMMR. Blood samples were extracted for genomic DNA. Variant detection was conducted for G6PD Chinese-4 (Quing Yang) (392 G>T), Kaiping (1388 G>A) and

Chinese-5 (1024 C>T). Throughout the analysis, out of 392 successful recruited samples, 10 (10/392; 2.6%) samples were G6PD deficient either in severely reduction of G6PD enzyme (in hemizygous or homozygous state) or moderately reduction of G6PD enzyme (in heterozygous state). G6PD Kaiping was found as the predominant variants (8/392; 2.04%) consisting of four hemizygous and four heterozygous. It was followed by G6PD Chinese-5 and Chinese-4 with one hemizygous case respectively (1/392; 0.3%). This study showed low prevalence (2.6%) of G6PD deficiency among blood donors. Therefore, G6PD screening might not be mandatory for blood donors. However, prevention of G6PD deficient individuals in donating blood is crucial to prevent any complication of hypoxia and anaemic condition to both donors and recipient.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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Lee Su Xian

APPROVAL SHEET

This project report entitled "**SCREENING AND MOLECULAR CHARACTERISATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY AMONG ELIGIBLE BLOOD DONORS"** was prepared by LEE SU XIAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

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I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(LEE SU XIAN)

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LIST OF ABBREVIATIONS

µL	Microlitre
µM	Micromolar
A260/A280	Assessment of Nucleic Acid Purity
bp	Base pair
CNSHA	Chronic Non-spherocytic Hemolytic Anemia
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium Bromide
FST	Fluorescent Spot Test
g	Gram
g	Gravity
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
GSSG	Oxidized glutathione
GSSG-R	Glutathione reductase
Hb	Haemoglobin
HMP	Hexose monophosphate pathway
L	Litre
L-DOPA	Levodopa
mL	Millilitre
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
nm	Nanometre

nt	Nucleotide
PCR	Polymerase Chain Reaction
PPP	Pentose phosphate pathway
R5P	Ribose-5-phosphate
RBC	Red Blood Cell
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
SPSS	Statistical Package for the Social Sciences
TAE	Tris-Acetic-EDTA
TE	Tris-EDTA
TPNH	Triphosphopyridine nucleotide
U/g	Unit per gram
UV	Ultraviolet
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The glucose-6-phosphate dehydrogenase gene is located on the long (q) arm of the X chromosome at position 28 (Xq28) and is important in synthesising glucose-6-phosphate dehydrogenase (G6PD) with 20 Kb in length consisting of 12 introns and 13 exons. This gene is also found located near to the genes encoding for Factor VIII and colour blindness. There are a total of 515 amino acid monomer encoded by G6PD gene with homo-dimers or tetramers constituting the active form of the G6PD enzyme (Francis, et al., 2013).

Lacking of G6PD enzyme production is known as G6PD deficiency. G6PD deficiency is a common enzymopathy which is estimated to affect over 400 million people worldwide. It is an X-linked inherited disease predominantly found in African, Asian, Mediterranean or Middle-Eastern population. Symptoms are shown when the erythrocytes are unable to counter the oxidative stress effectively, thus subsequently lead to haemolysis in the affected patient (Egesie, et al., 2013). According to Ainoon, et al., (2002), this deficiency is found to be more common in males compared to the females, which is attributed by the X-linked inheritance pattern of this disease. Male obtains the X-linked gene from the maternal side, whereby only one X chromosome could cause them

to be either normal or hemizygous. Female with two X chromosomes will present as either normal with normal G6PD gene, homozygous as G6PD deficient patient or heterozygous as G6PD deficient carrier. Carrier female with G6PD deficient would be asymptomatic throughout her life (Ainoon, et al., 2002).

The G6PD enzyme is a rate limiting enzyme that plays a crucial role in the pentose phosphate pathway. The G6PD enzyme activity also studied to aids in the cell growth through the redox reaction by providing NADPH. The NADPH is produced when the G6PD enzyme converts glucose-6-phosphate to 6-phosphogluconolactone and in the conversion from 6-phosphogluconolactone to ribulose-5-phosphate by the enzyme 6-phosphogluconate dehydrogenase (PGD, 6PGD) (Figure 1.1) (Tian, et al., 1998).

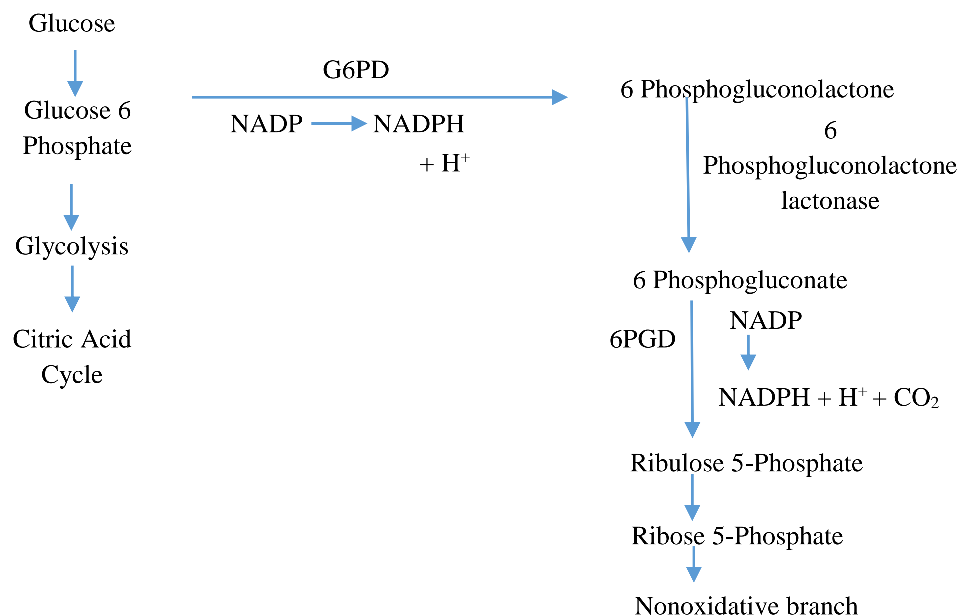

Figure 1.1: The Pentose Phosphate Pathway and the function of the rate limiting enzyme- G6PD enzyme in the pathway for the production of NADPH (Adapted from Tian, et al., 1998).

Other than the redox reaction, NADPH is also important to remove the oxidative stress which is arised in human cells after ingestion of fava beans or drugs by reducing the gluthathione which in turn will reduce the oxidative free radicals and hydrogen peroxide. In erythrocytes, the only source of NADPH is from the enzyme G6PD, thus it is very crucial for the enzyme to be sufficient in the erythrocytes. This would then help to protect the beta chain from oxidation which may lead to haemolysis event due to the breakdown of erythrocytes prematurely (McDonagh, et al., 2011).

Occurrence of extreme haemolysis event may lead to fatality if treatment such as blood transfusion is not provided. G6PD deficiency is also a serious issue in Malaysia. The recent report showed that annual mortality rate of this deficiency in Malaysia has been increasing from 1990 to 2010. Females reveal the highest mortality rate at the age of 80 years old and above while the lowest between the age of 45 to 49 years old. Meanwhile for males, the highest mortality rate was recorded at the age of 70 to 74 years old while the lowest recorded at the age of 40 to 44 years. In 2013, men have also recorded a higher mortality rate at 0.2 deaths per 100 000 men compared to women at 0.1 per 100 000 women (Health Grove, 2017).

In clinical setting, the routine screening test is Fluorescent Spot Test (FST). FST is able to pick up cases with severe reduction of G6PD enzyme which is less than 20% of the normal activity. Thus, cases with deficiency of G6PD between 20%

to 60% would be picked up as normal in which they should be categorised as intermediate deficiency of G6PD enzyme (Nadarajan, et al., 2011).

Individuals with the G6PD deficiency are asymptomatic when absence of haemolytic crisis, thus causing them to be unaware about their condition. This is not favourable particularly for those who are keen in donating their blood. However, when the G6PD deficient blood is transfused to a recipient, there is possibility that the recipient might face an episode of haemolysis when they are exposed to oxidative stress caused by certain drugs or fava beans ingestion especially to neonates. This would then require them to have another event of blood transfusion again to replace the haemolysed blood (Devi, et. al., 2010, Samantha, et al., 2009). In Malaysia, there is no documented report on the prevalence of G6PD deficiency among blood donors. Therefore, this study was conducted to investigate the prevalence of the G6PD deficiency individuals among eligible blood donors in Universiti Tunku Abdul Rahman (UTAR), Kampar Campus. In the present study, methods which included both the qualitative and quantitative screenings were incorporated with molecular characterisation for three G6PD variants- G6PD Chinese-4 (Quing Yang), Kaiping and the Chinese-5.

1.2 Research Objective

The general objective of this study was:

To characterise glucose-6-phosphate enzyme activity level and mutations among eligible blood donors.

The specific objectives were:

1. To qualitatively screen for G6PD using FST
2. To quantitate the enzyme activity level using quantitative enzyme activity assay
3. To characterise three G6PD variants at molecular level
4. To determine the association between the demographic data with the G6PD activity level
5. To determine the association between the G6PD activity level with G6PD variants

CHAPTER 2

LITERATURE REVIEW

2.1 Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency

Glucose-6-phosphate dehydrogenase is a crucial enzyme required in the redox reaction of aerobic cells for housekeeping functions (Luzatto, 2006). G6PD deficiency is the most common form of enzymopathy in human which estimating to affect 400 million individuals around the world. The geographical distribution of this disease is wide affecting certain part of the countries such as Africa, the Mediterranean and Asia at high frequency (Ainoon, et al., 2003).

2.1.1 Pathophysiology of G6PDDeficiency

According to Frank (2005), the most prevalent enzyme deficiency in the world is G6PD deficiency. The erythrocytes (or known as red blood cells) in individuals with G6PD deficiency, are more prone and susceptible to oxidative stress. This is contributed as G6PD is to catalyse the conversion from nicotinamide adenine dinucleotide phosphate (NADP) to NADPH which is in the reduced form in the pentose phosphate pathway (PPP). NADPH is important to maintain the reduced GSH which protect the erythrocytes from oxidative stress. However, NADPH can only be generated from G6PD through PPP but

not possible from other sources. Affected erythrocytes are detected with lower G6PD activity compared to normal erythrocytes. The G6PD activity in a normal erythrocyte without undergoing oxidative stress is approximately 2% (Frank, 2005).

2.2 Pentose Phosphate Pathway

The action of the G6PD enzyme when faced with an oxidative stress substance is depicted in Figure 2.1 together with detailed pathway and the flow of NADPH function in PPP.

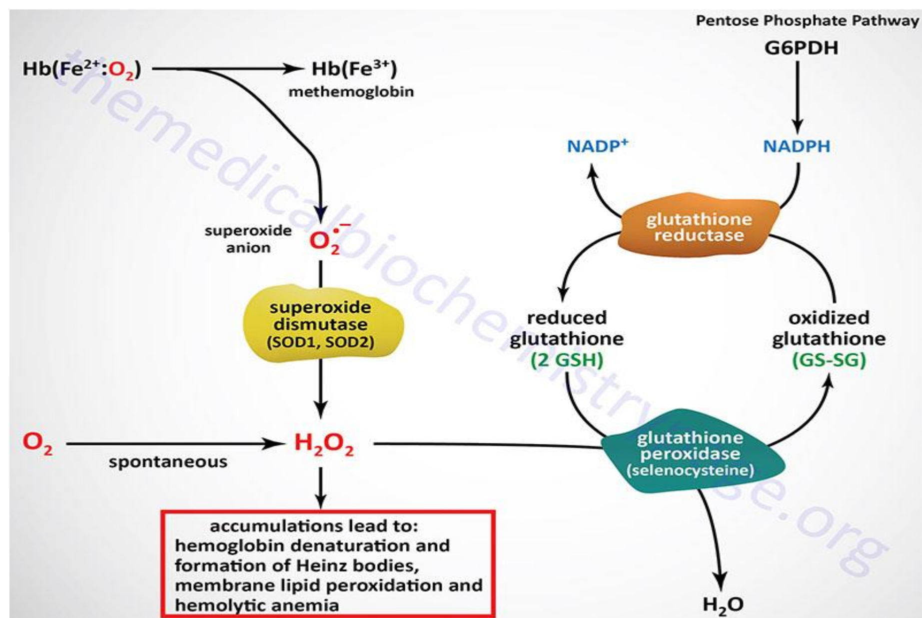

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Figure 2.1: The pathway of reactive oxygen species (ROS) removal in erythrocytes and the function of G6PD enzyme (Figure taken from King, 2017).

The reactive oxygen species such as H_2O_2 can be formed spontaneously from the oxygen or from the action of superoxide dismutase (SOD) on the superoxide anion. The superoxide anion is formed from the haemoglobin during the oxygen

oxidation of the ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) (King, 2017). The oxidative metabolism would produce the reactive oxygen species (ROS) as a product which in turn will cause damage and cell death. Therefore, reduced glutathione (GSH) with a structure of a tripeptide with a free sulfhydryl group is important to overcome the oxidative stress and to keep the cells in reduced state (Berg, Tymoczko and Stryer, 2002).

The G6PD enzyme plays an important role whereby it generates NADPH through the PPP in which the NADPH produces will reduce the oxidised glutathione (GSSG) to carry out the function to overcome oxidative stress. As the erythrocytes are lacking of mitochondria, the stress is most acute in the erythrocytes. In addition, they do not have other means of producing reducing power as well (Berg, Tymoczko and Stryer, 2002).

In a normal person, reticulocyte is found with five times more G6PD activity compared to old erythrocytes with only 10% G6PD enzyme activities. Among G6PD deficiency individuals, their erythrocytes are unable to produce sufficient G6PD enzyme leading to haemoglobin denaturation or proteolytic breakdown. Thus, this leads to shorter erythrocyte lifespan to about 60 days (Luzatto and Poggi, 2008).

2.3 Clinical classification of G6PD deficiency patients

According to the WHO working group, G6PD deficiency is classified based on the deficient of G6PD activity level. Until now, G6PD deficiency is classified into five groups. Class I is categorised for those with severe deficient of enzyme activity and is associated with chronic non-spherocytic haemolytic anaemia. Class II patients are those with severe G6PD deficiency with a residual activity between 1% to 10% and associated with acute haemolytic anaemia. As for class III, it is categorised as moderate G6PD deficiency with a residual activity between 10% to 60% and class IV is categorised as normal G6PD activity a residual activity between 60% to 150%. Lastly, class V category is with increased activity of more than 150% residual activity (Mehta, Mason and Vulliamy, 2000). Table 2.1 summarises the classification of G6PD deficiency with the prevalence ethnicity.

Table 2.1: Clinical classification of G6PD deficiency (Adapted from Frank, 2005).

Class	Level of Deficiency	Enzyme activity (%) and symptoms	Prevalence
Class I	Severe	Chronic non-spherocytic haemolytic anaemia in the presence of normal erythrocyte function	Uncommon, occurs across populations
Class II	Severe	<10 %	Varies, more common in Asian and Mediterranean populations
Class III	Moderate	10-60 %	10 percent of black males in the United States
Class IV	Mild to none	60-150%	Rare
Class V	None	>150%	Rare

2.4 Clinical manifestation

The most common manifestation of the G6PD deficiency is acute haemolytic anaemia. However, neonatal jaundice (NNJ) is another syndrome that is commonly seen in neonates. Chronic non-spherocytic haemolytic anaemia (CNSHA) is another rarer manifestation of G6PD is a life-long haemolytic process (Luzatto and Poggi, 2008).

2.4.1 Haemolytic Anaemia and its pathogenesis

Reduction of enzyme is seen in G6PD deficiency individuals, which leads to reduced production of NADPH and increased accumulation of toxic ROS in the blood. Thus, haemoglobin is prone to oxidative stress and denature in giving to the consequences of intravascular haemolysis (Schick, 2016).

The accompanying symptoms of haemolytic anaemia include malaise, low back pain, abdominal pain and haemoglobinuria (excretion of red or dark urine). When intravascular haemolysis occurred, changes of plasma colour from yellowish to red or brown indicate presence of haemoglobinuria or methemoglobinuria which also assist in more precise diagnosis. Other than that, high lactate dehydrogenase level, mild jaundice and blood films with polychromasia, anisocytosis and poikilocytes with various abnormal structure can be found during an early acute episode of haemolysis (Mason, Bautista and Gilsanz, 2007).

Few factors that may induce the occurrence of haemolysis crisis in G6PD deficiency individuals are drugs, fava beans, infections and diabetes mellitus. In the drug-induced haemolysis (Table 2.2) such as primaquine, the life span of the reticulocytes will be shortened in G6PD deficient patients. The urine of the patient will become dark in severe haemolysis and they will also experience back pain. However, in those mild G6PD deficiency patients (class III variants such as G6PD A-), the haemolytic event is limited whereby the young erythrocytes have normal or near to normal enzyme activity and only those old reticulocytes will be destroyed soon. As for those with severe deficiency from G6PD Mediterranean, young reticulocytes also have reduced activity and the haemolytic event can only be stopped with the removal of administration of drug. In addition, Asian people with G6PD deficiency when exposed to sulfamethoxazole, a combination of Septra and Bactrim, will cause reticulocytes life span shortening but no haemolysis occurred in patients with G6PD A- variant (Beutler, 1994).

Table 2.2: Drugs and chemicals to be avoided by G6PD deficient patients (Beutler, 1994).

Drugs and Chemicals		
Acetanilid	Niridazole (Ambilhar)	Sulfamethoxanole (Gantanol)
Furazolidone (Furoxone)	Isobutyl nitrite	Sulfanilamide
Methylene Blue	Nitrofurantoin	Sulpyridine
Nalidixic Acid (NegGram)	Phenazopyridine (Pyridium)	Thiazolesulfone
Napthalene	Primaquine	Toluidine blue
Phenylhydrazine	Sulfacetamide	Trinitrotoluene (TNT)

In addition, patients with favism are G6PD deficiency individuals who develop haemolysis after ingesting fava beans, *Vicia faba*. However, this symptom is not presented in all G6PD deficient individuals. It is attributed to the inherited genetic variant, which is related to the metabolism of the active ingredients in the beans. Majority cases with favism are seen among individual with G6PD class II variant but those in G6PD A- variant may also show occasional favism. Haemoglobinuria may occur for some days while haemolysis does not begin until 24 hours after the ingestion of the fava beans. The pathogenesis of haemolysis due to infection is not clearly reported but is suspected to be associated with the phagocytosis process. During phagocytosis, the reticulocytes are damaged by leukocytes by discharging active oxygen species (Beutler, 1994).

2.4.2 Formation of Heinz Bodies

Denatured haemoglobin can be viewed in blood smear as Heinz bodies due to unstable haemoglobin disorders or G6PD deficiencies (Schick, 2016). Not only that, according to Webster (1949), Heinz bodies is formed from the irreversible injury by a toxic agent producing newly formed particles either from the membrane of erythrocytes or the protoplasm. They also appear as large denatured proteins (Figure 2.2).

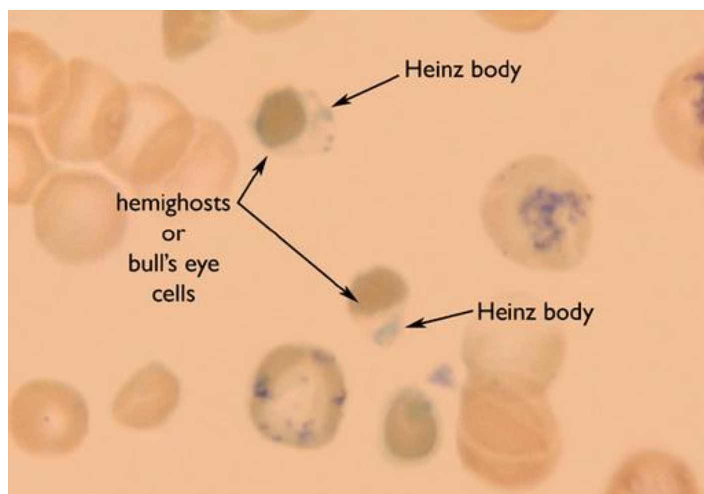


Figure 2.2: Formation of Heinz bodies due to haemolysis by deficiency of G6PD enzyme (Lawrence, 2002).

The clinical manifestation of the disorder is depending on the degree of G6PD deficiency. Those with minimally reduced enzyme levels will not experience haemolysis. However, those with high levels of deficiency may have episodes of brisk haemolysis. Due to the compensatory production of young reticulocytes with high G6PD activity, the haemolysis caused by oxidative stress is self-limited between eight to 14 days. Those with severe G6PD deficiency would be prone to chronic haemolysis and non-spherocytic haemolytic anaemia may occur in these patients (Schick, 2016).

2.4.3 Neonatal Jaundice (NNJ)

Neonatal jaundice (NNJ) in relation to G6PD deficiency is quite rare. NNJ has a peak incidence between day two and day three and the severity differs from being subclinical to kernicterus (chronic bilirubin encephalopathy). The specific

features in NNJ are erythrocytes with depressed glutathione reductase, elevated level of ascorbic acid and low levels of vitamin E, gluthathione peroxidase (GSHPX) and other enzymes (Luzatto and Poggi, 2008). Bilirubin is formed from the catabolism of heme which then bound to albumin in the circulation. Bilirubin is transported to the liver whereby it combines with glucuronic acid to form mono and diglucuronides. These are then excreted into the gut and bile. The enzyme uridine diphosphate glucuronosyl transferase catalyses this conjugation process. In newborns, the conjugated bilirubin in the gut tend to be hydrolysed back to the unconjugated form and reabsorbed into the blood stream through the enterohepatic circulation causing stress to the liver. In neonate, due to the bilirubin that was passed through the placenta, there is always a degree of hyperbilirubinaemia. G6PD deficient neonates have reduced ability to conjugate and clear bilirubin in the liver leading to the neonatal jaundice. NNJ is more common in G6PD Mediterranean but milder in the G6PDA^{-} (Mason, Bautista and Gilsanz, 2007).

2.4.4 Chronic non-spherocytic haemolytic anaemia

Chronic non-spherocytic haemolytic anaemia (CNSHA) occurs when the erythrocytes are unable to maintain a sufficient supply of NADPH to prevent the accumulation of toxic oxidising agents generated during the normal metabolism. Continuous presence of oxidising agents will cause damage to the erythrocyte membrane and cause haemolysis (Mason, Bautista and Gilsanz, 2007).

2.5 Glucose 6-Phosphate Dehydrogenase Gene

The gene encoding the G6PD is located on the long arm of the X chromosome (Xq28) consisting of 13 exons with a length of 18 kb. The G6PD locus is one of the most polymorphic loci with about 400 reported allelic variants (Nkhoma, et al., 2009). When a mutation occurs at the gene, it will be an abnormal G6PD, decreased or completely absence of G6PD production. However, gene alteration and deficiency of G6PD is not sufficient to cause clinical symptoms. Clinical features will be presented when interact with specific environmental factors (Youngster, 2010).

2.5.1 G6PD Variants in leading to G6PD deficiency

In 1992, over 400 G6PD variants have been identified (Beutler, 1992). Later in 2002, there are about 30 different G6PD variants reported. All of these variants cause a phenotypically altered enzyme, either by differing by quantity, activity, properties or a combination of these. However, three variants located at intron V at nucleotide 1116 and nucleotide 1311 are known for not causing any enzyme changes. Two common G6PD variants among African, G6PD A and G6PD A-, are found to produce normal G6PD with normal activity. G6PD A is a transition of nucleotide A to G at nucleotide 376 while G6PD A- with transition of nucleotide at two position of nucleotide G to A at position 202 and nucleotide transition from A to G at position 376. Schick (2016), reported G6PD A+ is associated with high G6PD enzyme level and therefore haemolysis does not occur. Meanwhile, G6PD A is associated with lower G6PD enzyme level and is

always associated with acute intermittent haemolysis. G6PD A- occurs mainly in Mediterranean, African and the Asian variants (Schick, 2016).

In Mediterranean, G6PD Mediterranean with a C to T nucleotide substitution at nucleotide 563 are commonly reported which causes a substitution of serine to phenylalanine at amino acid 188. G6PD Mediterranean is associated with severe enzyme deficiency compared to other variants such as G6PD A- and is classified under the class II deficiency (Zahraa, et al., 2015). Other common variants are G6PD Dallas, Cagliari, Sassari and Birmingham. Besides, G6PD Canton is commonly reported in Oriental region. G6PD Canton has a mutation at nucleotide 1376. Other than G6PD Canton, G6PD Gifu, Agrigento and Taiwan-Hakka are also commonly reported in this region.

Both G6PD Kaiping, Canton and Gaohe are commonly reported among Chinese which occurs up to 60% of the G6PD deficient patients. They are classified as class II variants by WHO (Bocchini, 2013). G6PD Kaiping involves the substitution of arginine-463 from histidine due to the G to A mutation at nucleotide 1388. Another two less common G6PD variants in Chinese are G6PD Qing Yuan, Nankang and Chinese-5. G6PD Qing Yuan or also known as Chinese-4 with G to T transversion in the cDNA nucleotide 392 (exon 5) which causes sunstitution of glycine to valine at amino acid 131. In G6PD Nankang, it occurs at nucleotide 517 from the T to C transition leading to a phenylalanine 179 to leucine substitution. As for the G6PD Chinese-5, there is a C to T

transversion at nucleotide 1024 resulting in the change of leucine to phenylalanine amino acid at location 342 (Beutler, et al., 1994).

In Thailand, the most prevalent variant is G6PD Viangchan which is also prevalent among Laotians, Malaysian Malays, Cambodians and Vietnamese. Whereas, G6PD Mahidol is predominantly seen among Burmese population. Most of the above mentioned G6PD variants are commonly resulted from transition mutations involving substitution of nucleotide throughout the 13 coding exons. However, there are some variants with deletion of single codon (G6PD Sunderland) or two codons (G6PD Stonybrook) (Phompradit, et al., 2011).

As for the mutation associated with CNSHA, the mutations are commonly located at exon 10 (Vuliamy, Beutler and Luzatto, 1993). Until 2002, Beutler and Vulliamy have discovered a total of 140 mutations which are depicted in Figure 2.3.

(A)

Variant name[a]	cDNA nucleotide substitution	Genomic nucleotide substitution	Amino acid substitution
Metaponto	172 G→A	10124A	58 Asp→Asn
Musashino	185 C→T	10137T	62 Pro→Leu
Asahi	**202 G→A**	**10154A**	**68 Val→Met**
A- Distrito Federal Matera Castilla Alabama Betica Tepic Ferrara Laghouat Kabyle	202 G→A 376 A→G	10154A 10877G	68 Val→Met 126 Asn→Asp
Namouru	208 T→C	10160C	70 Tyr→His
Murcia	209 A→G	10161G	70 Tyr→Cys
Swansea	224 T→C	10176C	75 Leu→Pro
Ube Konan	241 C→T	10193T	81 Arg→Cys
Lagosanto	242 G→A	10194A	81 Arg→His
Urayasu	281–283 del	10782–10784	95 Lys del
Vancouver	317 C→G 544 C→T 592 C→T	10818G 11715T 11763T	106 Ser→Cys 182 Arg→Trp 198 Arg→Cys
Hammersmith	323 T→A	10824A	108 Val→Glu
São Borja	337 G→A	10838A	113 Asp→Asn
A	376 A→G	10877G	126 Asn→Asp
Vanua Lava	383 T→C	10884C	128 Leu→Pro
Quing Yan	392 G→T	10893T	131 Gly→Val
Cairo	404 A→C	10905C	135 Asn→Thr
Valladolid	406 C→T	10907T	136 Arg→Cys
Acrokorinthos	**463 C→G** **376 A→G**	**10964T** **10877G**	**155 His→Asp** **155 Asn→Asp**
Santamaria	542 A→T 376 A→G	11713T 10877G	181 Asp→Val 126 Asn→Asp
Malaga	542 A→T	11713T	181 Asp→Val
Tsukui	561–563 del	11732–11734 del	188 or 189 Ser del
Mediterranean Dallas Birmingham Sassari Cagliari Panama	563 C→T	11734T	188 Ser→Phe
Coimbra Shunde	592 C→T	11763T	198 Arg→Cys
Santiago	593 G→C	11764C	198 Arg→Pro
Sibari	634 A→G	11805G	212 Met→Val
Minnesota Marion Gastonia LeJeune	637 G→T	11808T	213 Val→Leu
Harilaou	648 T→G	11996G	216 Phe→Leu
Radlowo	679 C→T	12027T	227 Arg→Trp
Mexico City	680 G→A	12028A	227 Arg→Gln
A-	680 G→T 376 A→G	12028T 10877G	227 Arg→Leu 126 Asn→Asp
North Dallas	683–685 del	12031–12033 del	229 Asn del
Asahikawa	695 G→A	12043A	232 Cys→Tyr
Durham	713 A→G	12061G	238 Lys→Arg
Stonybrook	724–729 GGCACT del	12072–12077	242–243 Gly&Thr del
Wayne	769 C→G	12117G	257 Arg→Gly
Aveiro	**806 G→A**	**12519A**	**269 Cys→Tyr**
Cleveland Corum	820 G→A	12533A	274 Glu→Lys
Sugao	**826 C→T**	**12539T**	**276 Pro→Ser**

Figure 2.3: G6PD variants Database (Beutler, Vulliamy and Luzatto, 2002).

(B)

Variant name[a]	cDNA nucleotide substitution	Genomic nucleotide substitution	Amino acid substitution
Papua	849 C→A	12562A	283 Asp→Glu
Osaka	853 C→T	12566T	285 Arg→Cys
Montalbano	854 G→A	12567A	285 Arg→His
Viangchan Jammu	871 G→A	13031A	291 Val→Met
West Virginia	910 G→T	13070T	303 Val→Phe
Seoul	916 G→A	13076A	306 Gly→Ser
Omiya	921 G→C	13081C	307 Gln→His
Ludhiana	929 G→A	13089A	310 Gly→Glu
Kalyan Kerala	949 G→A	13109A	317 Glu→Lys
Nara	953–976 del	13113–13136 del	319–326 del
Manhattan	962 G→A	13142A	321 Gly→Glu
Rehovot	**964 T→C**	**13124C**	**322 Tyr→His**
A- Betica Selma Guantanamo	968 T→C 376 A→G	13128C 10877G	323 Leu→Pro 126 Asn→Asp
Farroupilha	977 C→A	13137A	326 Pro→His
Chatham	1003 G→A	13163A	335 Ala→Thr
Fushan	1004 C→A	13164A	335 Ala→Asp
Torun	**1006 A→G**	**13166G**	**336 Thr→Ala**
Chinese-5	1024 C→T	13184T	342 Leu→Phe
Mira d'Aire	1048 G→C	13208C	350 Asp→His
Partenope	1052 G→T	13351T	351 Gly→Val
Ierapetra	1057 C→T	13356T	353 Pro→Ser
Iwatsuki	1081 G→A	13380A	361 Ala→Thr
Serres	1082 C→T	13381T	361 Ala→Val
Aachen	**1089 C→G**	**13388G**	**363 Asn→Lys**
Iowa Walter Reed Iowa City Springfield	1156 A→G	13455G	386 Lys→Glu
Guadalajara	1159 C→T	13458T	387 Arg→Cys
Mt. Sinai	1159 C→T 376 A→G	13458T 10877G	387 Arg→Cys 126 Asn→Asp
Beverly Hills Genova Worcester Yamaguchi Iwate Niigata	1160 G→A	13459A	387 Arg→His
Hartford	1162 A→G	13461G	388 Asn→Asp
Praha	1166 A→G	13465G	389 Glu→Gly
Wisconsin	1177 C→G	13476G	393 Arg→Gly
Nashville Anaheim Calgary Portici	1178 G→A	13447A	393 Arg→His
Alhambra	1180 G→C	13479C	394 Val→Leu
Bari	1187 C→T	13486T	396 Pro→Leu
Puerto Limon	1192 G→A	13491A	398 Glu→Lys
Anadia	1193 A→G	13492G	398 Glu→Gly
Clinic	1215 G→A	13514A	405 Met→Ile
Abeno	1220 A→C	13519C	407 Lys→Thr
Riverside	1228 G→T	13527T	410 Gly→Cys
Kawasaki	1229 G→C	13528C	410 Gly→Ala
Japan Shinagawa	1229 G→A	13528A	410 Gly→Asp

Figure 2.3: G6PD variants Database (Beutler, Vulliamy and Luzatto, 2002).

(C)

Variant name[a]	cDNA nucleotide substitution	Genomic nucleotide substitution	Amino acid substitution
S. Antioco	1342 A→G	13745G	448 Ser→Gly
Cassano	1347 G→C	13750C	449 Gln→His
Hermoupolis	**1347 G→C** **1360 C→T**	**13750C** **13763T**	**449 Gln→His** **454 Arg→Cys**
Harima	1358 T→A	13761A	453 Val→Glu
Union Maewo Chinese-2 **Kalo**	1360 C→T	13763T	454 Arg→Cys
Andalus	1361 G→A	13764A	454 Arg→His
Figueira da Foz	**1366 G→C**	**13874C**	**456 Asp→His**
Cosenza	1376 G→C	13884C	459 Arg→Pro
Canton Taiwan-Hakka Gifu-like Agrigento-like	1376 G→T	13884T	459 Arg→Leu
Yunan	**1381 G→A**	**13889A**	**461 Ala→Thr**
Kamiube **Keelung**	1387 C→T	13895T	463 Arg→Cys
Kaiping Anant Dhon Petrich-like Sapporo-like Wosera	1388 G→A	13896A	463 Arg→His
Neopolis	1400 C→G	13908G	467 Pro→Arg
Fukaya	1462 G→A	14067A	488 Gly→Ser
Campinas	1463 G→T	14068T	488 Gly→Val
Arakawa	1466 C→T	14071T	489 Pro→Leu
Brighton	**1488–1490 del**	**14093–14095 del**	**497 Lys del**
Bangkok Noi	1502 T→G 1376 G→T	14107G 13884T	501 Phe→Cys 459 Arg→Leu

Variant name[a]	cDNA nucleotide substitution	Genomic nucleotide substitution	Amino acid substitution
Sinnai	**34 G→T**	**34T**	**12 Val→Leu**
Lages	40 G→A	40A	14 Gly→Arg
Gaohe Gaozhou Sapporo-like Ube Bodia-like	95 A→G	95G	32 His→Arg
Honiara	99 A→G 1360 C→T	99G 13763T	33 Ile→Met 454 Arg→Cys
Sunderland	105–107 del	105–107 del	35 or 36 Ile del
Gidra	110 T→C	110C	37 Met→Thr
Orissa	131 C→G	9988G	44 Ala→Gly
Aures	143 T→C	10000C	48 Ile→Thr
Kozukata	159 G→C	10111C	53 Trp→Cys
Kamogawa	169 C→T	10121T	57 Arg→Trp
Ilesha	466 G→A	10967A	156 Glu→Lys
Mahidol	487 G→A	11658A	163 Gly→Ser
Plymouth	488 G→A	11659A	163 Gly→Asp
Taipei	493 A→G	11664G	165 Asn→Asp
Naone	497 G→A	11668A	166 Arg→His
Volendam	514 C→T	11684T	172 Pro→Ser
Nankang	517 T→C	11687C	173 Phe→Leu
Miaoli	519 C→G	11689G	173 Phe→Leu
Shinshu	527 A→G	11698G	176 Asp→Gly
Chikugo	535 A→T	11706T	179 Ser→Cys

Figure 2.3: G6PD variants Database (Beutler, Vulliamy and Luzatto, 2002).

(D)

La Jolla	**832 T→C**	**12545C**	**278 Ser→Pro**
Wexham	833 C→T	12546T	278 Ser→Phe
Chinese-1	835 A→T	12548T	279 Thr→Ser
Haikou	**835 A→G**	**12548G**	**279 Thr→Ala**
Seattle Lodi Modena Ferrara II Athens-like Mexico	844 G→C	12557C	282 Asp→His
Loma Linda	1089 C→A	13388A	363 Asn→Lys
Tenri	**1096 A→G**	**13395G**	**366 Lys→Glu**
Calvo Mackenna	1138 A→G	13437G	380 Ile→Val
Riley	1139 T→C	13438C	380 Ile→Thr
New York	**1141 T→A**	**13440A**	**381 Phe→Ile**
Olomouc	1141 T→C	13440C	381 Phe→Leu
Tomah	1153 T→C	13452C	385 Cys→Arg
Lynwood	1154 G→T	13453T	385 Cys→Phe
Madrid*	1155 C→G	13454G	385 Cys→Trp
Munich	**1231 A→G**	**13530G**	**411 Met→Val**
Tokyo Fukushima	1246 G→A	13545A	416 Glu→Lys
Georgia	1284 C→A	13560A	428 Tyr→End
Varnsdorf	3′ intron 10 splice site del	13689–13690 del	N/A
Surabaya	**1291 G→A**	**13694A**	**431 Val→Met**
Sumare	**1292 T→G**	**13695G**	**431 Val→Gly**
Pawnee	1316 G→C	13719C	439 Arg→Pro
Telti Kobe	1318 C→T	13721T	440 Leu→Phe
Santiago de Cuba Morioka	1339 G→A	13742A	447 Gly→Arg

Figure 2.3: G6PD variants Database (Beutler, Vulliamy and Luzatto, 2002).

2.6 Inheritance pattern of G6PD deficiency

As G6PD deficiency is an X-linked disorder, its inheritance pattern is gender dependent. As males have only one X chromosome, they are G6PD deficient with clinical presentation when the only X chromosome is mutated. However, as females have two X chromosomes, the mutated gene may be masked off by that of the other normal X chromosome and do not show any symptoms of the disease. This is known as random X chromosome inactivation. In body, one of the two X chromosomes will be inactivated or 'silenced' while the other X chromosome will be activated. This process occurs randomly and happens at a ratio of 50:50. However, two types of inactivations can happen. In favourable X inactivation, the affected X chromosome is silenced but in unfavourable X inactivation, the X chromosome which is silenced may be the unaffected X chromosome. Thus, in the unfavourable X inactivation, the female may develop symptoms for the disease as those seen in the males with the affected gene (Focus Information Technology, 2009). The inherited pattern of the X-chromosome from the parents are depicted in Figure 2.4.

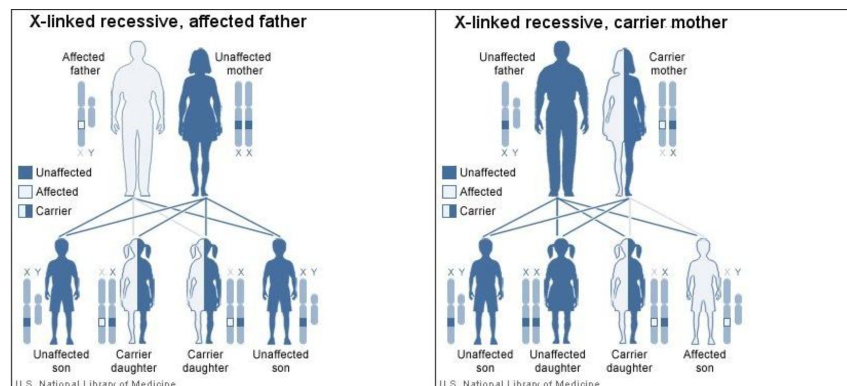


Figure 2.4: Inheritance Pattern of G6PD deficiency (X linked) (Figure taken from Focus Information Technology, 2009).

2.7 Diagnosis of G6PD deficient

G6PD diagnosis can be performed at two different levels which are screening and definitive diagnosis at genetic level. Screening of G6PD deficiency can be carried out either by semi quantitative test such as fluorescent spot test (FST) or quantitative test on G6PD enzyme activity. FST was first described by Beutler (1966) and further modified by Beutler and Mitchell (1968) is now widely used for population screening and diagnosis. The semi-quantitative test will be able to categorise individuals into either normal or G6PD deficient individuals (hemizygous males or homozygous females) under class I or II G6PD-deficient.

2.7.1 Qualitative Screening of G6PD deficient: Fluorescent spot test (FST)

Fluorescent spot test (FST) is done by fluorescence detection in which the nicotinamide adenine dinucleotide phosphate (NADP) is converted to its reduced form and showing fluorescence. Presence of fluorescence indicates the person with normal G6PD enzyme activity while absence of fluorescence indicates lacking of G6PD enzyme (Frank, 2005).

To conduct the FST, small amount of blood is first incubated with a substrate reagent containing glucose-6-phosphate and NADP. It is then spotted onto the filter paper. When the spot has dried, the paper is viewed under the long-wave ultraviolet (UV) light whereby the by-product of the reaction (NADPH) is fluorescent. The

fluorescent is directly proportionate to G6PD enzyme activity. The principle of detection is depicted and shown in Figure 2.5 (LaRue, Wittet and Pal, 2014).

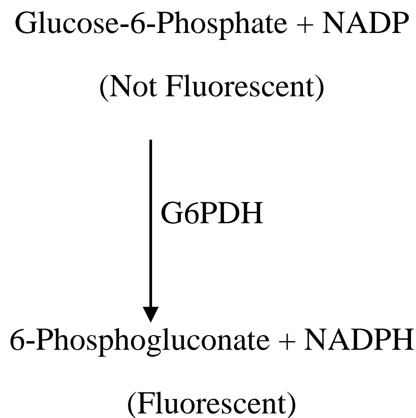


Figure 2.5: The formation of fluorescence in the G6PD qualitative screening-FST test (Adapted from LaRue, Wittet and Pal, 2014).

FST test is able to detect the normal G6PD activity as low as 2.1 U/ g Hb (Units/ gram haemoglobin) which is equivalent as 23.3% of the normal residual activity. Due to its ability to only pick up cases of deficiency less than 20% of normal, FST is unable pick up partially deficient cases which are 19.8% to 58% of those normal activity. This will lead to misdiagnosis especially those with partial deficient of enzyme activities (Ainoon, et al., 2003).

2.7.2 Quantitative Screening of G6PD deficient: Enzymatic Assay

Due to the limitation of the fluorescent spot test that can only pick up enzyme deficiency level below 20% compared to that of normal, Beutler (1991), came up with a new technique which is the enzymatic technique such as OSMMR2000-D G6PD assay.

The measurement of the enzyme activity is then done using a spectrophotometer. The principle of OSMMR2000-D G6PD assay is the G6PD enzyme from the study subjects will catalyse the conversion of glucose-6-phosphate to 6-phosphogluconate in the presence of NADP. The by-product, NADPH, is then reacted with a colour reagent and it will be measured spectrophotometrically at 550nm. Samples were also read at 405nm to get the haemoglobin concentration and the G6PD enzyme activity can be calculated. The enzyme activity would be directly proportional to the concentration of the G6PD enzyme that is present in the sample (R&D Diagnostics, 2016).

2.7.3 Definitive diagnosis at genetic level

With the development of the DNA technology, the G6PD mutation is possible to be detected prenatally. It can be detected by applying DNA amplification and restriction endonuclease digestion. When a mutation is present in a specific gene at

specific location of the nucleotides, it can be indicated when the specific base pair location of DNA is cleaved into two separate bands with different base pairs position (Beutler, et al., 1992).

2.8 Prevalence of G6PD worldwide

Glucose-6-phosphate dehydrogenase deficiency has been estimated to affect a worldwide population. According to Nabavizadeh and Anushiravani (2007), G6PD deficiency is estimated to affect about 35 million people worldwide. The regions with high prevalence include the tropical and subtropical regions in the eastern hemisphere which reach up to 35% in some areas. Not only that, WHO (2016) has estimated about 10% to 14% prevalence rate of G6PD deficiency in Iran. It was followed by Nkhoma, et al., (2009) who have estimated that about 400 million people in this world are with G6PD deficiency.

The prevalence of G6PD deficiency is found to be correlated globally with the population exposed to malaria including Mediterranean Europe, South East Asia, Latin America and Africa. Nkhoma et al. (2009), also reported that the overall random effects estimate for the total global prevalence of G6PD deficiency to be at 7.1% and 7.3% for males (Nkhoma, et al., 2009). According to Nkhoma's meta-analysis, the highest prevalence is in the sub-Saharan Africa with 8.5% followed by

the Middle East with 7.2%. Same prevalence rate of 5.2% was reported at America and Asia. Whereas, the prevalence rate of G6PD deficiency is quite similar at Europe and Pacific, with the rate of 3.8% and 3.4%, respectively. This data is depicted in Table 2.3.

Table 2.3: Estimate G6PD deficiency prevalence rate based on location (Nkhoma, et al., 2009).

Location	Number of estimates	Summary prevalence estimate (%)(95% CI)*
Africa	38	8.5 (7.9-9.1)
Americas	29	5.2 (4.7-5.8)
Asia	73	5.2 (4.7-5.6)
Europe	39	3.8 (2.9-4.7)
Middle East	50	7.2 (6.6-7.7)
Pacific	8	3.4 (2.7-4.1)

***CI = Confidence Interval.**

In North America, the largest group to be affected with G6PD deficiency is the African-American population which is up to 11% to 13%. This is then followed by other population subgroups such as the Italian (Sardinian ancestry), Greeks, Asians, Sephardic Jews (migrate from Southern Asians and Asia Minor) (Kaplan and Hammerman, 2000).

According to previous studies, the inherited G6PD variants are found geographical and ethnicity dependent. WHO (2016), reported that the main variant found in the Europe, northern India and central Asia is the Mediterranean variant. Not only that, G6PD African A- variant is often found among African Americans and the sub-Saharan African while Asians with G6PD Canton (WHO, 2016).

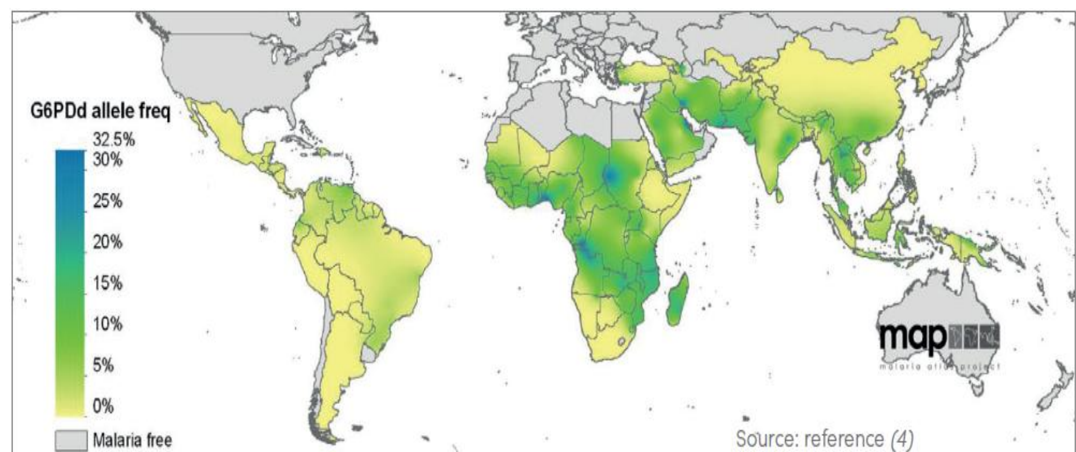


Figure 2.6: The prevalence of G6PD deficiency in the world (Figure taken from WHO, 2016).

2.9 Prevalence of G6PD in Malaysia

According to Ainoon and co-researchers (2002), the prevalence rate of G6PD deficiency in Malaysia is up to 3.4% with the overall prevalence rates among males and female are 5.3% and 1.05%, respectively. Different ethnicities demonstrate different prevalence rates in which the prevalence rates among Malays males and

females are 4.6% and 1.3%, respectively; Chinese males and females are 7.2% and 0.7%, respectively while Indian males and females are 2.7% and 0.7%, respectively. However, Malaysia is a multi-ethnic country that comprises of the Malay, Chinese, Indian and the aborigines (known also as Orang Asli). Each ethnicity is found to present different common variant. Malays are commonly seen with three G6PD variants, which are G6PD Viangchan (871 G>A) (37.2%), G6PD Mediterranean (563 C>T) (26.7%) and G6PD Mahidol (487 G>A) (15.1%) (Ainoon, et al., 2002). Other than the above three variants, the variants that are commonly seen in Cambodia and Myanmar are G6PD Viangchan (871 G>A, 1311 C>T, IVS11 nt93 T>C) and G6PD Mahidol (487 G>A) are also found among Malays. Meanwhile, G6PD Mediterranean with the Mediterranean subtypes (563 C>T, 1311 C>T, IVS11 nt93 T>C) and the Indo-Pakistan subtype (5623 C>T, 1311 C, IVS11 nt93 T) are also found among Malays. This can be explained that the Malay population could be from the similar origin as Myanmar, Cambodia and Indo-Pakistan (Wang, et al., 2008).

Malaysian-Chinese are found with the two most common variants which are G6PD Canton (1376 G>T) (4.7%), G6PD Kaiping (1388 G>A) (3.5%) and G6PD Gaohe (95 A>G) (Ainoon, et al., 2002; Wang, et al., 2008). G6PD Mediterranean (Indo-Pakistan subtype) and G6PD Namoru (208 T>C) are commonly seen among Malaysian-Indian (Wang, et al., 2008). Other variants such as G6PD Vanua Lava (383 T>C), G6PD Coimbra (592 C>T), G6PD Union (1360 C>T), G6PD Chatham (1003 G>A), G6PD Orissa (131 C>G) and G6PD Andalus (1361 G>A) are also found in Malaysia at lower percentage especially among aborigines' population.

As G6PD deficiency is an X-linked disorder, it is common among males compared to females in which Rafidah (2016) reported the prevalence rate of males with G6PD deficiency among Malays, Chinese and Indians are 4.6%, 6.0% and 1.3%, respectively. Meanwhile, female Malays, Chinese and Indians are reported with lower prevalence which are 1.4%, 1.65% and 0.49% (Table 2.4). The Negrito tribe of the Aborigines in Malaysia has shown a high prevalence of 9.0% (Rafidah, 2016).

Table 2.4: Tabulated percentage of prevalence of G6PD deficiency according to ethnics in Malaysia (Rafidah, 2016).

Ethnic	Male	Female
Malay	4.6%	1.4%
Chinese	6.0%	1.65%
Indian	1.3%	0.49%

2.10 Importance of G6PD testing in blood bank

In a previous study, it was found that the G6PD deficient red blood cells (RBC), respond less well in refrigerated storage as it is a form of oxidative stress to the RBC which leads to a slower recovery *in vivo* after transfusion. This case would be more severe in certain RBC transfusion dependent patient such as the sickle cell disease (SCD) patients. If these patients were to receive blood transfusion from a G6PD deficient donor and after the transfusion, the patients acquire infection or take medications which causes oxidative stress to the RBCs, it will lead to haemolysis in the patient (Francis, et al., 2013). In early study, if a patient was transfused with

blood from a G6PD deficient donor and the patient is given haemolytic drug or obtain hepatitis, it will lead to the lysis of the donor cells. The variant enzyme in the donor cells and the type of drug used will then determine the degree of lysis that occurs (McCurdy and Morse, 1975). Therefore, the donated blood from the donor would be a waste and in addition to it, the situation will lead to haemolytic event in the recipient as well.

In another study done to further support the importance of screening for G6PD deficient individuals is that a patient suffering from upper gastrointestinal bleeding developed rashes and itching throughout the whole body with transfusion of 50 mL of G6PD deficient blood. A test was carried out and the patient was said to undergo haemolytic anaemia reaction. When the transfusion was stopped, the haemoglobin level of the patient also fell to 7.7 gm/ dL from 9.0 gm/ dL. In the same study in neonates, the blood was transfused to the neonate during a surgery for ruptured meningomyelocoele. With the transfusion, the baby developed mottling and passed red urine. Therefore, the baby was suspected to undergo haemolytic transfusion event as the blood bag showed evidence of haemolysis. With this, the baby's haemoglobin also decreased from 12.2 gm/ dL to 8.0 gm/ dL. Besides decreased haemoglobin, mild increase in bilirubin and LDH can be seen when a patient is transfused with G6PD deficient blood (Devi, et al., 2010).

If the G6PD deficient blood is transfused to premature infants, severe hyperbilirubinaemia and haemolytic event may occur. In normal circumstances, the transfusion with G6PD deficient blood is harmless. However, complications will occur when the recipient is exposed to oxidative stress or drugs (Devi, et al., 2010). Thus, it drives to our study's attention on the determination of the prevalence of G6PD deficiency among blood donors in this region in order to measure the magnitude of the problem of G6PD deficiency in the population.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials preparation

3.1.1 Preparation of chemicals and reagents

Preparation of 50X TAE Buffer

For the preparation of the 50X TAE Buffer, 242 g of Tris Base with a molecular weight of 121.1 was dissolved in 600 mL of distilled water and mixed using a stir bar. Then, 100 mL of 0.5 M of EDTA and 57.1 mL of glacial acetic acid was added to the mixture. The final volume was added to 1 L using distilled water. The 50X TAE buffer was then stored at room temperature until further use.

3.1.2 Reagents and kits for G6PD screening and molecular detection

The reagents, chemicals and commercial kits used in this study is shown in Table 3.1 while the list of equipment used is shown in Table 3.2.

Table 3.1: Reagents, Chemicals and commercial kits used in this study.

Reagents, Chemicals and Commercial Kits	Manufacturer
Agarose Powder	PhileKorea Technology Inc, Daejeon, Korea
Absolute Ethanol (99%)	Merck, Germany
50X TAE Buffer	
- Ethylenediaminetetraacetic acid (EDTA)	SYTERM®, Selangor, Malaysia
- Glacial Acetic Acid	QRëC^{TM}, New Zealand
Tris Base	R&M Chemicals, Selangor, Malaysia
- Novel Juice – Midori Green Advance DNA/RNA stain for in gel or post staining	Nippon Genetics Europe, Deuren, Germany
6X DNA loading dye	Bioron Life Sciences, Ludwigshafen, Germany
2X Taq Master Mix	Bioron Life Sciences, Ludwigshafen, Germany
G-6-PDH Deficiency screening test kit (Fluorescent Spot Test)	RANDOX, Crumlin, Country Antrim, United Kingdom.
OSMMR2000-D/ 100C	R&D Diagnostics, Athens, Greece
FavorPrep^{TM} Blood Genomic DNA Extraction Mini Kit	FAVORGEN Biotech Corp, Taiwan
FavorPrep^{TM} Gel/ PCR Purification Kit	FAVORGEN Biotech Corp, Taiwan
100 bp DNA Ladder	Transgen Biotech, Beijing China
Restriction Enzyme BstEII	ThermoScientific, Malaysia

Table 3.2: Equipments used in this study.

Equipment	Manufacturer
Biometra TProfessional Thermocycler	Biometra GmbH, Germany
BIO-RAD T100^{TM} Thermal Cycler	BIO-RAD, USA
InGenius, Syngene Bio Imaging	Syngene, UK
NanoDrop^{TM} 2000/2000c	Thermo Fisher Scientific, USA
NanoPhotometer UV/Vis Spectrophotometer	Implen GmbH, Munich, Germany
FLUOstar Omega Microplate Reader	BMG Labtech, Germany
Horizontal Electrophoresis Set – Gel Tank	Ms Major Science, Saratoga, California, USA
Vortex Mixer VM-300	Gemmy Industrial Corp, Taiwan
Waterbath	Memmert, Germany
Weighing balance	Kern, Sigma Aldrich, USA.
Ultraviolet Box	Cole Parmer, Chicago, USA
Heraeus Fresco 21 Centrifuge Machine	Thermo Fisher Scientific, USA
BLooK LED Transilluminator	GeneDirex, Inc., Taiwan
InGenius, Syngene Bio Imaging with GeneSnap Software	Syngene International Ltd, India

3.1.3 Preparation of stock primers

All primers were synthesised by Integrated DNA Technologies, Coralville, Iowa, US. The primers were shipped in lyophilised form. The lyophilised primers were

appeared as translucent film or white powder. They were spun down before the addition of 1X Tris-EDTA (TE) buffer.

The 1X TE buffer was prepared from the 10X stock TE buffer solution (addition of one part of the stock 10X TE buffer with nine parts of autoclaved distilled water). In order to prepare 100 µM stock primer, specific volume of 1X TE buffer as indicated in the specification sheet provided by the IDT Company was added to the lyophilised primer. The mixture in the tube was mixed gently with pulse-vortex to ensure that the primers dissolved completely.

3.1.4 Preparation of working primers

Ten µM of working primer was prepared by adding 1 µL of the stock primer to 9 µL of autoclaved distilled water. Both stock and working primers were then stored in a -20 °C freezer to avoid the degradation of the primers. Primer sequences for detection of G6PD Quing Yang, Kaiping and Chinese-5 are listed in Table 3.3 and Table 3.4, respectively.

3.2 Experimental design

The study was conducted according to the experimental design described in Figure 3.1.

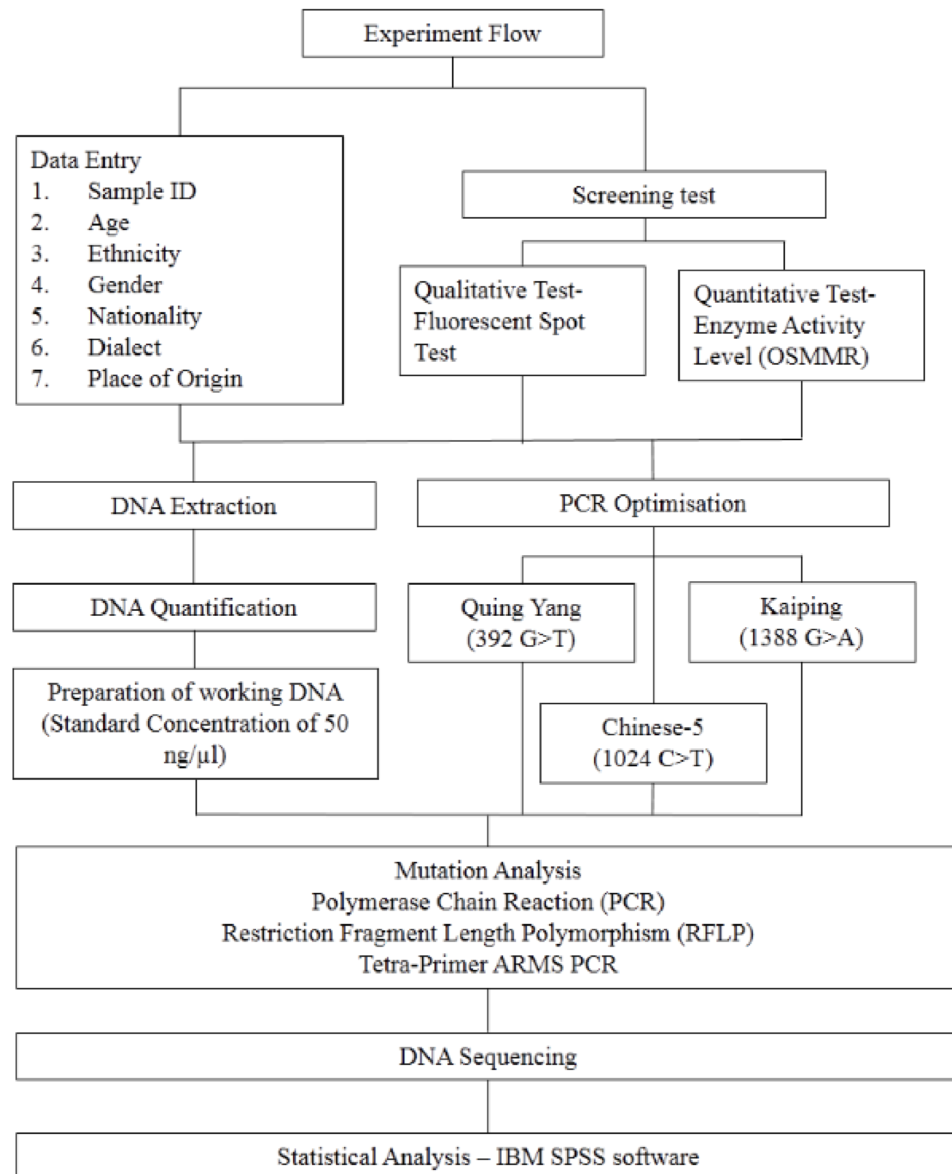


Figure 3.1: The experiment flow of this study.

3.3 *In silico* analysis of G6PD gene

In silico analysis was conducted for target G6PD gene in chromosome X. Nucleotide sequences in FASTA format for the target genes were reviewed through Genbank in National Centre for Biotechnology Information (NCBI) database.

All the G6PD Quing Yang (Chinese-4), G6PD Kaiping and G6PD Chinese-5 variants were found located in the X chromosome. The G6PD Quing Yang is found mutated at location nucleotide 392 G>T and located at exon 5. For G6PD Kaiping, it is mutated at nucleotide 1388 G>A and located at exon 12. As for G6PD Chinese-5, it is located at exon 9 and mutated at nucleotide 1024 C>T.

3.4 Ethics Approval

This epidemiology study was to investigate the prevalence of G6PD deficiency among the blood donors at the UTAR's blood donation campaign. Ethical approval was obtained from UTAR Scientific and Ethical Review Committee (SERC) [U/SERC/97/2016] (Appendix A) and sampling was done with informed consent (Appendix B). The study was conducted in concordance with the declaration of Helsinki.

3.5 Sample Recruitment

Sample recruitment was conducted at UTAR blood donation campaign located at Block A, UTAR, Kampar. Based on the largest anticipated population proportion of 0.5, the calculated sample size is 385 (Pourhoseingholi, Vahedi and Rahimzadeh, 2013). All the participants involved in this study must be eligible for blood donation after screening. Sample recruitment was on voluntary basis with informed consent before collection of 3 ml blood sample. Blood withdrawal and screening for donors were conducted by trained staff nurse from hospitals.

The sample size was calculated based on the formula as shown below (Pourhoseingholi, Vahedi and Rahimzadeh, 2013):

$$\text{Sample size, } n = \frac{z^2(P)(1-P)}{d^2} = \frac{1.96^2(0.5)(1-0.5)}{0.05^2}$$

$$= 384.16$$

$$= 385$$

where,

n= sample size

z= z statistic for the level of confidence, 5% (0.05)

P=anticipated population proportion (expected to be the largest; 0.5)

d= allowable error

3.6 G6PD screening for study subjects

3.6.1 Fluorescent Spot Test (FST)

Upon sample collection, FST was conducted on the same day to minimise false result due to enzyme degradation after storage. FST was conducted using Randox G-6-PDH Deficiency kit (by RANDOX, Crumlin, Country Antrim, United Kingdom) consisting of two reagents, R1a (substrate) and R1b (Buffer). R1a reagent contains the glucose-6-phophate, NADP and GGSG (oxidised glutathione) and R1b reagent contains the saponin and Tris (hydroxymethyl)-aminomethane.

The detailed protocol for the steps to conduct the fluorescent spot test was included in Appendix C. In this FST test, the whole blood was aspirated into a 1.5 ml microcentrifuge tube followed by the addition of reconstituted reagent R1a into the same tube and mixed thoroughly. The mixture was then incubated at room temperature and was spotted on the absorbent paper (Guthrie Test Paper). Then, the spots were allowed to dry. After the spots have dried, the paper containing the blood spots was viewed under the long wave UV light in a dark room and observations was done based on the fluorescence. (*Fluorescence= normal)

3.6.2 Quantitative analysis of G6PD enzyme activity

The G6PD enzyme activity level was determined using an enzymatic colorimetric method, OSMMR2000-D assay (R&D Diagnostics, Athens, Greece). This assay allows quantitative determination of the G6PD activity in both dried blood spots and whole blood specimens.

The elution buffer was first warmed to 30-37 °C prior to use. Ten parts of Colour Reagent was mixed with one part of Colour Booster to prepare ready-use Colour reagent. The lyophilised form of the normal and the deficient control was reconstituted by adding 0.5 ml of deionized water. The reagent mixture was also reconstituted from its lyophilised form by adding a volume of deionized water as indicated on the bottle label which is 2 mL.

As in Figure 3.2, position A1 and A2 were tested for 5 µL of Normal Control, A3 and A4 for 5 µL of the Deficient Control and A5 and A6 for Negative Control. The remaining 90 wells were allocated for 5 µL of whole blood samples from the study subjects.

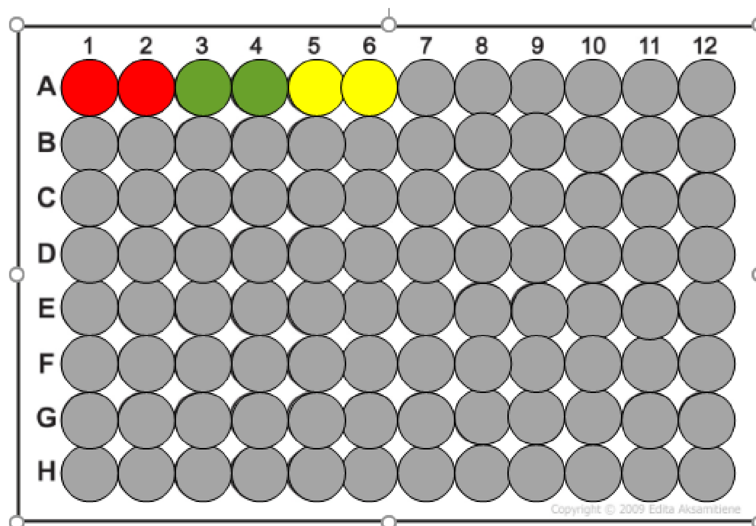


Figure 3.2: Plate layout design for G6PD quantitative analysis using 96 well microtiter (Red: Normal Control; Green: Deficient Control; Yellow: Negative Control; Grey:Samples).

The detailed protocol for the enzyme quantitative test can be obtained from Appendix D. Whole blood was added into the wells of the U-bottom microtiter plate (Elution Plate) followed by the addition of Elution buffer. The U-bottom microtiter plate was then placed on an orbital shaker at room temperature. The pre-warmed reagent mixture was added to a new F-bottom microtiter plate (Assay Plate). After incubation, eluent from the U-bottom microtiter plate was transferred correspondingly to the F-bottom microtiter plate and was mixed thoroughly. This was followed by the addition of prepared Colour Reagent Mixture (CRM) into each well respectively. This step was performed in the dark as the CRM is light sensitive. After addition of CRM, the microtiter plate was read using the microplate reader immediately. The plate was read at 550 nm using the kinetic mode for 15 minutes with 60 seconds (one minute) interval. Then, after the kinetic reading, the plate was

read again using the endpoint reading mode at 405 nm to obtain the haemoglobin content of each sample.

G6PD enzyme activity level was calculated for each sample using the formula as here:

$$\frac{(\ \delta \text{ OD Sample 550nm})/\ (\ \delta \text{ OD Control 550 nm})}{\text{OD sample 405 / OD Control 405 nm}} \times \text{Control Value}$$

$$= \text{Sample Value } (\frac{\text{U}}{\text{gHb}})$$

whereby,

δOD at 550 nm= (reading value at cycle 16)$_{550nm}$ – (reading value at cycle 0) $_{550nm}$
δOD at 450 nm= (reading value at cycle 16)$_{450nm}$ – (reading value at cycle 0) $_{450nm}$
Control value =13.6 (given by the manufacturer)

3.7 Extraction of genomic DNA

After the quantitative screening test was conducted, genomic DNA was extracted from the buffy coat layer of whole blood sample using FavorPrep^{TM} Blood Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corp, Taiwan). Before using the reagents, the W1 buffer was added with 45 mL of absolute ethanol (99%), Wash

buffer was added with absolute 200 mL ethanol and the lyophilised Proteinase K was dissolved in 1.1 mL of autoclaved distilled water.

A total of 200 µL of buffy coat was isolated from EDTA whole blood sample into a 1.5 mL microcentrifuge tube. Then, a volume of 20 µL of Proteinase K and 200 µL of FABG buffer were added to the tube containing the buffy coat. The mixture was mixed thoroughly by pulse vortexing. The sample was then placed into a 60°C water bath to lyse the cell membrane for 15 minutes. During the incubation, the sample was vortexed at each 5 minutes' interval time. The tube was then spun down to remove the water droplets from the cap. Then, a volume of 200 µL of absolute ethanol (96-100%) was added to the sample and the mixture was vortexed again thoroughly for 10 seconds. The tube was spun down again to remove droplets from the cap.

The FABG Mini Column was then placed to a collection tube and the mixture from the 1.5 mL tube was transferred completely into the FABG Mini Column. It was then centrifuged at 6 000 x *g* for one minute. The FABG Column was then placed into a new 1.5 mL collection tube and 400 µL of W1 Buffer was added to the column. The column was then centrifuged at the maximum speed of 18 000 x *g* for 30 seconds and the flow through was discarded. Then, a volume of 750 µL of Wash buffer was added to the FABG Mini Column and centrifuged at the maximum speed as well (18 000 x *g*) for 30 seconds followed by discarding the flow through. The

column was spun at full speed for an additional 3 minutes to dry the column. This step was to avoid the residual liquid to inhibit the subsequent enzymatic reaction.

After this, the FABG Mini Column was now placed to an Elution tube. A volume of 30 µL of pre heated Elution buffer was added to the centre of the membrane and was left to stand for 10 minutes at room temperature. This step was repeated with another 30 µL of pre heated Elution buffer. Lastly, the tube was centrifuged at full speed for one minute to elute all the extracted DNA. The direct extracted genomic DNA samples was the stock DNA samples and stored at the -20 °C freezer.

3.7.1 Quantification of Genomic DNA extracted from the samples

The extracted DNAs were quantified using the nanophotometer UV/Vis spectrophotometer (Implen GmbH, Munich, Germany). The concentration and the purity of each sample were recorded.

The spectrophotometer was blanked using 1 µL of autoclaved distilled water before quantitate the extracted genomic DNA. The lid was then cleaned with lens paper Multiple measurements were displayed including A230, A260, A280, A320, A260/A280, A260/A230 and also the concentration of the extracted DNA. Pure

DNA should have the reading of the A260/A80 between the range of 1.700-2.000. A purity of less than 1.7 indicates the presence of protein contamination.

3.7.2 Preparation of working DNA from the stock DNA extracted

As the extracted genomic DNAs were found with acceptable purity (A260/A280= 1.7-2.0), all the genomic DNAs were subjected for preparation of working DNA. This was to standardize their concentration to 50 ng/ µL with a final volume of 50 µL in order to ease the downstream PCR application. Stock DNA was then placed back into the -20 °C freezer to prevent DNA degradation due to repeated freezing and thawing processes and also to prevent contamination of the stock.

The working DNA was prepared by diluting the DNA stock with autoclaved distilled water using the formula as follow:

$$M1V1 = M2V2$$

where,

M1 = The concentration of the stock DNA

M2 = The concentration of the working DNA (50 ng/ µL)

V1 = The volume of the stock DNA required

V2 = The volume of autoclaved distilled water that need to be added to dilute

3.8 Genotyping of G6PD variants

3.8.1 Optimization of genotyping assay for G6PD Quing Yang, G6PD Kaiping and G6PD Chinese-5

To obtain an optimum cycling condition, gradient PCR was conducted at 8 different annealing temperatures from 55 °C to 68°C (Table 3.5 and Table 3.6).

Table 3.5: Range of Annealing Temperature (Ta) for optimizing G6PD Quing Yang.

Lane	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (°C)	68.0	67.0	65.4	62.9	59.9	57.5	55.9	55.0

Table 3.6: Range of Annealing Temperature (Ta) for optimizing G6PD Kaiping and Chinese-5.

Lane	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Temperature (°C)	65.0	64.0	62.4	59.9	57.0	54.5	52.9	52.0

3.8.2 Genotyping of G6PD Quing Yang (Chinese-4) variant using PCR-Restriction Fragment Length Polymorphism (RFLP)

All the samples were tested for G6PD Quing Yang in parallel with normal and negative controls. PCR component was prepared as shown in Table 3.7. Cycling condition was conducted for 30 cycles as shown in Table 3.8 using the BIO-RAD T100^{TM} Thermal Cycler (BIO-RAD, USA).

Table 3.8: Cycling condition of the RFLP method for the Quing Yang (Chinese-4) variant.

Steps	Temperature (°C)	Duration	Number of cycle(s)
Initial Denaturation	94	3 minutes	1
Denaturation	94	30 seconds	30
Annealing	60	30 seconds	30
Extension	72	1 minute	30
Final Extension	72	5 minutes	1
Infinite Hold	15	∞	1

All the amplified products were electrophoresed using a 2.0% of agarose gel at 90 volts for 35 minutes. Electrophoresed gel was stained and viewed by InGenius, Syngene Bio Imaging (Syngene, UK). Target region of G6PD gene was amplified at 202 bp amplicon size using the forward (392F) and reverse (392R) primers.

The amplified target region was then proceeded with restriction enzyme digestion using BstEII with the preparation as shown in Table 3.9. The digestion of amplified products with the BstEII was carried out in an incubation time of 90 minutes at 37 °C.

The recognition sites for Bst EII for cleavage is as shown in Table 3.10.

Table 3.10: Recognition site of Bst EII.

Restriction Enzyme	Manufacturer	Site of Cleavage
Bst EII	Thermo Scientific	5'-… G G T N A C C …-3' 3'-… C C A N T G G …-5'

After digestion, the digested products were proceeded for another round of gel electrophoresis using a 2.5% of agarose gel at 90 volts for 35 minutes. Samples were detected based on the amplicons size. Wild type for G6PD Quing Yang showed two amplicons with the size of 182bp and 20bp, respectively, homozygous or hemizygous sample showed an amplicon with the size of 202bp and heterozygous samples showed three amplicons with the size of 202bp, 182bp and 20bp, respectively.

3.8.3 Genotyping of G6PD Kaiping and Chinese-5 variants using Tetra primer ARMS PCR

Similar to PCR-RFLP for G6PD Quing Yang detection, all the samples detected for G6PD Kaiping and Chinese-5 were also run in parallel to the normal and negative controls. PCR primer mixes were prepared from respective inner and outer primers as shown in Table 3.11. PCR components were prepared as shown in Table 3.12. Cycling condition was conducted for 30 cycles as shown in Table 3.13 using the BIO-RAD T100^{TM} Thermal Cycler (BIO-RAD, USA).

Table 3.13: Cycling condition for the Kaiping / Chinese-5 variants.

Steps	Temperature (°C)	Duration	Number of cycle(s)
Initial Denaturation	95	30 seconds	1
Denaturation	95	15 seconds	30
Annealing	64	30 seconds	30
Extension	68	16 seconds	30
Final Extension	68	5 minutes	1
Infinite Hold	15	∞	1

PCR product was subjected to gel electrophoresis using a 2.5% agarose gel at 90 volts for 35 minutes. Genotype was determined based on the amplicon size obtained. For genotyping of G6PD Kaiping, a wild type sample shows a band at 411bp and 275bp; homozygous or hemizygous shows two bands (411 bp and 192 bp) and heterozygous shows three bands (411bp, 275bp and 192 bp). For genotyping of G6PD Chinese-5, a wild type sample will show two bands (372 bp and 252 bp); homozygous or hemizygous will show two bands at 372 bp and 175 bp while heterozygous will show three bands at 372 bp, 252 bp and 175 bp.

3.8.4 Gel electrophoresis

A 2.0% agarose gel was prepared by using 0.6 g of agarose powder dissolved in 30 mL of 1X Tris-Acetate-EDTA (TAE) buffer. The 1X TAE buffer was prepared from the 50X stock TAE buffer by mixing 10 mL of stock TAE with 490 mL of autoclaved dH_2O.

Five μL of the PCR product was mixed with 2 μL of 1X loading dye which is prepared from the 6X loading dye by diluting with autoclaved dH_2O in a ratio of 1 mL 6X loading dye with 5 mL of dH_2O. The mixture of the PCR product and the loading dye was loaded into the respective wells with the first well filled with 2.5 μL 100 bp of ladder. Then, the gel was run at 90 volts for 35 minutes.

For G6PD Kaiping and the Chinese-5 detection, a 2.5% agarose gel was prepared by dissolving 0.75 g of agarose powder in 30 mL of 1X TAE buffer. As the master mix used comes along with loading dye, the PCR product was loaded directly into the wells instead of mixing with the 1X loading dye and 2.5 μL of 100 bp ladder was loaded into the first well of each gel followed by the samples. The gel electrophoresis was run at 90 volts run for 35 minutes.

3.9 Preparation of DNA sequencing

Normal and mutant samples were randomly selected for sequencing analysis in order to confirm the variant. PCR component was prepared at a final volume of 50 µL. Target regions were amplified with respective primers to amplify the target region (Table 3.14). Cycling condition was run as the cycling condition for respective variant genotyping.

Table 3.14: The respective primers for the amplification of the target region with their amplicon size

Variants	G6PD Quing Yang	G6PD Kaiping	G6PD Chinese-5
Forward primer (ID)	392F	Outer Forward Kaiping	1024F
Reverse primer (ID)	392R	Outer Reverse Kaiping	1024R
Amplicon size (bp)	202	411	187

Amplified region was checked for successful amplification using gel electrophoresis with 2.5% agarose gel and loaded with 3 µL of PCR product with 2 µL of loading dye at 90 volts for 35 minutes. Then the gel was stained using the Novel Juice and was viewed using the BLooK LED transilluminator which uses the blue light. All the amplified products were subjected for PCR purification before sending for sequencing analysis.

3.9.1 PCR Purification

Polymerase chain reaction (PCR) purification was done using FavorPrep™ Gel/ PCR Purification Kit (FAVORGEN Biotech Corp, Taiwan) with detailed protocol in Appendix E. Before conducting the purification, the wash buffer was first added with 100 mL of absolute ethanol (99%). Then, PCR product was transferred into a 1.5 mL microcentrifuge tube which makes up to 100 µL in an Eppendorf tube. Then, five volumes of FADF buffer was added to the same tube and vortexed thoroughly (100 µL PCR product + 500 µL FADF buffer).

A FADF column was placed into a Collection tube. The sample mixture was then transferred to the FADF column and centrifuged at 11 000 x *g* for 30 seconds and the flow through was discarded. Next, 750 µL of Wash buffer was added to the FADF column and was centrifuged again at 11 000 x *g* for 30 seconds. The flow through was discarded as well. The FADF column then undergoes centrifugation at the full speed of 18 000 x *g* for an additional 3 minutes to ensure that the column was dried to remove the residual liquid thoroughly. The FADF column was placed to a microcentrifuge tube and 40 µL of elution buffer was added to the centre of the FADF column. The FADF column was left to stand for 10 minutes. After 10 minutes, the tube was centrifuged at full speed which was 18 000 x *g* for 1 minute to elute the DNA.

3.9.2 Sequencing analysis

Quality and quantity of the purified DNA products were analysed using Nanophotometer™ UV/Vis Spectrophotometer (Implen GmBH, Munich, Germany) and agarose gel electrophoresis. Pure PCR products were sent for automated Sanger sequencing service (First BASE Laboratories Sdn Bhd, Selangor, Malaysia) using BigDye® terminator v3.1 Cycle sequencing kit (Applied Biosystems, California, US) and analysed using ABI PRISM 3730xl Genetic Analyser (Applied Biosystems, California, US). Sequence alignment and assembly was done using DNA baser sequence assembly software v6.0 (Heracle Biosoft SRI, Arges, Germany).

3.10 Statistical Analysis

Data entry was done for the genotypes of three variants (G6PD Quing Yang, Kaiping, Chinese-5) and also the demographic data of the blood donors such as gender, age, ethnicity, nationality, dialect, as well as G6PD screening result obtained from FST qualitative test and OSMMR quantitative test. Statistical analyses were conducted by using SPSS version 68 software. Analysis of variants (ANOVA) was conducted by comparing the genotype and G6PD enzyme activity. G6PD enzyme activity was used as dependent variable, while G6PD genotypes were used as the independent variables. Association analysis (chi-square) was also conducted between the demographic data (gender, ethnicity and dialect) with the G6PD genotype.

CHAPTER 4

RESULT

4.1 Study Subjects

A total of 397 samples were recruited in this study with sampling done during the blood donation campaign held at the Heritage Hall of UTAR- Kampar campus on the first week of the October 2017. However, out of the 397 samples, only 392 samples were proceeded with subsequent analysis. Five samples were excluded due to incomplete demographic data or insufficient sample for analysis. Demographic data such as age, gender, nationality, ethnicity and dialect were tabulated in Table 4.1.

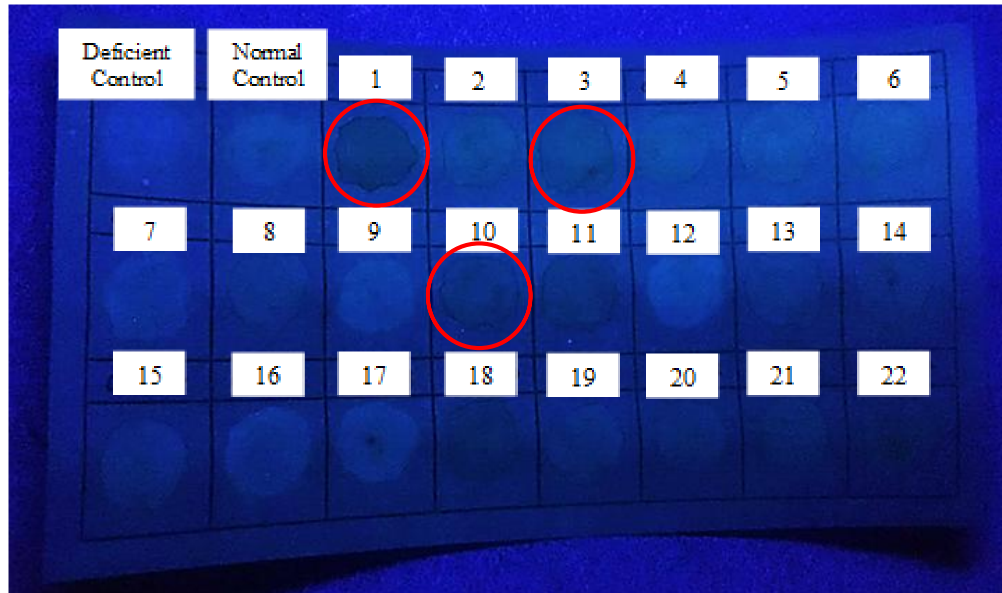
4.2 G6PD screening for study subjects

4.2.1 Qualitative test: Fluorescent Spot Test (FST)

The first screening test done was the common qualitative test known as the fluorescent spot test abbreviated as FST. In this test, the reagents were mixed with the blood sample and spotted on the paper. The paper was then viewed under the UV light when it's dried. Fluorescent was presumptively diagnosed as normal while

no fluorescent was presumptively diagnosed as G6PD deficiency. All 392 samples underwent this qualitative test and then followed by the quantitative assay for a better and more accurate result. The reason was because FST kit is able to pick up samples with a deficiency less than 20%. Samples with G6PD deficiency in between 20% to 60% might be missed out from the diagnosis.

Each round of FST analysis was conducted in parallel with both deficient and normal control. To ease the differentiation of the fluorescence signal especially the intermediate cases with fluorescence intensity in between the normal and deficient control. As shown in Figure 4.1, no fluorescence was seen in sample 1, 3 and 10; sample 11 and 18 showed intermediate fluorescence while samples 16 and 17 showed strong fluorescence and therefore regarded as normal.



*The circles indicate positive cases (no fluorescence)

Figure 4.1: Representative result for Fluorescent Spot Test (FST). The first two columns represent the deficient (negative) control and the normal (positive) control while the columns numbered 1 to 22 represent FST for 22 study subjects.

Table 4.2 tabulates the FST for 392 study subjects. Out of the analysis, 266 showed normal fluorescence, 71 showed intermediate fluorescence while the remaining 55 samples, showed no fluorescence.

4.2.2 Quantitative analysis of G6PD enzyme activity

Quantitative analysis of G6PD enzyme activity was conducted next using OSMMR2000-D/100 C kit. In this assay, presence of NADP reacted with the G6PD enzyme to oxidise the glucose-6-phosphate to 6-phosphogluconate and NADPH. The NADPH produced would then react with the colour reagent to produce a distinct

colour that was measured kinetically. The haemoglobin concentration of the sample was also measured.

All the samples which were further classified into three categories such as totally deficient, partially deficient and normal. Table 4.3 demonstrates the G6PD enzyme activity for 392 study subjects after categorizing them into three groups. A total of 346 samples out of 392 samples fell within the normal range of residual activity (6.6 -17.0 U/ g Hb) making up 88.3%. This was then followed by those with partial deficient with residual activity range of 2.5 – 6.5 U/ g Hb and those in the total deficient group with residual activity range of less than 2.5 U/ g Hb. Both the groups have 10.7% and 1.0%, respectively.

Table 4.3: G6PD enzyme activity level for 392 study subjects

Category	**Residual Activity Range (U/ g Hb)**	**Number of samples (n)**	**Percentage (%)**
Normal	6.6 – 17.0	346	88.3
Partially Deficient	2.5 – 6.5	42	10.7
Totally Deficient	< 2.5	4	1.0
Total		392	100

Results for both FST and OSMMR test were compared. Concordance results are demonstrated in Table 4.4 as grey boxes. Concordance results were seen in 256 out of 392 study subjects (65%) with 243 with normal G6PD level, nine with partially

deficient and four with total deficient. In-concordance results were seen in 136 subjects (34.7%) with the false diagnosis found in normal, intermediate and also deficient in G6PD.

4.3 Optimization of genotyping assay for three G6PD variants

4.3.1 Optimization of genotyping assay for G6PD Quing Yang

Gradient PCR was conducted to determine the optimum annealing temperature for the particular variant. Gradient PCR enables one to set a range of temperatures to conduct PCR and to obtain the best temperature with the highest specificity to the band of interest. The gel electrophoresis was done using a 2.5% gel at 90 volts for 35 minutes and the gel was viewed after staining.

Figure 4.2 shows that the optimum annealing temperature for detection of the Quing Yang variant is at 60.0 ºC when the targeted region was amplified using the primers of 392F and 392R.

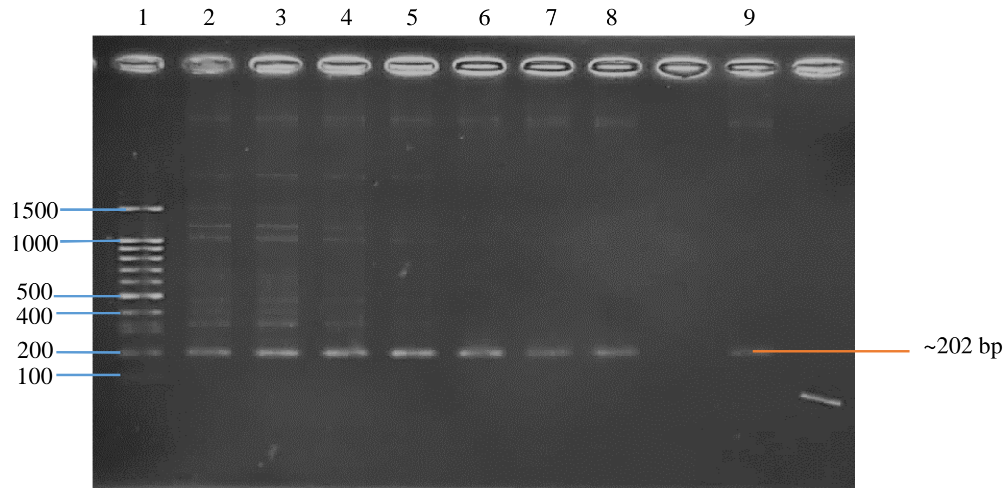


Figure 4.2: Gradient PCR for detection of Quing Yang variant with a range of annealing temperature.
Lane 1 represents 100 bp Ladder. Lane 2 to lane 9 represent samples with similar cycling condition but different annealing temperatures (Lane 2= 55 ℃; Lane 3= 55.9 ℃; Lane 4= 57.5 ℃; **Lane 5= 59.9 ℃**; Lane 6= 62.9 ℃; Lane 7= 65.4 ℃; Lane 8= 67.0 ℃ and Lane 9= 68.0 ℃)

4.3.2 Optimization of genotyping assay for G6PD Kaiping

Based on the representative gel image in Figure 4.3, the specific targeted regions were amplified with four primers (Kaiping Outer Forward, Outer Reverse, Inner Forward and Inner Reverse). The pair of outer primers (Kaiping Outer Forward - Outer Reverse) gave the amplification of the internal control at 411 bp. The Kaiping Inner Forward and Outer Forward primers amplified the specific wild type or mutant allele and allowing differentiation of wild type, heterozygous or homozygous / hemizygous. The most optimum T_a temperature was observed at 64 ℃ with the best amplification of both wild type allele and internal control (Figure 4.3).

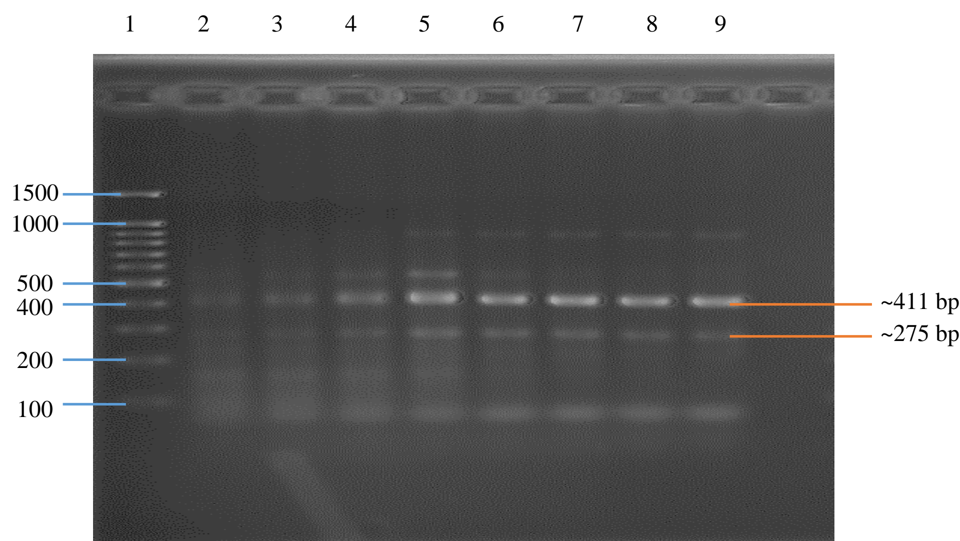


Figure 4.3: Gradient PCR for detection of Kaiping variant with a range of annealing temperature.
Lane 1 represents 100 bp Ladder. Lane 2 to lane 9 represent samples with similar cycling condition but different annealing temperatures (Lane 2= 52 ºC; Lane 3= 52.9 ºC; Lane 4= 54.5 ºC; Lane 5= 57.0 ºC; Lane 6= 59.9 ºC; Lane 7= 62.4 ºC; **Lane 8= 64.0 ºC** and Lane 9= 65.0 ºC).

4.3.3 Optimization of genotyping assay for G6PD Chinese-5

Based on the representative gel image in Figure 4.4, the specific targeted regions were amplified well with four primers (Chinese-5 Outer Forward, Outer Reverse, Inner Forward and inner Reverse) at the optimum annealing temperature at 64 ºC. The primers pair of Chinese-5 Outer Forward and Outer Reverse allowed the internal control amplification at 372 bp. The primer Chinese-5 Inner Forward and Outer Forward primers allowed the amplification of either mutant or wild type allele.

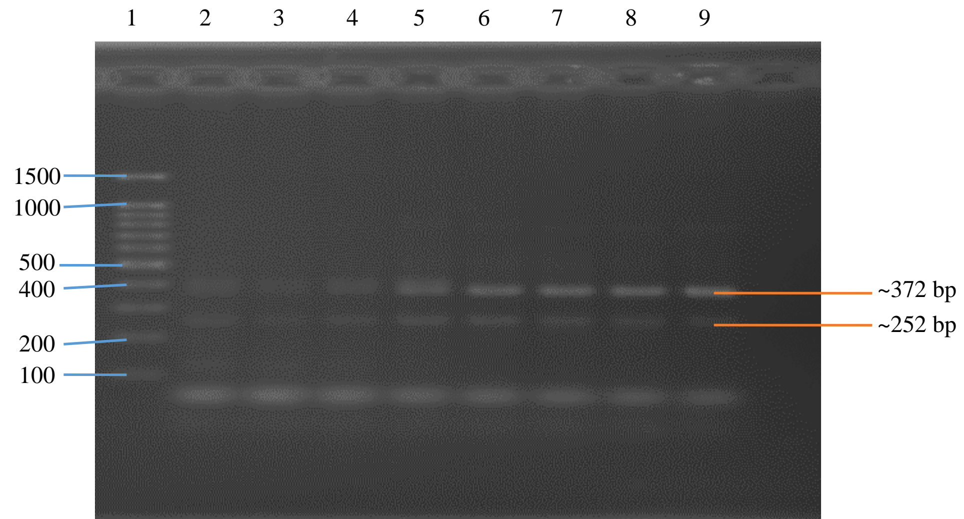


Figure 4.4: Representative gel image of the PCR optimization for detection of Chinese-5 variant.
Lane 1 represents 100 bp Ladder. Lane 2 to lane 9 represent samples with similar cycling condition but different annealing temperatures (Lane 2= 52 ºC; Lane 3= 52.9 ºC; Lane 4= 54.5 ºC; Lane 5= 57.0 ºC; Lane 6= 59.9 ºC; Lane 7= 62.4 ºC; **Lane 8= 64.0 ºC** and Lane 9= 65.0 ºC).

4.4 Genotyping of G6PD variants

4.4.1 Genotyping analysis of G6PD Quing Yang (Chinese-4) variant

Figure 4.5 demonstrates the amplification of target region and the amplicon size after digested with restriction enzyme. Successful amplification of both 392F and 392R primers resulted in an amplified region at 202 bp as shown in Figure 4.5A. Successful amplicon for target region was preceded for restriction enzyme digestion using B*stE*II with specific recognition site. Restriction enzyme recognized the wild type allele and gave 182 bp after cleavage while mutant allele was remained intact at 202 bp without cleavage as shown in Figure 4.5B.

One sample was randomly selected for sequencing analysis to confirm G6PD Quing Yang variant. Chromatogram was shown in Figure 4.6 using forward primer. In the detection of the Quing Yang variant, transition was shown from the nucleotide G (wild type) to nucleotide T (mutant) at the position of 181 bp.

4.4.2 Genotyping analysis of G6PD Kaiping variant

Figure 4.7 shows the representative gel in genotyping analysis for G6PD Kaiping using two pairs of primers (Kaiping Outer Forward, Outer Reverse, Inner Forward and Inner Reverse). Based on Figure 4.7, three bands were shown in heterozygous samples while hemizygous in male or homozygous samples showed two bands at 411 bp and 192 bp. Meanwhile, normal samples demonstrated two bands at 411 bp and 275 bp.

In the detection of the Kaiping variant through sequencing analysis, there was a transition from the nucleotide G (wild type) to nucleotide A (mutant). This is shown in the Figure 4.8 with the transition of G to A at base position 246.

4.4.3 Genotyping analysis of G6PD Chinese-5 variant

Figure 4.9 shows the representative gel in genotyping analysis for G6PD Chinese-5 using 2 pairs of primers (Kaiping Outer Forward, Outer Reverse, Inner Forward and Inner Reverse). Based on Figure 4.9, three bands were shown in heterozygous samples while hemizygous in male or homozygous samples showed two bands at 372 bp and 175 bp. Meanwhile, normal samples demonstrated two bands at 411 bp and 252 bp.

In the detection of the Chinese-5 variant through sequencing analysis, there was a transition from the nucleotide C (wild type) to nucleotide T (mutant). This is shown in the Figure 4.10 with the transition from C to T at base position 159.

4.5 Genotyping analysis of three G6PD variants (G6PD Quing Yang, Kaiping & Chinese-5)

Genotyping analysis was conducted for all the 392 study subjects for three G6PD variants and the result was tabulated as shown in Table 4.5. From the genotyping analysis, there were 10 positive cases that was picked up with 4 hemizygous Kaiping and heterozygous Kaiping respectively and one hemizygous Quing Yang and hemizygous Chinese-5 respectively. The remaining 382 samples were found wild type for these three G6PD variants.

4.6 Statistical Analysis using the One-Way ANOVA

Statistical analysis was conducted to analyse the difference between genotype and the enzyme activity level by using genotype of G6PD variant as independent variable and G6PD enzyme activity level as dependent variable. There was a statistically significant difference between groups as determined by one-way ANOVA ($F = 7.978$, $p = 0.000$) with p-value smaller than 0.05. Therefore, the enzyme activity level of an individual is dependent on the G6PD variant of the G6PD patient. G6PD Chinese-5 in hemizygous state was found with the highest G6PD enzyme activity level compared to hemizygous of G6PD Quing Yang and Kaiping (Table 4.6).

Distribution of G6PD enzyme activity level is demonstrated as box plot in Figure 4.11 together with the post hoc comparison analysis using least significant different (LSD). There was significant difference between study subjects with hemizygous Kaiping and heterozygous Kaiping and also between the study subjects with hemizygous Kaiping and wild type subjects with *p*-value less than 0.05.

4.7 Association Analysis between the demographic data of gender, ethnicity and dialect with the G6PD genotype

Chi-square test or Pearson's chi-square was conducted to determine the association between two categorical variables. Chi-square test was selected as data met two assumptions in which involves of two categorical variables and two independent variables. Association analysis was conducted to determine the association between gender, ethnicity and dialect with G6PD genotype.

CHAPTER 6

CONCLUSION

In conclusion, precautions in the screening of G6PD deficiency should be provided in order to prevent any mishandling of the samples either during the collection, transportation or due to long storage period of sample from the time of collection which might lead to inconsistent finding. This study incorporated of two screening tests, qualitative test (FST) and quantitative assay, with molecular characterisation to have comprehensive detection of G6PD deficiency. Sequencing analysis is possible to be included to rule out some unknown variants or as double confirmatory of variant detection. Blood transfusion from a G6PD deficient donor to the patients is unsafe as the haemolysis event may occur when exposed to oxidative stress. This condition could then harm the patient unintentionally if they are transfused with G6PD-deficient blood. Thus, it is crucial to detect G6PD deficiency among blood donors.

In this study, prevalence rate of G6PD deficiency was found up to 2.6% (10/392) among the blood donors in a university community, consisting of four hemizygous G6PD Kaiping, four heterozygous G6PD Kaiping, one hemizygous Quing Yang (Chinese-4) and one hemizygous G6PD Chinese-5. Most of the positive cases were

found among males (6/10) instead of the females (4/10). This was supported as G6PD deficiency is an X linked inherited disorder and males have only one X chromosome causing them to be more prone to be affected by the disease. Study showed that the positive cases would have no fluorescence in the FST test and depicted low enzyme activity level in the quantitative test. Different genotypes would affect the G6PD enzyme activity at different extent, thus leading to different severity of haemolysis. However, due to the low prevalence found in this study, it is not mandatory to perform all the tests.

To conclude, the FST test and the quantitative test shows that it might not be sufficient enough to diagnose the G6PD deficiency. It should be followed by the molecular analysis for a more proper and accurate result. It can also be followed by the sequencing process to further reconfirm the result. As this study was conducted for only three common G6PD variants among the blood donors, the possibility to variants other than that the tested three variants was unable to rule. Thus, this study suggests to include more variants detection for the complete delineation of G6PD variants.

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Appendix A

Ethical Approval form obtained from the UTAR Scientific and Ethical Review Committee (SERC).

UNIVERSITI TUNKU ABDUL RAHMAN
Wholly Owned by UTAR Education Foundation (Company No. 578227-M)

Re: U/SERC/97/2016

30 December 2016

Dr Teh Lai Kuan
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti
Bandar Baru Barat
31900 Kampar
Perak

Dear Dr Teh,

Ethical Approval For Research Project/Protocol

We refer to your application dated 5 December 2016 which was circulated for the consideration of the UTAR Scientific and Ethical Review Committee (SERC). We are pleased to inform that your application for ethical approval for your research project involving human subjects has been approved by SERC.

The details of your research project are as follows:

Research Title	Prevalence of α-, β-thalassaemia and G6PD Deficiency Disorders in a University Community
Investigator(s)	Dr Teh Lai Kuan Lim Li Fang (UTAR Undergraduate Student) Lee Kok Mun (UTAR Undergraduate Student)
Research Area	Science
Research Location	Kampar, Perak
No of Participants	350 participants
Research Costs	Self-funded
Approval Validity	30 December 2016 - 29 December 2017

The conduct of this research is subject to the following:

(1) The participants' informed consent be obtained prior to the commencement of the research.

(2) Confidentiality of participants' personal data must be maintained; and

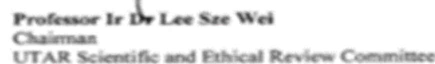
(3) Compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for Research Involving Humans and other related policies/guidelines.

Should you collect personal data of participants in your study, please have the participants in the research signed the attached Personal Data Protection Statement for your records.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,



Professor Ir Dr Lee Sze Wei
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Science
Director, Institute of Postgraduate Studies and Research

Appendix B

The consent form filled by volunteering donors in this study.

UNIVERSITI TUNKU ABDUL RAHMAN

Form Title : **VOLUNTEER INFORMATION AND CONSENT FORM**

Form Number : **FM-IPSR-R&D-057** | Rev No : 2 | Effective Date: **19/10/2015** | Page No : **1 of 4**

Application No. (Official use only)

(PARTICIPATION IN THIS RESEARCH IS VOLUNTARY)

1. Investigator's Name	:	**Faculty** :
Title of research project	:	Prevalence of α-, β-thalassaemia and G6PD deficiency disorders in a university community
Purpose of study	:	This study is to determine the prevalence of α-, β-thalassaemia and G6PD deficiency disorders and the predominant mutations in a university community.
Procedure	:	Venipuncture to collect 3 ml whole blood into EDTA vacutainers.
Risk and Discomfort	:	None except there may be some slight pain, bruising or bleeding on the site of venipuncture.
Benefit	:	Information obtained from this study will help to improve the prevention of α-, β-thalassaemia and G6PD deficiency disorders. The mutations causing α-, β-thalassaemia and G6PD deficiency disorders will be identified. Result of the identification will be sent to the respective respondents for healthcare awareness.
Payment	:	NA
Alternatives	:	NA
Contact Person	:	TEH LAI KUAN (tehlk@utar.edu.my; +6016-5580938)

Note: 1. All volunteers involved in this study will not be covered by insurance
2. Contact person must be the principal investigator/supervisor

2. Particulars of Volunteer (Volunteer Identifier/Label)
(Please use separate form if more than one volunteer)

Full Name :

Chinese character *(if applicable)* :

New Identity Card/ Passport No. : Gender :

Contact No. :

Email :

UNIVERSITI TUNKU ABDUL RAHMAN

Form Title : **VOLUNTEER INFORMATION AND CONSENT FORM**

Form Number : **FM-IPSR-R&D-057** | Rev No : 2 | Effective Date: **19/10/2015** | Page No : **2 of 4**

3. Medical History

A brief medical history will be taken as detailed in **Appendix A**, if applicable.

4. Voluntary participation

You understand that participation in this study is voluntary and that if you decide not to participate, you will experience no penalty or loss of benefits to which you would otherwise be entitled. If you decide to participate, you may subsequently change your mind about being in the study, and may stop participating at any time. You understand that you must inform the principal investigator of your decision immediately.

5. Available Medical Treatment

If you are injured during your participation or in the course of the study or whether or not as a direct result of this study, UTAR will not be liable for any loss or damage or compensation or absorb the costs of medical treatment. However, assistance will be provided to you in obtaining emergency medical treatment.

6. Confidentiality

All information, samples and specimens you have supplied will be kept confidential by the principal investigator and the research team and will not be made available to the public unless disclosure is required by law.

7. Disclosure

Data, samples and specimens obtained from this study will not identify you individually. The data, samples and specimens may be given to the sponsor and/or regulatory authorities and may be published or be reused for research purposes not detailed within this consent form. However, your identity will not be disclosed. The original records will be reviewed by the principal investigator and the research team, the UTAR Scientific and Ethical Review Committee, the sponsor and regulatory authorities for the purpose of verifying research procedures and/or data.

By signing this consent form, you authorize the record review, publication and re-utilisation of data, information and sample storage and data transfer as described above

8. Declaration

I have read or have the information above read to me, in the language understandable to me. The above content has been fully explained to me.

I have asked all questions that I need to know about the study and this form. All my questions have been answered. I have read, or have had read to me, all pages of this consent form and the risks described. I voluntarily consent and offer to take part in this study. By signing this consent form, I certify that all information I have given, including my medical history, is true and correct to the best of my knowledge. **I will not hold UTAR or the research team responsible for any consequences and/or liability whatsoever arising from my participation in this study.**

9. Consent

If you wish to participate in this study, please sign below.

Signature of Volunteer — IC/Passport No.

Name of Volunteer — Date

Signature of witness — IC/Passport No.

Name of witness — Date

10. Statement of Principal Investigator/Supervisor

I have fully explained to the volunteer taking part in this study what he / she can expect by virtue of his / her participation. The volunteer who is giving consent to take part in this study

- Understands the language that I have used.
- Reads well enough to understand this form, or is able to hear and understand the contents of the form when read to him or her.
- Is of the age of majority of 18 or above.

To the best of my knowledge, when the volunteer signed this form, he or she understands:

- That taking part in the study is voluntary.
- What the study is about.
- What needs to be done.
- What are the potential benefits.
- What are the known risks.

A copy of this consent form has been given to the volunteer.

Teh Lai Kuan — 840808-08-5118

Name of Principal Investigator/Supervisor — IC/Passport No.

Signature of Principal Investigator/Supervisor — Date

Note: *1. The principal investigator/supervisor conducting the informed consent process, must sign* ***and date form at the same time as the volunteer.***

Appendix A

Project Title :	Prevalence of α-, β-thalassaemia and G6PD deficiency disorders in a university community	Application No. (As provided by UTAR)	
		Volunteer Identifier / Label	

Medical History of Volunteer			
Have you ever had any of the following:		**Yes**	**No**
a	a serious illness or accident?		
b	an operation/ investigative procedure?		
c	yellow jaundice or hepatitis?		
d	tuberculosis?		
e	malaria?		
f	a tattoo?		
g	a blood transfusion?		
h	contact with any infectious disease?		
i	heart disease?		
j	high blood pressure (>140/90 mmHg)?		
k	asthma?		
l	kidney disease?		
m	diabetes?		
n	a stomach ulcer?		
Do you or family have any of the following:			
o	Cancer?		
p	Is a HIV carrier?		
q	psychiatric disease/ mental problem?		

If **human tissues samples** collected (*please complete the following*)

Sample		**Sampling**			
Identification	Type	Date	Location	Process (*if applicable*)	
				Random	Others (*please specify*)

Signature of Principal Investigator/Supervisor

Appendix C

Detailed protocol of Fluorescence Spot Test (FST).

RANDOX

G-6-PDH DEFICIENCY (G-6-PDH)

SCREENING TEST MANUAL

INTENDED USE

Screening test for glucose-6-phosphate dehydrogenase (G-6-PDH). This product is suitable for Manual use.

Cat. No.

PD 2616	R1a.	Substrate	3 x 25 ml
3 x 250 tests	R1b.	Buffer	1 x 80 ml
		Test paper	34 sheets

PRINCIPLE

$$\text{Glucose-6-P} + NADP^+ \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-P} + NADPH + H^+$$

The production of NADPH causes fluorescence under long wave UV light. If G-6-PDH is not present or strongly deficient no fluorescence will be observed.

SAMPLE COLLECTION AND PREPARATION

Whole blood dried on filter paper(1) (a drop of blood on absorbent paper ("Guthrie Test Paper") Schleicher and Schuell No: 2992) when completely dry is stable for one week at +20 to +25°C.

Whole blood (instead of dried blood). Heparin, citrate, oxalate and EDTA are suitable anticoagulants. Blood stable for up to 7 days. Use 0.005 ml for the assay.

REAGENT COMPOSITION

Contents	Concentrations in Test
R1a. Substrate	
Glucose-6-P	1 mmol/l
NADP	0.75 mmol/l
GGSG (oxidised glutathione)	0.8 mmol/l
R1b. Buffer	
Saponin	0.2%
Tris (hydroxymethyl)-aminomethane	225 mmol/l ; pH 7.8

SAFETY PRECAUTIONS AND WARNINGS

For *in vitro* diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Solution R1b contains Sodium Azide. Avoid ingestion or contact with skin or mucous membranes. In case of skin contact, flush affected area with copious amounts of water. In case of contact with eyes or if ingested, seek immediate medical attention.

Sodium Azide reacts with lead and copper plumbing, to form potentially explosive azides. When disposing of such reagents flush with large volumes of water to prevent azide build up. Exposed metal surfaces should be cleaned with 10% sodium hydroxide.

Health and Safety Data Sheets are available on request.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

STABILITY AND PREPARATION OF REAGENTS

Reconstitute the contents of one vial of substrate (R1a) with 25 ml of buffer (R1b). Stable for 4 weeks at +4°C or 3 months at -20°C.

MATERIALS PROVIDED

Substrate
Buffer
Test paper

MATERIALS REQUIRED BUT NOT PROVIDED

Long-wave U.V.-lamp
Randox G-6-PDH Controls, Deficient (Cat. No PD 2617) and Normal (Cat. No. PD 2618)

SAMPLE PREPARATION

Punch out a disk of blood stained paper of 5 mm diameter (3 mm is also suitable).

PROCEDURE(2)

Add the sample disk of blood stained filter paper to a small vial (1-3 ml) containing 100 µl of working reagent. Mix well and incubate for 10 minutes at 25°C. Then take 10 µl of the resulting solution and place on the paper provided and leave to dry.

NOTES

1. In this method it is necessary to separate older erythrocytes from the young ones which exhibit normal enzyme activity in some forms of G-6-PDH deficiency. For this reason, it is not recommended that this assay be performed after a severe haemolytic crisis, since G-6-PDH levels may appear falsely elevated. Blood from patients must first be treated according to the method described by Herz *et al* [3]. Use 0.005 ml of the resulting suspension for the assay.
2. This test is clinically significant only 30 days after a blood transfusion, as the donors erythrocytes will exhibit a normal G-6-PDH activity and will therefore give an elevated result.
3. U.V. lamps emitting long wave UV-light are suitable for this procedure.

QUALITY CONTROL

Randox G-6-PDH Controls, Deficient and Normal are recommended for daily quality control. Two levels of controls should be assayed at least once a day. If the results are not as expected, contact Randox Laboratories Customer Technical Support, Northern Ireland +44 (0) 28 9442 2413.

EVALUATION OF RESULTS

When the filter paper is completely dry (approximately 1 hour) view under a long wave UV-lamp in a darkened room.

Samples with normal or slightly depressed G-6-PDH activity will show strong fluorescence.

No fluorescence after a 10 minute incubation period, suggests complete lack or marked deficiency of G-6-PDH.

Randox Laboratories Limited, 55 Diamond Road, Crumlin, County Antrim, BT29 4QY, United Kingdom
T +44 (0) 28 9442 2413 F +44 (0) 28 9445 2912 E applications@randox.com I www.randox.com

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Appendix D

Detailed protocol of quantitative enzyme activity level- OSMMR.

10. Assay Procedure

1. Punch blood spots 4.7 mm (3/16") diameter [or 2 x 3.2 mm (1/8")] in U-bottom microtiter plate (Elution Plate).
   Alternatively you can use 5 µl of whole blood (with anticoagulant).
   Use position A1 and A2 for 5 µl of Normal Control.
   Use position A3 and A4 for 5 µl of Deficient Control.
2. Add 75 µl of Elution Buffer to each well.
3. Place U-bottomed microtiter plate on an orbital plate shaker for 30 min at room temperature (10 min for whole blood samples). This step should be performed at room temperature.

4. During the elution, reconstitute Reagents to prepare The Reagent Mixture. The reagents should be warmed up slowly to 30 or 37°C prior to use if this is the temperature the assay is going to be performed at.
5. Add 75 µl of the Reagent Mixture to the corresponding wells of a new F-bottomed microtiter plate (Assay Plate).
6. Transfer 15 µl of the eluant from each U-well to the corresponding well in the Assay Plate and mix thoroughly.
7. Add 100 µl of the prepared Color Reagent Mixture (CRM) to each well.
8. Read the plate in a plate reader at **550 nm** (500-570 nm) for 12-15 minutes with 60 seconds intervals (kinetic mode). Alternatively an endpoint mode can be used, taking two measurements one at time = 0 and a second one at time = 12-15 minutes later. The latter protocol is encouraged if your microplate reader is not equipped with kinetic software. If an incubator is used, it is advised to place the microplate in during measurements.
9. After the final reading at 550 nm is taken, read the plate at wavelength 405 nm (to get the Hb content of each sample)

Appendix E

Detailed protocol of DNA purification for sequencing.

FavorPrep™ GEL/ PCR Purification Kit

- For extraction of DNA fragments from agarose gel
- For purification of PCR products or reaction mixtures (concentration and desalination of reaction mixtures)

Cat. No.: FAGCK 000
FAGCK 001
FAGCK 001-1

(For Research Use Only)

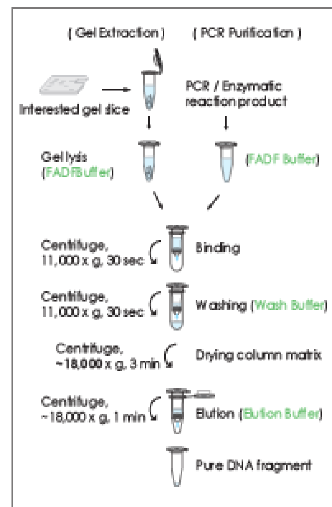
Kit Contents:

Cat. No:	FAGCK 000 (4 preps_sample)	FAGCK 001 (100 preps)	FAGCK 001-1 (300 preps)
FADF Buffer	3 ml	80 ml	240 ml
Wash Buffer (concentrate)[a]	1 ml	25 ml	50 ml
Elution Buffer	0.5 ml	6 ml	30 ml
FADF Column	4 pcs	100 pcs	300 pcs
Collection Tube	4 pcs	100 pcs	300 pcs
User Manual	1	1	1
Preparation of Wash Buffer by adding ethanol (96 ~ 100%)			
Ethanol volume for Wash Buffer[a]	4 ml	100 ml	200 ml

Specification:

- Principle: spin column (silica matrix)
- DNA Binding capacity of spin column: 20 μg
- Sample size: up to 300 mg of agarose gel
  up to 100 μl of reaction solution
- DNA size: 65 bp ~ 10 kbp
- Recovery: 70% ~ 85% for Gel extraction
  90% ~ 95% for PCR clean-up
- Operation time: 10 ~ 20 min
- Elution volume: 40 μl

Brief procedure:



Important Notes:

1. Buffer provided in this kit contain irritants. Wear gloves and lab coat when handling these buffer.
2. Add the required volume of ethanol (96~ 100%) to Wash Buffer before use.
3. Centrifugation steps are done by a microcentrifuge capable of the speed at 11,000 ~1,8000 x g.

Gel Extraction Protocol: For extraction of DNA fragments from agarose gel

Please Read Important Notes Before Starting Following Steps.

HINT: Prepare a 55 °C dry bath or water bath for step 4.

1. **Excise the agarose gel with a clean scalpel.**
   - Remove the extra agarose gel to minimize the size of the gel slice.
2. **Transfer up to 300 mg of the gel slice into a microcentrifuge tube.**(not provided).
   - The maximum volume of the gel slice is 300mg.
3. **Add 500 μl of FADF Buffer to the sample and mix by vortexing.**
   - For > 2% agarose gels, add 1000 μl of FADF Buffer.
4. **Incubate at 55 °C for 5 ~10 minutes and vortex the tube every 2 ~ 3 minutes until the gel slice dissolved completely.**
   - During incubation, interval vortexing can accelerate the gel dissolved.
   - Make sure that the gel slice has been dissolved completely before proceed the next step.
   - After gel dissolved, make sure that the color of sample mixture is yellow. If the color is violet, add 10 μl of sodium acetate, 3M, pH 5.0. Mix well to make the color of sample mixture turned to yellow.
5. **Cool down the sample mixture to room temperature. And place a FADF Column into a Collection Tube.**
6. **Transfer 800 μl of the sample mixture to the FADF Column. Centrifuge at 11,000 x g for 30 seconds, then discard the flow-through.**
   - If the sample mixture is more than 800 μl, repeat this step for the rest of the sample mixture.
7. **Add 750 μl of Wash Buffer (ethanol added) to the FADF Column. Centrifuge at 11,000 x g for 30 seconds, then discard the flow-through.**
   - Make sure that ethanol (96-100 %) has been added into Wash Buffer when first use.
8. **Centrifuge again at full speed (~ 18,000 x g) for an additional 3 minutes to dry the column matrix.**
   - **Important step !** The residual liquid should be removed thoroughly on this step.
9. **Place the FADF Column to a new microcentrifuge tube (not provided).**
10. **Add 40 μl of Elution Buffer or ddH_2O to the membrane center of the FADF Column. Stand the FADF Column for 1 min.**
    - **Important step !** For effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely.
    - **Important :** Do not elute the DNA using less than suggested volume (40 μl). It will lower the final yield.
11. **Centrifuge at full speed (~ 18,000 x g) for 1 min to elute the DNA.**

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PCR Clean-Up Protocol: For purification of PCR products or reaction mixtures

Please Read Important Notes Before Starting Following Steps

1. **Transfer up to 100 µl of PCR product (excluding oil) to a microcentrifuge tube (not provided) and add 5 volumes of FADF Buffer, mix well by vortexing.**
   - For example, Add 250 µl of FADF Buffer to 50 µl of PCR product.
   - The maximum volume of PCR product is 100 µl (excluding oil). Do not excess this limit. If PCR product is more than 100 µl, separate it into multiple tubes.
2. **Place a FADF column into a Collection Tube.**
3. **Transfer the sample mixture to the FADF Column. Centrifuge at 11,000 x g for 30 seconds, then discard the flow-through.**
4. **Add 750 µl of Wash Buffer (ethanol added) to the FADF Column. Centrifuge at 11,000 x g for 30 seconds, then discard the flow-through.**
   - Make sure that ethanol (96-100 %) has been added into Wash Buffer when first open.
5. **Centrifuge again at full speed (~18,000 x g) for an additional 3 minutes to dry the column matrix.**
   - **Important step !** The residual liquid should be removed thoroughly on this step.
6. **Place the FADF Column to a new microcentrifuge tube (not provided).**
7. **Add 40 µl of Elution Buffer or ddH_2O to the membrane center of the FADF Column. Stand the FADF Column for 1 min.**
   - **Important step !** For effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely.
   - **Important :** Do not elute the DNA using less than suggested volume (40 µl). It will lower the final yield.
8. **Centrifuge at full speed (~18,000 x g) for 1 min to elute the DNA.**

Troubleshooting

(For Gel Extraction)

Problems	Possible reasons	Solutions
The gel slice is hard to dissolve	Agarose gel of high percentage (> 2 %) is used	Add 1000 µl of FADF Buffer to 1 volume of the gel slice.
	The size of the gel slice is too large	If the gel slice is more than 300 mg, separate it into multiple tubes.
Low or none recovery of DNA fragment	The column is loaded with too much agarose gel	The maximum volume of the gel slice is 300 mg per column.
	Elution of DNA fragment is not efficient	Make sure the pH of Elution Buffer or ddH_2O is between 7.0- 8.5.
		Make sure that the elution solution has been completely absorbed by the membrane before centrifuge.
	The size of DNA fragment is larger than 5 Kb	Preheat the elution solution to 60 °C before use.
Eluted DNA contains non-specific DNA fragment	Contaminated scalpel	Using a new or clean scalpel.
	DNA fragment is denatured	Incubate eluted DNA at 95 °C for 2 min, then cool down slowly to reanneal denatured DNA.
Poor performance in the downstream applications	Salt residue remains in eluted DNA fragment	Wash the column twice with Wash Buffer.
	Ethanol residue remains in eluted DNA fragment	Do discard the flow-through after washing with Wash Buffer and centrifuge for an additional 3 min.

(For PCR Clean-Up)

Problems	Possible reasons	Solutions
Low or none recovery of DNA fragment	Apply more than 100 µl of PCR product	If PCR product is more than 100 µl, separate it into multiple tubes.
	Elution of DNA fragment is not efficient	Make sure the pH of Elution Buffer or ddH_2O is between 7.0- 8.5.
		Make sure that the elution solution has been completely absorbed by the column membrane before centrifugation.
	The size of DNA fragment is larger than 5 Kb	Preheat the elution solution to 60 °C before use.
Poor performance in the downstream applications	Salt residue remains in eluted DNA	Wash the column twice with Wash Buffer.
	Ethanol residue remains in eluted DNA	Do discard the flow-through after washing with Wash Buffer and centrifuge for an additional 3 min.