STUDY OF DNA FOR MOLECULAR ANALYSIS AND BIOCHEMICAL MARKERS FROM NON-INVASIVE SAMPLES IN BETA-THALASSAEMIA MAJOR PATIENTS

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DESSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Registration/Matric No: MGN090018

Name of Degree: MASTERS OF MEDICAL SCIENCE

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

STUDY OF DNA FOR MOLECULAR ANALYSIS AND BIOCHEMICAL MARKERS FROM NON-INVASIVE SAMPLES IN BETA-THALASSAEMIA MAJOR PATIENTS

Field of Study: MOLECULAR GENETICS AND BIOCHEMISTRY

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ABSTRACT

Beta-thalassaemia is a common genetic disorder in Malaysia. It is a haemolytic anaemia which is caused by mutations within the β-globin gene complex, affecting the synthesis of β-globin chains. This will result in excessive free α-globin chains causing α-globin chain toxicity. Erythropoiesis is also impaired leading to chronic anaemia. Beta-thalassaemia major babies may appear healthy at birth. However, symptoms such as jaundice and anaemia will start to develop as they reach six months of life. They require frequent transfusions to maintain haemoglobin levels which lead to iron-overload. Although chelation therapy is recommended, the patients are still under oxidative stress. Patients need to be monitored during their therapy to prevent any organ damage and mortality due to oxidative injuries. The current sampling method used to diagnose and monitor the β-thalassaemia major patients involved the use of venous blood. The sampling method is invasive and requires a phlebotomist to perform the procedure with minimal pain to paediatric patients. Assessment of non-invasive methods as alternative sampling procedure will be advantageous for the molecular and biochemical analysis of β-thalassaemia. The present study aims to genotype purified DNA extracted from non-invasive samples including mouthwash, saliva and buccal cytobrush samples and to assess the biochemical markers from saliva samples. Samples were collected from β-thalassaemia major patients in University Malaya Medical Centre and healthy individuals. DNA was extracted using two alkaline lysis DNA extraction methods followed by organic purification to compare the concentration and purity. The purified DNA was amplified using various DNA amplification methods available to detect β-globin gene mutation present in the Malaysian population. Saliva samples were assessed for total non-enzymatic antioxidant capacity, level of protein and lipid peroxidation, activity of glutathione peroxidase (GPx), level of uric acid (UA) and

cytokines tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6). Biochemical parameters were further analysed using parametric and non-parametric statistical analysis. Saliva samples provided highest amount of purified DNA compared with mouthwash and buccal cytobrush samples. In contrast, the DNA purity was the highest from mouthwash samples. DNA extraction Method 2, which used higher concentration of lysis agents and additional purification steps compared with Method 1, provided purified DNA with better reproducibility. The surface area of sample collection site and the amount of leukocytes may have contributed to the high purified DNA concentration while the amount of mucin contributed to the purity. The level of GPx was higher in β-thalassaemia major patients. Strong correlation was also observed between ferric reducing antioxidant power (FRAP) assay and UA. When the β-thalassaemia major patients group was further sub-divided, there was a notable difference in the level of AOPP between genetic classification of β-thalassaemia and the level TNF-α between ethnicity and age groups. Better chelation due to combination therapy, compliance, education and patient management may have helped in improving the oxidative stress status in β-thalassaemia major patients. In conclusion, mouthwash and saliva can provide high quality purified DNA for reproducible molecular analysis and biochemical parameters in saliva samples are within detectable limits for biochemical assays.

ABSTRAK

Beta-talasemia merupakan penyakit genetik yang lazim di Malaysia. Ia merupakan penyakit anemia hemolitik yang disebabkan oleh mutasi di kompleks gen β-globin, yang memberi kesan kepada penghasilan rantaian β-globin. Kecacatan ini mengakibatkan lebihan rantaian α-globin terbebas dan keracunan α-globin. Eritropoesis turut terganggu lalu menyebabkan anemia yang berpanjangan. Bayi β-talasemia kelihatan sihat ketika lahir. Namun, tanda-tanda seperti jaundis dan anemia mula kelihatan apabila bayi mencecah umur enam bulan. Mereka memerlukan pemindahan darah yang kerap untuk mengekalkan tahap hemoglobin, yang akhirnya membawa kepada masalah lebihan zat besi. Walaupun rawatan kelasi disarankan, masalah tekanan oksidatif masih dapat diperhatikan pada pesakit. Mereka perlu dipantau sepanjang rawatan bagi mengelakkan kerosakan organ dan kematian akibat kecederaan oksidatif. Kaedah pengambilan sampel yang masih digunakan kini untuk mendiagnos dan memantau pesakit melibatkan pengambilan darah vena. Kaedah ini adalah invasif dan memerlukan ahli flebotomi untuk melakukannya dengan kesakitan minima terhadap pesakit pediatrik. Penilaian terhadap sampel tidak invasif pastinya akan memberi faedah dalam analisis molekular dan biokimia β-talasemia. Kajian ini bertujuan untuk menganalisa DNA dari sampel kumuran, air liur dan kesatan mulut dan menganalisa penanda biokimia dalam air liur. Sampel dikutip dari pesakit β-talasemia major di Pusat Perubatan Universiti Malaya dan individu sihat. DNA diasingkan menggunakan dua kaedah lisis beralkali diikuti penulenan menggunakan pelarut organik bagi membandingkan kepekatan dan ketulenannya. DNA tertulen diganda dengan pelbagai kaedah untuk mengesan mutasi gen β-globin yang lazim dalam populasi Malaysia. Air liur dianalisa untuk mengesan jumlah kapasiti antioksida bukan enzimatik, tahap peroksidaan protein dan lemak, aktiviti glutation peroksidase (GPx), tahap asid urik

(UA) dan sitokin ‘tumor necrosis factor-α’ (TNF-α) dan ‘interleukin-6’ (IL-6). Parameter biokimia dinilai dengan ujian statistik parametrik dan bukan parametrik. Air liur menghasilkan DNA berkepekatan tinggi berbanding kumuran dan kesatan pipi. Sebaliknya, DNA paling tulen diperoleh dari sampel kumuran. Kaedah pengasingan DNA ke-2, yang menggunakan agen lisis berkepekatan lebih tinggi dan penambahan langkah penulenan berbanding kaedah pertama, menghasilkan DNA tertulen yang lebih mudah disalin semula. Luas permukaan kawasan pengambilan sampel dan kandungan leukosit mungkin memberi kesan terhadap kepekatan DNA manakala kandungan musin mempengaruhi ketulenan DNA. Tahap aktiviti GPx lebih tinggi di kalangan pesakit β-talasemia major. Hubungan kukuh dapat diperhatikan antara ‘ferric reducing antioxidant power’ (FRAP) dan UA. Apabila kumpulan pesakit β-talasemia major dipecahkecilkan, terdapat perbezaan ketara pada tahap AOPP mengikut klasifikasi genetik β-talasemia dan tahap TNF-α mengikut kumpulan etnik dan umur. Rawatan kelasi yang lebih baik melalui terapi gabungan, kesesuaian, pengetahuan terhadap terapi dan pengurusan pesakit mungkin membantu memperbaiki status tekanan oksidatif di kalangan pesakit β-talasemia major. Kesimpulannya, sampel kumuran dan air liur mampu membekalkan DNA berkualiti tinggi bagi analisis molekular dan kepekatan parameter biokimia dalam air liur berada di tahap yang mampu dikesan bagi analisa biokimia.

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ACKNOWLEDGEMENTS

"By the name of Allah, The Most Beneficent and The Most Merciful"

All praise due to Allah, I was able to complete this dissertation and the Degree of Master of Medical Science.

First and foremost, I would like to express my gratitude to my advisors Professor Dr Mary Anne Tan Jin Ai and Professor Dr Umah Rani Kuppusamy from the Department of Biomedical Science, Faculty of Medicine, University of Malaya for their advice and guidance in understanding the molecular genetics and biochemistry research in thalassaemia.

My sincere appreciation to the University of Malaya for the support by providing the financial aid, including tutorship and research grant (PS199/2009C) and well equipped laboratory for the research.

Special acknowledgement is addressed to staff of Department of Biomedical Science, Faculty of Medicine, University of Malaya and Peadiatrics Day Care 6, University Malaya Medical Centre for their guidance and cooperation on laboratory equipment and subject recruitment during the course of this research.

Special thanks are also extended to my parents, Abd Rahim bin Yang and the late Normala binti Shuib and my family for their moral support and understanding which enabled me to complete my study.

Last but not least, my sincere appreciation to my colleagues from Thalassaemia Genetics Laboratory, Biochemistry Laboratory and the Department of Biomedical Science, Faculty of Medicine, University of Malaya for their support and advice throughout the study.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
<	Less than
>	More than
±	Plus/minus
°C	Degree Celsius (centigrade)
µg	Microgram
µg/µL	Microgram per microlitre
µg/L	Microgram per litre
µg/mL	Microgram per mililitre
µL	Microlitre
µL/mL	Microlitre per mililitre
µM	Micromolar
•OH	Hydroxyl radical
3'	3 prime
5'	5 prime
A_{230nm}	Absorbance at 230 nm
A_{260nm}	Absorbance at 260 nm
A_{280nm}	Absorbance at 280 nm
ANOVA	Analysis of Variance
AOPP	Advanced oxidation protein product
ARMS	Amplification Refractory Mutation System
Avidin-HRP	Avidin-horseradish peroxidase
bp	Base pair
BSA	Bovine serum albumin
C-ARMS	Combine-Amplification Refractory Mutation System
CD	Codon
cm	Centimeter
DFO	Deferoxamine
DFP	Deferiprone
DFS	Deferasirox
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiotrietol
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al	*et alia*, (Latin) and other
Fe^{2+}	Ferrous (iron (II)) ion
Fe^{3+}	Ferric (iron (III)) ion
$FeCl_3.6H_2O$	Ferric chloride hexahydrate

$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
fL	Femtolitre
FRAP	Ferric reducing antioxidant power
g	Gram
g	Gravity
g/dL	Gram per decilitre
GCF	Gingival crevicular fluid
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HbA	Haemoglobin A
HbA_2	Haemoglobin A2
HbE	Haemoglobin E
HbF	Haemoglobin F
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
in utero	Latin, in the womb
IVS	Intervening sequence
kb	Kilobase
KI	Potassium iodide
LOOH	Lipid hydroperoxide
M	Molar (mole per litre)
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscualr Volume
mg/kg	Miligram per kilogram
mg/mL	Miligram per microlitre
$MgCl_2$	Magnesium chloride
min	Minute
mL	Mililitre
mM	Milimolar
MPI	1-methyl-2-phenyl-indole
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NaCl	Sodium chloride
NADP+	Nicotinamide adenine dinucleotide phosphate

NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium acetate
$NaOAc.3H_2O$	Sodium acetate trihydrate
nm	Nanometer
nmol	Nanomole
nmol/µL	Nanomole per microlitre
nmol/min/mL	Nanomole per minute per mililitre
nmol/mL	Nanomole per mililitre
$O_2•^-$	Superoxide anion
p	*p* value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR-Restriction fragment length polymorphism
pg	Picogram
pg/mL	Picogram per mililitre
pmol	Picomole
r	Pearson's coefficient
RE	Restriction enzyme
RM	Ringgit Malaysia
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
r_s	Spearman's rho
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TEP	1,1,3,3-tetraethoxypropane
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TPTZ	2,4,6-tripyridyl-triazine
Tris	Tris(hydroxymethyl)aminomethane
UA	Uric acid
UMMC	University Malaya Medical Centre
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X	Times
α	Alpha
β	Beta
β^+	Beta plus

β^0	Beta nought
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
χ^2	Chi-squared

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Thalassaemia is a public health problem in Malaysia. About 4.5% of the Malaysian population are β-thalassaemia carriers and the estimated prevalence of β-thalassaemia major children is 2.1 in 1000 births (George, 2001). Until 2009, 4541 patients were registered in the Malaysian Thalassaemia Registry, with more than 3000 patients identified with Haemoglobin E (HbE)/β-thalassaemia and β-thalassaemia major (Abdul Wahab *et al.*, 2011).

Beta-thalassaemia occurs when there are point mutations, additions or deletions in the β-globin gene complex which is located on the short arm of chromosome 11. The effects of mutation on gene transcription result in reduced or absence of β-globin chain production. The reduced amount of β-globin chain production indirectly increases the level of unpaired α-globin chains. Insoluble free α-globin chains adhere easily to the cellular membranes of red blood cells and disrupt the integrity of the cell membranes. Red blood cells thus become more fragile and lead a shortened life span (Weatherall & Clegg, 2001).

Beta-thalassaemia carriers present as asymptomatic to mildly anaemic individuals. This asymptomatic manifestation causes β-thalassaemia carriers to not realise that they are carriers of a genetic disorder. Couples with β-thalassaemia have a 25% risk of producing a β-thalassaemia major child.

Beta-thalassaemia major babies appear to be normal in the early months of life. The effects of reduced or zero β-globin chain synthesis is not prominent as yet, since the most dominant haemoglobin for oxygen circulation in the early months of life is foetal haemoglobin (HbF). The symptoms begin to appear after the adult haemoglobin (HbA) replaces the HbF, at around six months of life. Beta-thalassaemia major babies develop jaundice, are anaemic, and require regular blood transfusions for survival. Molecular characterisation for β-thalassaemia mutations in parents who are carriers and prenatal diagnosis are performed to avoid the birth of thalassaemia major children (Cao & Kan, 2012).

The most common approach to obtain DNA for molecular analysis is blood sample collection. The most common site for blood drawing is the median cubital vein in the elbow. A skilful phlebotomist is required to perform the collection safely without causing discomfort or side effects such as haematoma and nerve injury (Rayegani & Azadi, 2007; Stitik *et al.*, 2001). Paediatric thalassaemia major patients are often frail and their veins collapse easily, thus making blood collection more difficult. Another option for blood collection is from the dorsal metacarpal vein but this is generally uncomfortable for the patients.

Recent studies involving non-invasive sampling for molecular and biochemical studies have gained popularity. Besides being painless, the procedure is simple and can be carried out by the patients or subjects themselves. Previous studies showed that the analytes in saliva were significantly correlated to that in serum or plasma (Sculley & Langley-Evans, 2002), and that DNA was successfully isolated from buccal cells for genomic studies (Lum & Marchand, 1998). The common non-invasive samples include mouthwash, saliva, and buccal swab.

The presence of oxidative stress in disease conditions has been well documented (Kassab-Chekir *et al.*, 2003; Livrea *et al.*, 1996). However most of these studies were based on blood oxidative stress indices levels. The assessment and evaluation of oxidative stress particularly in β-thalassaemia major patients is very crucial as they are susceptible to oxidative damage induced by iron overload. Regular assessment of oxidative stress status will be useful in the clinical management and proper intervention in order to improve the quality of life, reduce complications and mortality in β-thalassaemia patients.

A study on oxidative stress indices level on β-thalassaemia major patients attending blood transfusion in University of Malaya Medical Centre was previously carried out. The level of advanced oxidative protein products (AOPP), lipid hydroperoxide (LOOH), and enzyme activities such as glutathione peroxidase (GPx) and catalase were measured in plasma and peripheral blood mononuclear cell lysate (Kuppusamy & Tan, 2011).

The use of non-invasive techniques to obtain biological samples for DNA evaluation and biochemical assessment in paediatric patients will be well received by both patients and their parents. This study will establish techniques to effectively extract DNA from mouthwash, saliva and buccal cells for genotyping purposes and to assess the oxidative stress levels in thalassaemia major patients.

1.1 Objectives

The objectives of this study are:

1.1.1 Genotyping of DNA from non-invasive samples

i. To optimise and establish DNA extraction techniques from mouthwash, saliva and buccal swab samples from β-thalassaemia major patients
ii. To determine and compare the concentrations and purities of extracted DNA from the different samples
iii. To carry out molecular characterisation of DNA extracted from mouthwash, saliva and buccal cells using different DNA amplification techniques.

1.1.2 Assessment of biochemical markers in saliva samples

i. To estimate oxidative stress levels via measurement of lipid hydroperoxide, advanced oxidation protein products, ferric reducing antioxidant power, uric acid and glutathione peroxidase activity.
ii. To determine salivary inflammatory markers TNF-α and IL-6.

CHAPTER 2: LITERITURE REVIEW

2.1 Cooley's anaemia

Thalassaemia was first reported by Thomas Benton Cooley in 1925, following observation of four anaemic paediatric patients with hepatosplenomegaly and discoloration of the skin and sclera (white eye). Blood analysis showed that these patients presented with nucleated red blood cells, leukocytosis and resistance of red cells towards hypotonic lysis. The patients also presented with enlargement of facial and cranial bones described as "mongoloid appearance". These patients with Cooley's anaemia were later confirmed with homozygous β-thalassaemia (Cooley & Lee, 1925).

The term thalassaemia originated from the Greek words - 'thalassa' which means 'the sea', referring to the Mediterranean Sea and 'emia' which means 'blood'. This 'sea blood' referred to the high frequency of thalassaemia found in populations in the Mediterranean region. The thalassaemia genes are widely distributed among the Mediterranean population, including the populations in the Middle East and Southeast Asia.

In the early times, thalassaemia major patients did not survive even to the first decade of life. With the introduction of blood transfusion treatments, thalassaemia major patients can now live up to their third decade of life and longer by maintaining normal haemoglobin (Hb) levels (Piomelli *et al.*, 1969; Prabhu *et al.*, 2009).

2.2 Basics of red blood cells synthesis

2.2.1 Haematopoiesis and erythropoiesis in normal individuals

Haematopoiesis takes place during the first few weeks of gestation in the yolk sac of embryos. Starting from the sixth week until the sixth to seventh month *in utero*, production of blood cells is predominated by the liver and spleen and continues until the second week after birth.

The bone marrow takes over the process of haematopoiesis beginning from the sixth to seventh week after birth. During infancy, the bone marrow is involved in production of blood cellular components and production is more focused in the central skeleton and proximal ends of femurs and humeri towards adulthood. This is due to the progression of fatty/yellow marrow replacement starting in early childhood and involving marrow in the long bones (Hoffbrand & Pettit, 2000).

In a normal state, human erythrocytes are produced and develop to maturity in the red bone marrow. Differentiation of pluripotential stem cells to anucleated erythrocytes are regulated by various growth factors such as erythropoietin. Erythropoietin controls the erythrocyte production by maintaining the number of circulating erythrocytes (Besa *et al.*, 1992).

2.2.2 Beta-globin gene cluster and haemoglobin synthesis

The β-globin gene complex is located on the short (*p*) arm of chromosome 11 (Figure 2.1a). The cluster is approximately 34 kb long and consists of five functional globin genes, located from the 5' to 3' end; epsilon (ε)-, gamma-G ($^{G}\gamma$)-, gamma-A ($^{A}\gamma$)-, delta (δ)-, and β-globin genes. The genes located in this cluster are involved in production of globin chains of the β-globin family, and pair with the globin chains from the α-globin family to form functional haemoglobin (Weatherall & Clegg, 1979). The β-globin gene is 1.606 kb in length and consists of 3 exons and 2 introns (Figure 2.1b).

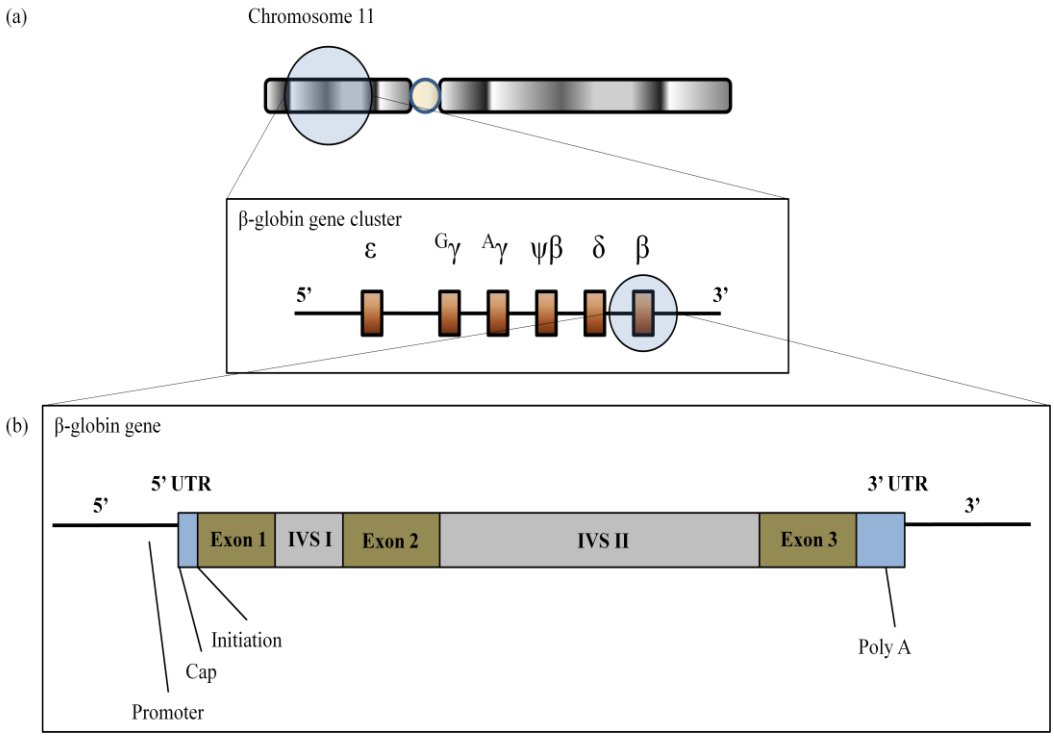

Figure 2.1a & b The β-globin gene complex on chromosome 11 and the β-globin gene
(Adapted from Thein (1998); Weatherall & Clegg (2001))

Haemoglobin molecules are tetrameric structures, made up of two pairs of different globin molecules attached together with one haem molecule in each globin chain. The genes present in the cluster are arranged according to the order of expression at different stages of life (Hoffbrand & Pettit, 2000). Table 2.1 summarises the type of haemoglobin present in human throughout the different stages of life.

Table 2.1 Haemoglobin synthesised at various stages of human life

Stage	**Haemoglobin**	**Globin chain**
Embryonic (Up to 6 weeks)	Hb Gower I	$\zeta_2\varepsilon_2$
	Hb Gower II	$\alpha_2\varepsilon_2$
	Hb Portland I	$\zeta_2\gamma_2$
	Hb Portland II (minor Hb)	$\zeta_2\beta_2$
	Hb Portland III (minor Hb)	$\zeta_2\delta_2$
Foetal	HbF	$\alpha_2\gamma_2$
Adult	HbA (97%)	$\alpha_2\beta_2$
	HbA_2 (2% - 3%)	$\alpha_2\delta_2$
	HbF (<1%)	$\alpha_2\gamma_2$

(Reference: Bunn & Forget (1986); Weatherall & Clegg (2001))

Starting from the thirteenth week of gestation, β-globin chain synthesis has already started with the production of adult haemoglobin (HbA) which comprises of two α-globin chains and two β-globin chains ($\alpha_2\beta_2$). Production increases gradually *in utero* until it reaches 20 - 40% of the total haemoglobin in the foetal circulation at birth, while HbF ($\alpha_2\gamma_2$) still functions as the main circulating haemoglobin (Turgeon, 2005).

After birth, the HbA level continues to increase while HbF reduces as HbA starts to take over the oxygen transport function. After 6 months of birth, HbA is the main functioning haemoglobin for cellular respiration and comprises over 95% of the total adult haemoglobin (Hoffbrand & Pettit, 2000).

In order to function properly, the amount of β-globin chains produced needs to correspond to the amount of α-globin chains. Disturbance in α- or β-globin chain synthesis will result in globin chain imbalance and produce abnormal haemoglobin. The abnormal haemoglobin will not be able to transport oxygen effectively and will lead to physiological problems in the affected individuals (Besa *et al.*, 1992).

2.3 Beta-thalassaemia

Beta-thalassaemia is a condition where productions of functional β-globin chains are reduced or absent. This leads to a condition of excessive amounts of free α-globin chains, which will precipitate in the form of inclusions. The inclusions damage the erythroid precursor cells, indirectly reducing the efficacy of erythropoiesis leading to anaemia (Cao *et al.*, 2000 ; Weatherall & Clegg, 2001).

2.3.1 Cause of β-thalassaemia

Beta-thalassaemia is mainly caused by point mutations. This includes single base substitutions, deletions and insertions within the β-globin gene (Weatherall & Clegg, 2001).

The effect of gene mutations depends on the location of the point mutation. For example, changes at the promoter site may reduce the β-globin chain synthesis by altering the mRNA transcription rate. On the other hand, mutations altering splicing sites may lead to improper mRNA translation and totally disrupt β-globin chain synthesis.

Beta-thalassaemia can also be caused by gene deletions. Large size deletions such as Filipino β-deletion and Thai (3.5 kb) deletions remove the entire β-globin gene. Thus, β-globin chains are not synthesised and this results in anaemia (Lynch *et al.*, 1991; Motum *et al.*, 1993; Ziffle *et al.*, 2011).

2.3.2 Pathophysiology of β-thalassaemia

A reduction of β-globin chain synthesis results in excessive amount of free α-globin chains, which is insoluble and will precipitate intracellularly. The precipitations will disturb DNA synthesis and halt mitosis of the precursor cells. Degradation products of α-globin chains can disturb cellular membranes and cause cells to be removed from circulation. Indirectly, this affects the efficiency of erythropoiesis (Weatherall & Clegg, 2001).

Erythropoietin levels are significantly elevated when haemoglobin levels drop to 7 g/dL or are reduced as a response to anaemia (Hammond *et al.*, 1962). Its action towards precursor cells in bone marrow stimulates production of erythrocytes. However, since erythropoiesis is ineffective, production is continuously stimulated to overcome hypoxia due to anaemia. Indirectly, the stimulation leads to bone marrow expansion and deformity, especially in facial bones (thalassaemia facie) (Hoffbrand & Pettit, 2000).

The spleen acts as a filter to remove defective blood cells and foreign bodies (Chen & Weiss, 1973; Kashimura & Fujita, 1987; Moghimi, 1995; Wandenvik & Kutti, 1988). The overproduction of defective erythrocytes due to excess free α-globin chains may cause the spleen to overwork and leads to splenomegaly. With all the formed elements trapped in the spleen due to congestion, anaemia may become more severe, and thrombocytopaenia and neutropaenia may occur.

Other health problems associated with β-thalassaemia major include expansion of plasma volume due to marrow expansion, iron overload, hepatomegaly, bone disease, and increased risk of blood-borne infections (Weatherall & Clegg, 2001).

2.3.3 Clinical classification of β-thalassaemia

Beta-thalassaemia is commonly classified according to the clinical manifestations presented by the patients. There are three classes of β-thalassaemia – β-thalassaemia minor, intermedia and major.

The minor form of β-thalassaemia usually presents as asymptomatic or mild anaemia. Another term commonly used to address β-thalassaemia minor is β-thalassaemia trait or carrier. Identification of a β-thalassaemia minor individual is through haematological screening and since only one of the β-globin genes is affected, the reduction of β-globin chain synthesis is not severe enough to cause severe anaemia. There is a group termed as 'silent' carriers and these patients appear asymptomatic and have normal haematology indices. They are heterozygotes for the thalassaemia mutations and are only identified by molecular screening.

The major form is the most severe manifestation of β-thalassaemia. With both β-globin genes affected, β-globin chain production is severely impaired or terminated. Patients usually start to present severe anaemia at the age of six months and require monthly blood transfusions. Insufficient production of HbA leads to elevation of HbF and slight increase in HbA_2.

Beta-thalassaemia intermedia is a condition where the patient does not present anaemia as severe as the major form, but still requires occasional blood transfusions to maintain the haemoglobin level at around 7 g/dL. The 'intermedia' term is mainly used in clinical practice as the disorder involves a different treatment regime based on the patient phenotype (Weatherall & Clegg, 2001).

2.3.4 Genetic classification of β-thalassaemia

Genetic classification of thalassaemia refers to the extent of reduction of globin chain synthesis. In β-thalassaemia, the main genotypes are β^0-thalassaemia and β^+-thalassaemia (Weatherall & Clegg, 2001).

In β^0-thalassaemia, β-globin chain synthesis does not occur. There is absence of HbA production. Individuals who are homozygous or compound heterozygotes exhibit β^0-thalassaemia phenotype. HbA is at zero percent and HbF can increase up to 98% (Telen & Kaufman, 1999).

On the other hand, β^+-thalassaemia shows a reduced amount of β-globin chain production. Homozygotes or heterozygotes that possess one of the many β^+-thalassaemia mutations show HbA production, but at a reduced level. There is also β^{++}-thalassaemia where the defects in β-globin production are less severe compared to β^+-thalassaemia.

2.4 Detection of β-thalassaemia

2.4.1 Haematological study for detection of β-thalassaemia

Globin chain accumulation and inadequate amount of haemoglobin production lead to destruction of erythrocytes and anaemia with hypochromasia and microcytosis. Peripheral blood film is used to observe the presence of abnormality in erythrocyte appearance and cell counting. Beta-thalassaemia patients usually present peripheral blood films with microcytic and hypochromic red blood cells, anisopoikilocytosis and presence of codocytes (target cells). In β-thalassaemia major patients, an abundance of nucleated red blood cells can be observed in the stained peripheral blood preparations.

Haematological indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) are evaluated and compared with values from normal individuals. Values of MCV and MCH are affected by cellular size and haemoglobin concentration, respectively. Thus, microcytosis and hypochromasia produce an impact on both index levels (Turgeon, 2005). The value of MCHC may be slightly reduced or remain at a normal level since both haemoglobin and haematocrit are reduced and produce a slight effect on the ratio. A comparison of values of the haematological indices is presented in Table 2.2.

Table 2.2 Comparison of haematological indices between normal individuals (male and female), β-thalassaemia carriers and β-thalassaemia major patients

Haematological Indices	Normal		β-thalassaemia carrier	β-thalassaemia major
	Male	Female		
Haemoglobin (Hb, g/dL)	15.9±1.0	14.9±0.9	Male: 11.5-15.3 Female: 9.1-14.0	<7.0
Mean corpuscular volume (MCV, fL)	89.1±5.01	87.6±5.5	<79	50-70
Mean corpuscular haemoglobin (MCH, pg)	30.9±1.9	30.2±2.1	<27	12-20

(Reference: Galanello *et al.* (1979))

However, microcytic and hypochromic anaemia does not always indicate thalassaemia. Various underlying factors may also lead to these symptoms – anaemia of chronic disease, iron deficiency anaemia and sideroblastic anaemia (Dacie & Lewis, 1994). Thus, subsequent testing must be carried out depending on the preliminary results obtained on cellular appearance and haematological indices. A general scheme of investigation carried out for microcytic anaemia screening and expected findings is represented in Figure 2.2.

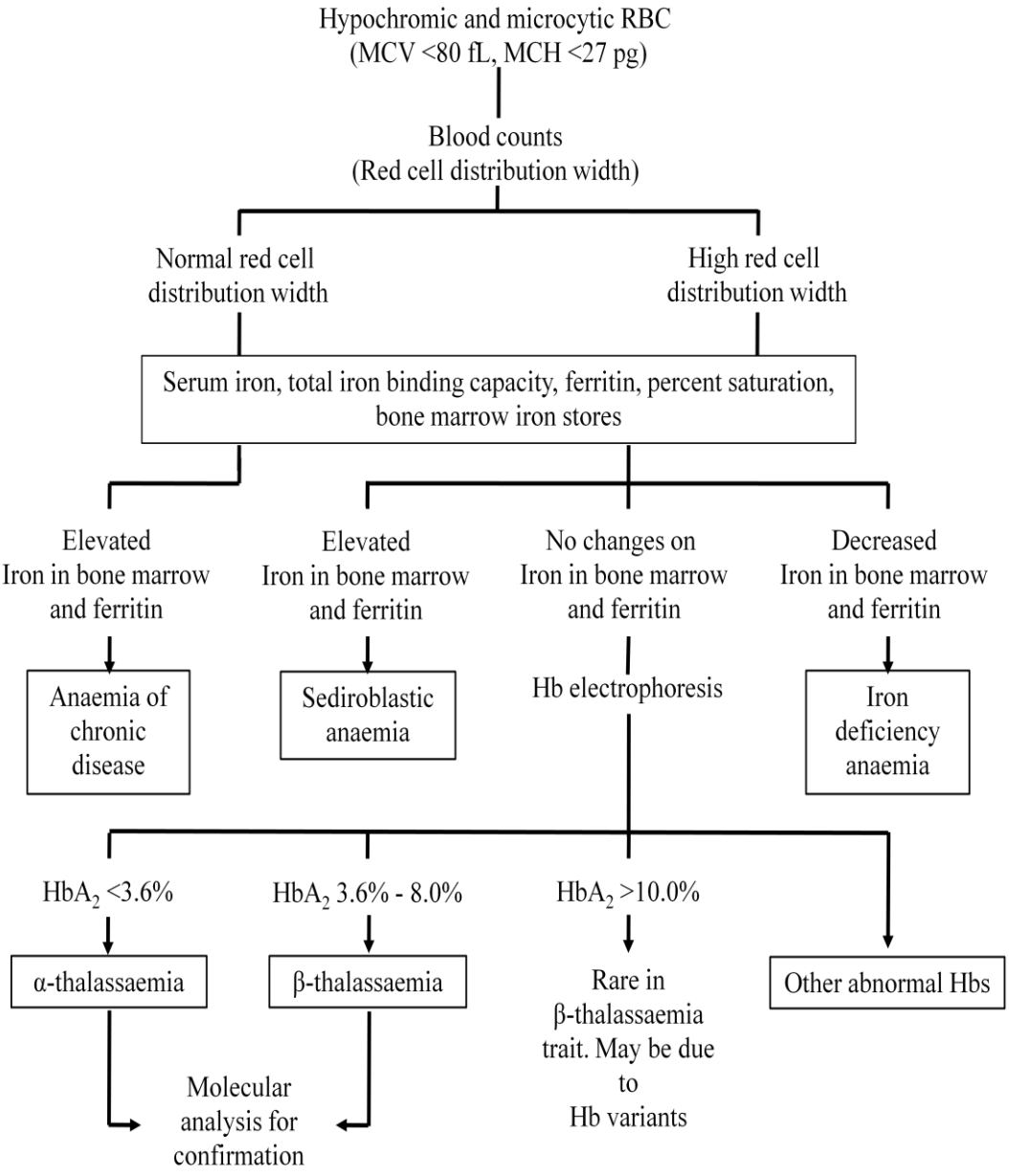

Figure 2.2 Investigation scheme for thalassaemia and abnormal haemoglobins (Reference: Besa *et al.* (1992); Dacie & Lewis (1994))

2.4.2 Specific haematology tests

Determination of iron status is carried out on subjects with MCV levels lower than 80 fL and MCH less than 27 pg. This includes determination of serum ferritin level, total iron binding capacity and estimation of soluble transferrin protein. Performing these tests is crucial to rule out iron deficiency anaemia, since iron deficiency may also lower the HbA_2 level and mask the presence of β-thalassaemia in the subjects (Bates & Bain, 2006).

Further investigation to detect haemoglobin subtypes are carried out using haemoglobin electrophoresis on cellulose acetate at alkaline pH (pH 8.2 - 8.6). In alkaline pH, haemoglobin molecules have a net negative charge and will move towards the anode of the electrophoresis system. Haemoglobin variants have different net charges, causing the molecules to migrate at different rates. This indirectly allows differentiation of variants present in the subject's blood. This method can be used to detect the presence of HbE and Hb Lepore (Kohn, 1969; Turgeon, 2005).

Quantitation of HbA_2 is confirmed by high performance liquid chromatography (HPLC) and microcolumn chromatography. Estimation of HbF levels can be carried out by haemoglobin denaturation at alkaline pH or through HPLC. Elevated levels of HbA_2 and HbF suggest that the subject may have β-thalassaemia. The haemoglobin subtype levels in β-thalassaemia patients are summarised in Table 2.3.

Table 2.3 Haemoglobin subtype fractions in normal individuals, β-thalassaemia carriers and major patients

Haemoglobin subtypes	Normal	β-thalassaemia carrier	β-thalassaemia major	
			β^+/β^+ or β^+/β^0	β^0/β^0
HbA	96% - 98%	92% - 95%	10% - 30%	0%
HbA_2	2% - 3%	>3.5%	2% - 5%	2% - 5%
HbF	<1%	0.5% - 4%	70% - 90%	95% - 98%

(Reference: Telen & Kaufman (1999))

2.5 Sample collection for genomic DNA and biochemical studies

2.5.1 Sampling for analysis of β-thalassaemia

Common sample used for molecular analysis of thalassaemia is collected from venous blood. Leukocytes present in the blood are harvested and extracted to obtain high quality DNA for molecular analysis. High quality DNA yield and purity is an advantage since it can be used for multiple molecular analyses with optimal amplification. These factors are important to ensure rapid and cost effective molecular analysis.

Plasma samples are used for a wide array of biochemical analysis. Since blood carries nutrients and metabolites throughout the human body, any abnormal accumulation of metabolites can be observed in plasma or serum samples. This includes enzyme activity, intermediate and end-products of metabolism.

However, the skill requirement in phlebotomy can limit sample collection by researchers in the field. Adverse effect such as haematoma due to blood leakage to surrounding tissue, infection from needle prick, discomfort and even nerve injury can be acquired if the procedures are not carried out accordingly or by a trained technologist (Hold *et al.*, 1995; Rayegani & Azadi, 2007; Stitik *et al.*, 2001). Subjects especially children are often reluctant to cooperate when it comes to blood sample collection due to their previous experience in phlebotomy. Collection of non-invasive samples is more convenient for researcher and patients. With sufficient information and guidance on sample collection, the process can be done with minimal supervision. The non-invasive process is painless and more comfortable to the patients.

2.5.2 Stratified squamous epithelium of oral cavity as the source of DNA

Stratified squamous epithelium is the most widely present stratified epithelium on the human body. It forms the skin and the mucous membrane of the upper digestive system, cornea, vagina and anal canal (Junqueira & Carneiro, 2003). It consists of multiple layers of epithelial cells resting on a basement membrane, with various shapes from cuboidal or columnar at the bottom to irregular and flat squamous cells at the outermost layer (Applegate, 2002).

The basal layer lying on the top of the basement membrane is the most active in mitotic activity. Addition of cell numbers through mitosis pushes the cells upwards, changing the cell configuration and making them flatter than the ones present at the bottom. The cells move farther from the blood vessels and receive fewer nutrients and lose mitotic activity. Later the cells start to lose their nuclei, died and desquamated from the tissue.

This tissue can be further divided into two - keratinised and non-keratinised stratified squamous epithelium. Keratinised stratified squamous epithelium is found in thick and thin skin. The basal layer (stratum basale/germinativum) mainly consists of keratinocytes. These cells will grow older and move up to the outermost layer of the epithelium (stratum corneum) and undergo keratinisation, which provides a dry and rough barrier. The nuclei and organelles of the cells are hydrolysed and disintegrated. The cytoplasm is then filled with keratin and form a barrier on the epidermal surface (Eroschenko, 2008).

The non-keratinised variant is found in moist surface such as upper digestive tract. The upper digestive tract includes the inner cheeks, palate and oesophagus. Just like the keratinous type of this epithelium, this tissue sheds the outermost layer daily due to abrasion and replaces them with newer cells from the lower strata. However, due to lack of keratinocytes, the cells do not undergo keratinisation as they age. The outermost cellular layer which consists of mature cells still maintains the nuclear structure and the organelles (Junqueira & Carneiro, 2003).

Due to the intact nuclear structure in the outermost layer of non-keratinised stratified squamous epithelium, it is possible to collect buccal cells to obtain genomic DNA. Collection of the sloughed off buccal cells can be carried out indirectly through collection of mouthwash and saliva, or directly through swabbing using cotton swab or cytological brushes.

2.5.2.1 Collection of epithelial cells from mouthwash

Previous studies have shown that DNA from human buccal cells has been successfully isolated and amplified for genomic studies. Collection of buccal cells from mouthwash involves rinsing the subject's mouth with a suitable solution for a given time. The mouthwash is then expectorated into a container and processed to collect the cells by centrifugation (King *et al.*, 2002; Lum & Marchand, 1998). Solutions such as antiseptic mouthwash solution, sterile water or isotonic solution such as 0.85% - 0.9% normal saline is utilised as the suspension medium (Garcia-Closas *et al.*, 2001; Mulot *et al.*, 2005).

Collection is performed after a resting period if the subjects have just finished eating, smoking, drinking, or brushing teeth (Aidar & Line, 2007; de Vries *et al.*, 2006). Collection of samples directly after eating may increase the amount of contaminants originating from food particles. This might affect the purified DNA when the undigested particles are not properly removed during purification. On the other hand, sample collection right after brushing teeth may reduce the number of collected cells (Feigelson *et al.*, 2001). Thus, a lag between brushing teeth and sample collection is given to allow the recovery of sloughed off cells.

Antiseptic mouthwash solution acts as both mechanical and chemical agents for mouth cleansing and bactericidal agent. Thus, the number of oral normal flora vegetative cells can be reduced, and prevent the DNA degradation due to their metabolic activity (Pandeshwar & Das, 2014). In spite of that, the usage of antiseptic mouthwash, even the alcohol-free mouthwash is not encouraged for children due to their tendency to swallow the solution.

The burning sensation due to the presence of alcohol also serves as a limitation of usage of antiseptic mouthwash as a suspension medium. Additional rinsing is needed to completely remove the solution. In addition, leaving the alcohol-containing mouthwash residue in the oral cavity can be carcinogenic to the epithelial cells lining the oral cavity (Lachenmeier *et al.*, 2008).

Sterile water or other isotonic solution is another option for mouthwash solution. The use of these solutions is safer and more acceptable especially to children since the solutions used are non-toxic. These solutions may only remove the bacteria by mechanical means. Thus, an initial rinsing with tap water or the same solution is added to reduce the oral bacteria load (King *et al.*, 2002). Addition of DNA preservative such as ethylenediamine tetraacetic acid (EDTA) and proper storage will keep the DNA integrity until the extraction is performed (Aidar & Line, 2007; Lahiri & Schnabel, 1993).

2.5.2.2 Collection of epithelial cells from saliva

Whole saliva consists of fluid and cellular component. The cellular components are composed of normal flora of the oral cavity, epithelial cells and also leukocytes that have migrated through the gingival crevices (Kaufman & Lamster, 2000; Kumar *et al.*, 2014; Pandeshwar & Das, 2014; Schiott & Loe, 1970). The friction between the oral cavity and the teeth and tongue desquamate the epithelial cells from the oral cavity and transfer them into the salivary fluid. Salivary fluid can be collected by expectoration of stimulated or unstimulated saliva (Dizgah & Hosseini, 2011; Sculley & Langley-Evans, 2002; Zalewska *et al.*, 2014).

To expectorate unstimulated saliva, the subject needs to sit down calmly and prevent him/herself from making any movement in the oral cavity and from swallowing the fluid (Dizgah & Hosseini, 2011; Zalewska *et al.*, 2014). Saliva is pooled in the mouth and then spitted out from the subject's mouth into a container. The cells present in the fluid are later collected by centrifugation process. Unstimulated saliva collection process may require a long time to complete. The process can also be stunted if the subject does not rehydrate before the collection procedure was performed.

Stimulation of saliva can be carried out by introducing external material into the subject's mouth such as a piece of paraffin wax, cotton roll or chewing gum (Ash *et al.*, 2014; Dizgah & Hosseini, 2011). The material will be expectorated out at the same time during the collection. Further processing such as centrifugation will separate the sample from the inducing material prior to DNA extraction. The other way of inducing is through the use of chemical such as citric acid or chewing motion (Zalewska *et al.*, 2014). This method takes less time since the saliva flow rate is increased. Nevertheless, improper rehydration may halt the process as in unstimulated saliva collection.

Other than extracting DNA from the whole saliva sample, treated cards made of filter paper pre-treated with antibiotics can be used to collect the cellular component for the saliva. After the subject has expectorated the salivary fluid into a sterile container, the treated card is then placed into the saliva. The card is then air-dried and stored until DNA extraction is performed. The card can be separated into a number of pieces and DNA can be extracted using various methods and in multiple batches (Milne *et al.*, 2006; Mulot *et al.*, 2005).

2.5.2.3 Collection of epithelial cells using buccal swabs and cytobrushes

The use of buccal swabs or cytobrushes to collect the buccal cells is well accepted for non-invasive sampling for molecular studies. The most common tool used for the method is cotton swabs (Bennet *et al.*, 2000; Cheng *et al.*, 2010; Milne *et al.*, 2006). Other than that, another option for buccal cells collection is by using sterile cytological brushes (Aldave *et al.*, 2004; King *et al.*, 2002; Said *et al.*, 2014).

The collection involves performing a few firm strokes on the oral mucosa for a given time. The swabs are then air-dried or stored in sterile stabilising buffer or saline solution (Hansen *et al.*, 2007; Swinfield *et al.*, 2009; Zhou *et al.*, 2012). The soft swab and bristles in cotton swabs and buccal cytobrush made the procedure comfortable and convenient to use. However, despite being economical, cells tend to get trapped between the cotton fibers or bristles. The trapped cell may be excluded from the extraction procedure and this may result in reduction of DNA recovery after the extraction process.

Various tools are also introduced for buccal cells collection, such as foam tipped applicator stick and tongue depressor (Burger *et al.*, 2005; Hansen *et al.*, 2007; Moore *et al.*, 2001). Collection of samples using a foam-tipped applicator stick involves collection of saliva present around the cheek and gum line. The use of foam-tipped applicator sticks is usually paired with antibiotic and stabiliser treated cards, where the foam is squeezed onto the card to retrieve as much as cells and saliva that is collected in the foam. For tongue depressors, this involves scraping the buccal cells from cheek and transferring the device into storage buffer or solution.

Contamination by microbial DNA from oral cavity normal flora cannot be avoided completely in all of the buccal cell collection methods mentioned above. Proper procedure planning such as initial mouth rinsing can indirectly help to reduce the amount of microbial DNA present in the sample and produce better results in determining DNA concentration and purity for molecular analysis.

2.5.3 Saliva as an alternative biological fluid

Fluid components of whole saliva are composed of – gingival crevicular fluid (GCF), liquid released from salivary glands, serum, traces of blood from intra-oral bleeding and additional fluids of bronchial and nasal origin (Kaufman & Lamster, 2000; Sculley & Langley-Evans, 2002).

The GCF is the transudate and exudates of the gingival (gum) tissue interstitial fluid (Alfano, 1974; Brill & Krasse, 1958; Griffiths, 2003; Uitto, 2003). The fluid is released through the gingival crevices present between the teeth and the gum line. In normal physiological conditions, the fluid is the filtrate from capillaries in the gum tissue released to the oral cavity under the influence of osmotic gradient. In pathological conditions, the increased permeability of the capillary wall increases the amount of interstitial fluid. The increased amount of interstitial fluid indirectly increases the GCF flow rate.

The GCF contains a mixture of components from blood, host tissue and subgingival plaque. This includes small organic molecules, protein, cytokines and enzymes originating from the host and the bacteria present in the oral cavity (Delima & Van Dyke, 2003). Thus, it can be concluded that the GCF has the composition of serum. However, the concentration may be reduced due to dilution by the salivary gland secretions.

Other investigators have conducted antioxidant level measurements in salivary samples in patients with medical conditions, such as type 2 diabetes mellitus, renal failure, hypertension and heart disease (Al-Rawi, 2011; Bibi *et al.*, 2008 ; Soukup *et al.*, 2012; Wolfram *et al.*, 2005). Salivary uric acid concentration was reported to be similar to that of serum, while other antioxidants such as ascorbic acid were present at a lower concentration (Sculley & Langley-Evans, 2002).

Collection of samples can be carried out using the same methods performed for collection of buccal cells from saliva – collection of whole saliva whether stimulated or unstimulated, direct collection from specified salivary gland and collection of GCF from gingival crevices (Michishige *et al.*, 2006). Although stimulated saliva can be used for biochemical analysis, unstimulated saliva provides more accurate observations (Sculley & Langley-Evans, 2002).

2.6 Molecular analysis of β-thalassaemia

Confirmation of β-thalassaemia is carried out by molecular studies to specify the β-globin gene mutation(s) involved. Genotyping of β-thalassaemia for diagnosis and research purpose is commonly carried out by the Amplification Refractory Mutation System (ARMS), gap-polymerase chain reaction (gap-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP) and reverse dot-blot hybridisation.

2.6.1 Detection of point mutations using the Amplification Refractory Mutation System

In 1989, the Amplification Refractory Mutation System (ARMS) was introduced in place of normal PCR for confirmation of the mutation status for α1-antitrypsin deficiency patients. The system allows rapid analysis of known point mutations present in genomic DNA sequences (Newton *et al.*, 1989). It was later applied for molecular analysis in carrier detection and prenatal diagnosis for various genetic disorders including α- and β-thalassaemia (George & Tan, 2010; Old *et al.*, 1990; Tan *et al.*, 2004; Wee *et al.*, 2009).

The primers in ARMS are designed such that a mismatch at the 3' end will not allow the amplification process to continue. The mutant primers are designed with the 3'-nuclotide complementary to the point mutation present on the DNA sequence. This makes the 'mutant' primers refractory (resistant) to PCR on 'normal' DNA templates. The same basis is applied for normal primers, where the 3'-nucleotide is complementary to the normal DNA sequence.

The ARMS primers need to be paired with a second primer, called common primer, to generate allele-specific amplification products. As an indicator of DNA amplification, two internal control primers are included in every reaction. These primers will amplify DNA sequences located in other regions of the DNA template that is not directly related to the point mutations. Amplification of the internal control indicates that the reaction mixture has been prepared correctly and DNA amplification has taken place. Figure 2.3 shows the basis of ARMS and the primer design.

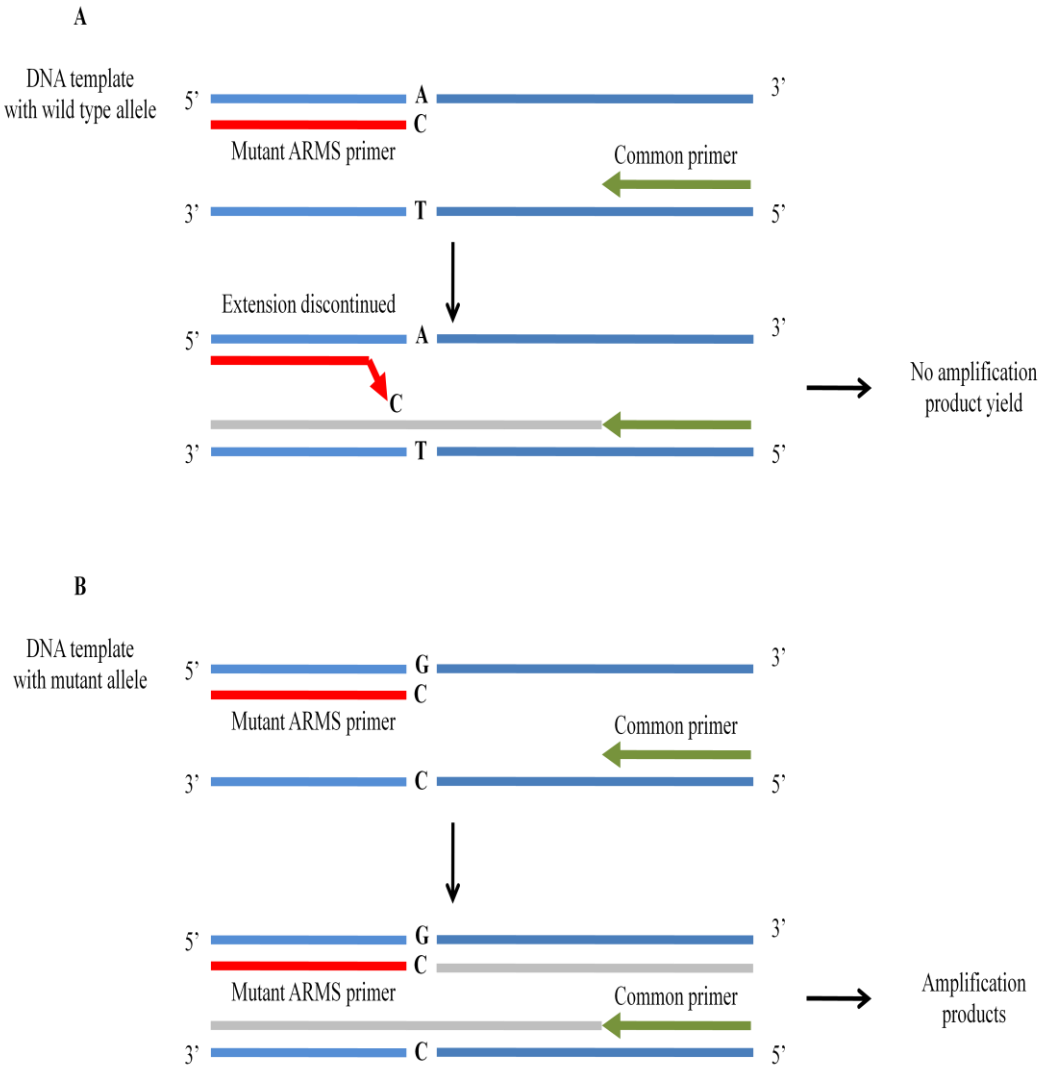

Figure 2.3 Basic concept of primer designed for ARMS and their behaviour with different DNA templates

(Adapted from Kitching & Seth (2005))

In some instances, a single 3'-nucleotide mismatch will still allow DNA amplification to progress. Purine/pyrimidine mismatches are less refractory when compared with purine/purine and pyrimidine/pyrimidine mismatches when extended with *Taq* DNA polymerase. Non-specific amplification may thus arise due to this and lead to false-positive results. To remove the problem, adding one more mismatches before the 3' end may cause the primer/template complexes to destabilise. The primers are thus more refractory and specific when compared with only a single mismatch at the 3' end.

Provided that the amplification product sizes are different and can be distinguished after gel electrophoresis, presence of both wild type and mutant alleles can be detected in a single reaction (Chen *et al.*, 2007). Figure 2.4 represents the schematic diagram of a single-tube ARMS for mutant and wild type allele identification.

Combined-ARMS (C-ARMS) has also been developed to detect two different mutations in a single PCR reaction. Two to three primers with mutant sequences are combined in a single-tube reaction mixture. In this procedure, the ARMS primers need to share the common primer, or they can be paired with different common primers that will not hybridise with each other. This allows rapid and more cost-effective prenatal diagnosis compared with individual ARMS reactions (Tan *et al.*, 2001; Thedsawad *et al.*, 2012; Wee *et al.*, 2009).

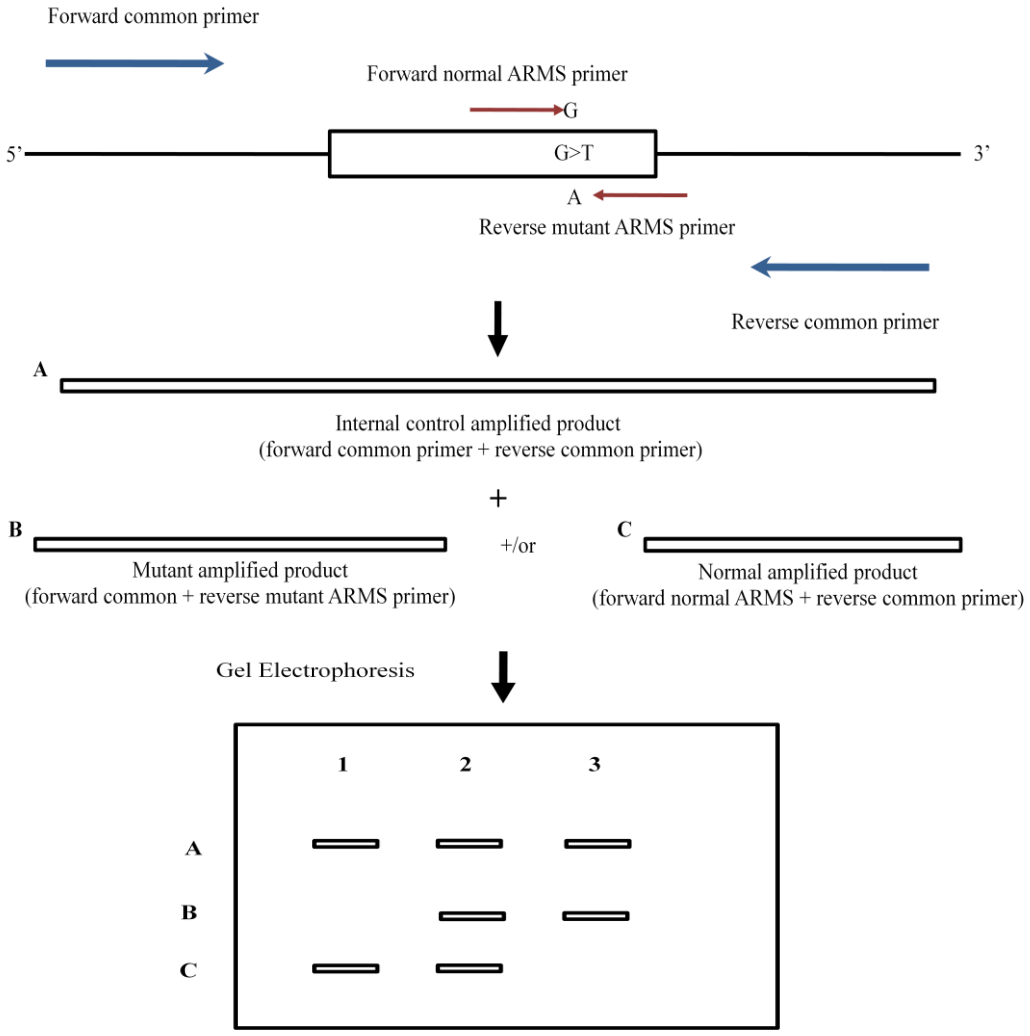

Figure 2.4 Example of primer design for single-tube ARMS for detection of mutant and normal allele of one point mutation involving substitution of a single base from G to T

The amplification products are of different molecular weight and thus distinguishable from one another. Amplification product on (1) normal individual: A + C; (2) carriers or compound heterozygous individual: A + B + C; (3) β-thalassaemia major: A + B

(Adapted from Chen *et al.* (2007))

2.6.2 Detection of large size deletions by gap-PCR

Beta-thalassaemia can be caused by large deletions in the β-globin gene sequence. However, amplification using normal PCR is difficult to detect the presence of the genes of interest due to annealing time constraints and limited amount of nucleotides and reagents. When the deletion breakpoints are known, gap-PCR can be performed to detect deletions of large gene sequences by utilising primers flanking the deleted region (Clark & Thein, 2005).

The primers are designed to specifically anneal to the complementary sense and antisense sequences on either ends of the deleted sequence. Presence of the gene sequence in a normal individual renders the sequence resistant to DNA amplification. Thus, there is no product yield from templates with undeleted sequences. However, the presence of a deletion will shorten the gene sequence and allow amplification to occur as the primers are brought close together. Significant copies of amplified gene sequence are thus produced and confirmed through gel electrophoresis.

To detect the presence of the normal sequence, a third primer or another pair of primers can be included to amplify the sequence present within the deleted region. The observation of an amplified product after gel electrophoresis confirms the presence of the normal sequence. A combination of these primers will enable the laboratory to determine whether the patient is homozygous or heterozygous for the specified deletion in a single PCR reaction. A diagrammatic representation of gap-PCR is shown in Figure 2.5

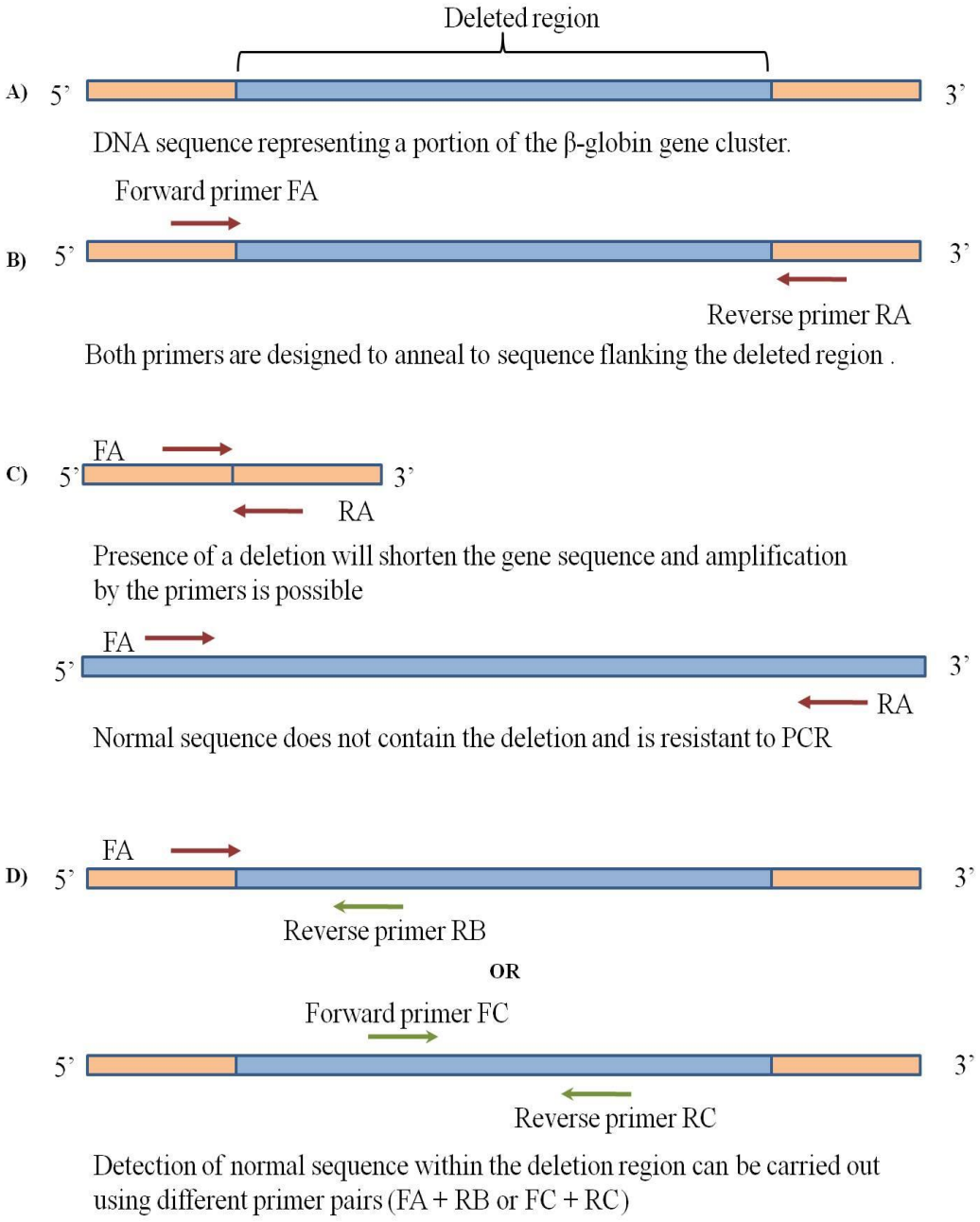

Figure 2.5 Positions of forward and reverse primers designed for analysis of large-sized deletions using gap-PCR

2.6.3 Detection of mutation by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Restriction endonuclease is an enzyme produced by prokaryotic organisms as a mechanism of defence against viral invasion. The enzymes recognise the particular sequence termed as a restriction site and cleave the DNA strand. The most commonly used enzymes are type II enzymes which cleave the DNA at the recognition site itself (Berg *et al.*, 2002). A report in 1989 states that 50% of mutations in the β-globin gene in the Mediterranean region creates or removes restriction endonuclease sites in the β-globin gene sequence (Pirastu *et al.*, 1989).

Detection of mutations involving PCR-RFLP begins with the amplification of DNA sequences containing the mutations. The amplicon obtained is then treated with restriction endonuclease that is able to recognise the creation or removal of a restriction site in the amplified DNA product. Confirmation of restriction enzyme analysis is carried out using gel electrophoresis.

PCR-RFLP is an inexpensive method with no requirements of high-technical instrumentation. However, the time constrains due to multiple electrophoretic separation steps and the possibility that several SNPs may affect the same restriction site can limit the usability of this procedure (Rasmussen, 2012).

2.7 Oxidative stress

Oxidative stress is a condition caused by imbalance of pro-oxidant and antioxidant in the biological system (Halliwell & Gutteridge, 1999). Pro-oxidants induce oxidative stress by forming reactive oxygen species or suppressing the activity of antioxidants. While antioxidants serve as molecules which protect the biological system from being oxidised and produce more free radicals.

Free radicals are molecules or molecular fragments containing one or more unpaired electron in the outermost molecular orbital (Halliwell & Gutteridge, 1999). The unsatisfied electron valence pair made the molecules highly reactive. Free radicals readily react with macromolecules such as protein, lipids and DNA, damaging the molecules.

Free radicals are produced in normal and pathological condition. Aerobic organisms use oxygen to oxidise molecules for metabolism. Even though oxidative phosphorylation in eukaryotic cells manage to fully utilise oxygen without producing intermediary molecules, trace amount of reactive oxygen species (ROS) formation is unavoidable (Berg *et al.*, 2002).

Antioxidants as cellular defence mechanism will try to delay or prevent oxidation of target substrates. The prevention is carried out by stopping the chain reaction or converting the free radicals into less injuring molecules, before being excreted or utilised by the cell itself (Ghone *et al.*, 2008).

Increase in pro-oxidant activity may lead to various macromolecular damage and tissue damage (Low, 2005). Living organisms may counteract reactive species by having complex antioxidant systems. However, the accelerated production of reactive species depletes cellular antioxidants rapidly and this will result in oxidative stress.

2.7.1 Oxidation of macromolecules

Oxidation of macromolecules such as DNA, lipid and protein usually involves abstraction of hydrogen atom by radicals such as hydroxyl radicals (•OH) (Berlett & Stadtman, 1997; Cooke *et al.*, 2003; Gutteridge, 1995). The molecules produced after hydrogen atom abstraction may become the new reactive molecules, continuing the oxidation chain reaction.

2.7.1.1 Oxidation of lipid

Lipid peroxidation can occur through enzymatic or non-enzymatic pathway, with non-enzymatic pathway being initiated with or without free radicals (Niki *et al.*, 2005). Main target for peroxidation is polyunsaturated fatty acids which contain double covalent bonds. Presence of double covalent bond weakens the carbon-hydrogen bond of the adjacent carbon atom. This factor facilitates the removal of hydrogen atom from the methyl group (Gutteridge, 1995). Membrane phospholipids which are rich in polyunsaturated fatty acids are very susceptible to lipid peroxidation.

2.7.1.2 Oxidation of protein

Protein oxidation occurs with the same basics; abstraction of α-hydrogen atom by •OH which initiates chain reactions that further oxidise the amino acid residues. All amino acid residues are prone to oxidation by •OH. The most susceptible amino acid residues are aromatic amino acids such as tryptophan and tyrosine. However, some damages are reversible, such as the oxidative damage which occurs on sulphur containing residues like methionine (Berlett & Stadtman, 1997).

2.7.1.3 Oxidation of DNA

Oxidation of DNA strands may cause structural changes, leading to mutations and cellular destruction. Abstraction of hydrogen atom usually happens at methyl group on sugar base of nucleotide (Cadet & Douki, 1999; Cooke *et al.*, 2003). Cellular replication and repairs cannot be carried out due to this disruption. Cell will be wasted and marked for destruction by macrophage.

2.7.2 Oxidative stress in β-thalassaemia

2.7.2.1 Iron overload

Majority (67%) of the body iron storage is in haemoglobin, followed by ferritin and haemosiderin which add up to 27% of the storage. Almost all (up to 95%) of the iron for haemoglobin synthesis originate from the old erythrocytes. The remaining is obtained through diet (Besa *et al.*, 1992).

Due to the rapid destruction of the red blood cells and hypertransfusion, the iron storage in the patient's body exceeds ferritin storage and detoxification capacity limit. Multiple transfusions increase transferrin saturation and later give rise to free iron species that is unbound to transferrin. This non-transferrin-bound iron (NTBI) is taken up by parenchymal cells such as hepatocytes and myocardium readily if compared with transferrin-bound iron (Hershko, 2010; Prabhu *et al.*, 2009).

Hypoxia condition favours the production of superoxide anion ($O_2\bullet^-$) due to 'slowed down' electron transport chain (Misra & Fridovich, 1971). Increased amount of $O_2\bullet^-$ can induce separation of iron from its carrier protein. Free iron (Fe^{2+}) will participate in Fenton and Haber-Weiss reaction, inducing the production of •OH (Gutteridge *et al.*, 1981). Peroxides formed through the reaction will start cross-linking with macromolecules, reducing their functionality or making the molecules totally non-functional (Figure 2.6).

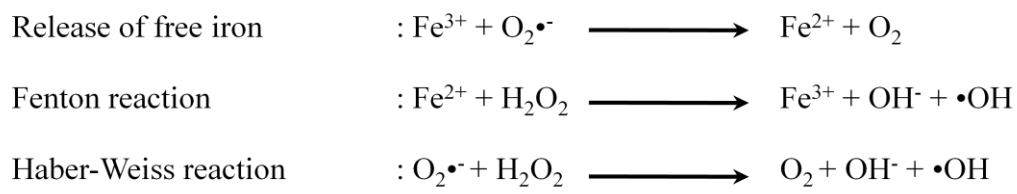

Figure 2.6 Production of •OH through Fenton and Haber-Weiss reaction involving iron
(Reference: Haber & Weiss (1932); Koppenol (2001))

Oxidation targeted on red blood cells give rise to serious problems, since the absence of nuclei in red blood cells means they cannot initiate cellular repair by themselves. Lipid peroxidation might disfigure the cells while protein oxidation can produce protein clusters on the membrane, making it prone to be targeted as abnormal and removed from circulation (Prabhu *et al.*, 2009). Oxidation can worsen thalassaemia patient's condition, since haemolysis can be enhanced and indirectly increase the level of iron in circulation.

Other than that, the excess iron also affects the endocrine system leading to reduced endocrinal function (Galanello *et al.*, 2010). This can be observed in female patients where their puberty and physical development was delayed due to hypogonadotropic hypogonadism (Roussou *et al.*, 2013).

2.7.2.2 Iron chelation therapy

Accumulation of iron in parenchymal tissue already started at the first year of regular transfusion. Normal homeostasis in human cannot control the excessive iron present in the system. To reduce the amount of iron accumulated in the body, it need to be removed therapeutically, such as through iron chelation therapy (Hershko, 2010). Chelated iron is later excreted through urine and faeces.

Deferoxamine (DFO) was introduced in 1960's as a subcutaneous chelating agent. Chelation therapy is usually started after serum ferritin level has exceeded 1000 µg/L. Patients need to infuse 20 to 60 mg/kg of body weight DFO for 8 to 12 hours a day, 5 to 7 days a week (Hershko *et al.*, 2003). Daily parenteral infusion might cause discomfort and the expensive cost of the drugs and equipment might limit the usage in several developing countries (Prabhu *et al.*, 2009).

Deferiprone (DFP) is introduced as an oral iron chelator, and usually prescribed in combination with DFO (Olivieri *et al.*, 1992). DFP is reported to have better efficacy in chelating iron. However, side effects such as arthropathy, agranulocytosis, neutropaenia, and gastrointestinal disturbance have also been reported (Cohen *et al.*, 2003).

Newer oral iron chelators such as deferasirox (DFS), commercially known as Exjade has been introduced and approved for use in various countries including Malaysia. It was reported in short-term trial that it is able to remove iron from heart (Cohen, 2006). However, long-term report is still limited. Other oral chelator such as deferitrin is still in development (Eckes, 2011).

2.7.2.3 Cytokine associated to hypoxia

Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are pleiotropic cytokines which have various biological activities. TNF-α is produced by various types of immune and non-immune cells in response to various stimuli, such as viral and parasitic infection, bacterial endotoxin, enterotoxin, nitric oxide, hypoxia and oxygen radicals (Wang *et al.*, 2003).

On the other hand, IL-6 is produced by lymphoid and non-lymphoid cells and plays a role in immunological reactivity regulation, acute-phase response, inflammation, oncogenesis and haematopoiesis. In haematopoiesis, IL-6 triggers the dormant progenitor cells to exit G_0 phase and enter cell cycle (Kishimoto, 2003).

Hypoxia and overproduction of oxygen radicals may also induce the production of TNF-α in the circulation. TNF-α will also cause the secretion of other cytokines including IL-6 (Beyaert & Fiers, 1998). Persistence of the hypoxic condition and the continuous overproduction of radicals might cause overproduction of TNF-α. Even though TNF-α is supposed to induce apoptotic cell death of tumor cells, overproduction of TNF-α will exhibit its pro-inflammatory effects. This might lead to hypotension, diffuse coagulation and widespread tissue damage (Wang *et al.*, 2003).

Overproduction of IL-6, on the other hand plays a role in autoimmune diseases (Beyaert & Fiers, 1998). Previous studies showed that excess production of IL-6 impairs erythropoietin, disrupting erythroid development and increasing the severity of anaemia (Raj, 2009).

2.8 Methods of assessing oxidative stress

2.8.1 Ferric reducing antioxidant power

Ferric reducing antioxidant power assay measures the ferric reducing ability of metabolites in the sample. The colorimetric assay measures the level of overall non-enzymatic antioxidants present. In acidic condition, ferric 2,4,6-tripyridyl-triazine complex (Fe^{3}-TPTZ) will react with antioxidant present in the sample. Reaction will reduce Fe^{3+} to Fe^{2+}, which the complex formed with TPTZ and produced intense blue colour (Benzie & Strain, 1996).

2.8.2 Advance oxidation protein products

Advanced oxidation protein products (AOPP) is produced during oxidative stress by chlorinated oxidants such as chloramines or hypochlorous acid reaction. The oxidative stress marker was detected in abundance in individuals with kidney failure (Li *et al.*, 2007; Witko-Sarsat *et al.*, 1996). The assay reported by Witko-Sarsat (1996) involves measuring sample absorbance after mixing with potassium iodide (KI) in acidic condition in comparison to chloramine-T level.

2.8.3 Lipid hydroperoxide assay

There are various assays present to assess lipid peroxidation. However, there is no single assay that can be used to thoroughly explain the overall reaction that occurs (Halliwell & Chirico, 1993). One of the methods described is measurement of lipid hydroperoxide (LOOH), which is an intermediate of lipid peroxidation. LOOH indicates whether the primary reaction is initiated by singlet oxygen or oxyradicals (Girotti, 1998).

Colorimetric assay previously described in 1990 used 1-methyl-2-phenyl-indole (MPI) as chromogen (Esterbauer & Cheeseman, 1990). MPI prepared in acetonitrile is mixed with samples to react in acidic condition at 45°C. Presence of LOOH is observed by the presence of blue-coloured chromophore, which can be measured spectrophotometrically.

2.8.4 Glutathione peroxide activity

Glutathione peroxidase (GPx) is another method assessing lipid peroxidation. It reacts with H_2O_2 and oxidise glutathione from its reduced form (GSH) to oxidised form (GSSG). Addition of glutathione reductase and NADPH will reduce GSSG back to GSH, while oxidising NADPH to $NADP^+$. Changes in absorbance occurred due to reduced amount of NADPH which can be monitored by spectrophotometry. The decreasing absorbance rate is directly proportional to the GPx activity in the sample (Forman & Boveris, 1982).

2.8.5 Uric acid level

Uric acid (UA) is a major antioxidant present in human blood and may protect against aging and oxidative stress. Human have a high basal level of plasma UA level. However, elevated UA level may lead to various conditions such as cardiovascular diseases (Johnson *et al.*, 2007).

Measurment of UA can be carried out through colorimetric method by measuring the reduction of chromogenic substance by uric acid. However, the method is not specific as it measures substances other than UA itself. Using the enzyme uricase to convert UA to allantoin produce more specific result even though it cost more than the former method (Barr, 1990).

2.8.6 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a variant of immunoassay used to detect analytes in biological samples. Innovation of ELISA from the commonly used radioimmunoassay allows quantitative detection of antibody or antigen in the sample without using expensive and radioactive labelling (Lequin, 2005; Van Weemen & Schuurs, 1971).

A capture antibody is used to coat a stationary phase such as microtiter plate to bind with antigen of interest which increases specificity. Secondary biotinylated antibody is added after reaction of sample and primary antibody. Biotin-binding protein labelled with enzymes such as avidin-horse radish peroxidase is added before adding enzyme substrate to produce colour. The colour production is measured by spectrophotometry and compared to standard curve to determine the concentration of antigen. The absorbance reading is directly related to the concentration of antigen.

In competitive ELISA, competition between unlabelled antigens with enzyme-labelled antigen will determine the concentration. Less amount of antigen in sample will increase the binding of enzyme-labelled antigen, increasing the absorbance values. Thus, absorbance reading is inversely related to antigen concentration.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study population

3.1.1 Beta-thalassaemia major patients

Participation of patients and control subjects were on a voluntary basis. The β-thalassaemia major patients were recruited from Paediatric Ward 6 Day Care, Level 4, Women and Children Health Complex, University Malaya Medical Centre (UMMC). Patients with ages ranging from 4 to 25 years old were recruited during their monthly visits for blood transfusion.

3.1.2 Normal controls

Normal and healthy volunteers with ages within the β-thalassaemia patient study group were recruited as control subjects. Subjects with severe anaemia due to other causes, serious and chronic diseases and those following serious drug treatments were excluded.

Participants were briefed about the objectives of the study, method of sample collection and any risks associated with the sample collection procedures. Informed and written consent was obtained from all patients and control subjects. In patients and control subjects below 18-years old, informed and written consent was obtained from their parents/guardians. Patients and control subjects were asked about their history of ancestry, intake of supplements, medication and health status.

This project was approved by the Medical Ethics Committee of University Malaya Medical Centre (MEC Ref. No: 727.1).

3.1.3 Recruitment of β-thalassaemia major patients for molecular analysis of β-thalassaemia using mouthwash, saliva and buccal cytobrush samples

For optimisation of DNA extraction protocols and molecular analysis of β-thalassaemia, mouthwash, saliva and buccal cytobrush samples were collected from 50 β-thalassaemia major patients. The patients include 27 Malays, 14 Chinese and 9 from other ethnic groups. Patients classified from other ethnic groups include Indians and patients with a history of interracial marriage in their ancestry. The β-thalassaemia major patients group consisted of 24 males and 26 females. Three of the patients were aged below 5-years, 17 patients with ages ranging from 6 – 10-years, 21 patients were aged 11 – 15-years and 9 patients with ages ranging from 16 – 20-years. The summary of the ages of the patients are shown in Table 3.1.

Table 3.1 Age group of β-thalassaemia major patients recruited in the study for optimisation of DNA extraction protocols and molecular analysis of β-thalassaemia

Age (years)	Number of patients (n)
< 5	3
6 – 10	17
11 – 15	21
16 – 20	9
Total	50

The β-globin gene mutations in all β-thalassaemia major patients in this study had been previously characterised using blood samples. Table 3.2 shows the list of patients with their genotype in this study.

Table 3.2 List of patients, ethnic groups and β-thalassaemia mutations

	Ethnicity	Beta-thalassaemia mutations		Ethnicity	Beta-thalassaemia mutations
1	Malay	Hb Lepore/Hb Lepore	16	Chinese	IVS2-654/CD41/42
2	Malay	CD26/CD71/72	17	Malay	CD26/IVS1-5
3	Malay	CD26/CD35	18	Malay	CD26/IVS1-5
4	Malay	IVS1-5/IVS1-5	19	Malay	CD26/IVS1-5
5	Chinese	IVS2-654/IVS2-654	20	Chinese	CD41/42/CD17
6	Malay/Chinese	CD26/CD71/72	21	Malay	CD26/IVS1-5
7	Malay	CD26/IVS1-5	22	Malay	CD26/IVS1-5
8	Indian	IVS1-5/Poly A	23	Malay	CD26/IVS1-1
9	Malay/Chinese	IVS1-5/IVS2-654	24	Chinese	CD41/42/CD41/42
10	Chinese	CD41/42/CD27/28	25	Malay	CD26/IVS1-5
11	Chinese	CD26/Filipino β-deletion	26	Malay	CD26/IVS2-654
12	Chinese	CD41/42/CD41/42	27	Chinese	CD41/42/CD41/42
13	Chinese/Siamese	CD41/42/IVS1-1	28	Malay/Siamese	CD41/42/CD26
14	Chinese	CD41/42/CD41/42	29	Malay	CD26/IVS1-5
15	Malay	CD26/IVS1-5	30	Malay	CD26/IVS1-1

Table 3.2 List of patients, ethnic groups and β-thalassaemia mutations (cont.)

	Ethnicity	Beta-thalassaemia mutations		Ethnicity	Beta-thalassaemia mutations
31	Indian	IVS1-5/Cap+1	41	Malay	CD26/IVS1-1
32	Malay	CD26/IVS1-5	42	Malay	CD26/IVS1-5
33	Chinese	CD41/42/$^{G}\gamma(^{A}\gamma\delta\beta)^{o}$	43	Malay	CD26/IVS2-654
34	Chinese	CD41/42/-28	44	Malay	CD26/IVS2-654
35	Chinese	IVS2-654/IVS2-654	45	Malay	CD26/IVS1-5
36	Chinese	-28/-28	46	Malay	IVS1-5/IVS1-1
37	Chinese	IVS2-654/-28	47	Malay	CD19/CD8/9
38	Chinese/Siamese	CD26/CD17	48	Malay	CD26/IVS1-5
39	Chinese/Siamese	CD26/CD41/42	49	Malay	CD26/IVS1-5
40	Pakistani	CD8/9/CD16	50	Malay	IVS1-5/CD19

3.1.4 Recruitment of β-thalassaemia major patients and healthy controls for biochemical analysis using saliva samples

For the analysis of biochemical parameters, saliva samples were collected from 65 patients and 55 healthy controls. Beta-thalassaemia major patients consisted of 37 Malays, 20 Chinese and 8 from other ethnic groups. With regards to gender, 25 out of the 65 patients were males. In aspects of age distribution, 20 patients were below 10-years, 34 patients were between 11 – 20-years and 11 patients were between 21 – 30-years.

With regards to genetic classifications of β-thalassaemia major, the β-thalassaemia major patients were further divided into three groups based on their β-globin gene mutations. Group 1 consisted of patients who have been characterised as β^+-thalassaemia homozygotes (β^+/β^+) which consisted of 9 patients, Group 2 was β^+/β^0-compound heterozygotes with 39 patients and Group 3 was β^0-thalassaemia homozygotes (β^0/β^0) with 17 patients.

Healthy controls were recruited from the public, with age matching the age range of the β-thalassaemia major patients. The healthy controls consisted of 35 Malays, 16 Chinese and 4 individuals from other ethnic groups. Twenty-three out of the 55 healthy individuals were males. In aspects of age distribution, 7 individuals were with aged below 10-years, 18 were between 11 – 20-years and 30 were between 21 – 30-years. Summary of the ages of the patients and the healthy individuals are shown in Table 3.3.

Table 3.3 Age group of participants recruited in the study for analysis of biochemical parameters using non-invasive samples

Age (years)	Number of participants (n)	
	Patients	Healthy controls
< 10	20	6
11 – 20	34	15
21 – 30	11	34
Total	65	55

3.2 Sample collection

Patients were given four sample containers which were clearly labeled prior to sample collection: (1) and (2) – are two wide-mouth containers containing 15 mL of sterile normal saline (0.85% NaCl) for mouthwash sample collection, (3) – one empty 50 mL centrifuge tube (Labcon, USA) for saliva sample collection and (4) – one 15 mL centrifuge tube (Labcon, USA) containing 5 mL normal saline with one sterile, individually packed cytological brush (Cross Protection, Malaysia) for buccal cytobrush sample collection. The procedures for sample collection were explained and demonstrated to the patients.

Healthy individuals were given container (3) for saliva sample collection for biochemical analysis.

Containers used for sample collection are shown in Figure 3.1.

Figure 3.1 Containers 1, 2, 3 and 4 used for sample collection with sterile individually packed buccal cytobrush

3.2.1 Mouthwash samples

Patients were asked to rinse their mouth with sterile normal saline in container (1) as provided to remove food debris from their oral cavity. They were then instructed to rub their cheeks against their molars for 30 seconds. They were then required to rinse their mouth for the second time with fresh sterile normal saline solution in container (2) for 1 minute while rubbing the cheeks against their molars occasionally. The mouthwash solution was then expectorated into the container.

3.2.2 Salivary fluid collection

Patients were asked to spit at least 5 mL of saliva slowly into container (3) without forcing the fluid flow. They were allowed to drink if they felt their mouth becoming dry during the process. However, they were reminded to gargle and swish the oral cavity with clean water prior to continuing. They were asked to minimise the time needed to collect the samples to prevent loss of analytes in the samples.

For healthy controls, collection of saliva samples was performed after initial mouth rinsing using sterile normal saline in container (1).

3.2.3 Buccal cytobrush samples

Buccal cytobrush samples were collected using the sterile cytological brush provided with container (4). Patient's inner cheeks were rubbed by twirling the brush up and down for at least 15 seconds on each side. The brush was then dipped and agitated in 5 ml of fresh sterile normal saline in the container to loosen the buccal cells obtained. The process was repeated three times.

After the final collection, the brush was agitated and left in the solution and the tube was sealed.

3.2.4 Sample storage and processing

All samples were immediately kept on ice after collection was completed. Sample processing was carried out on the same day as sample arrival in the laboratory.

The saliva samples were centrifuged at 2,500 x *g* for 10 minutes at 4°C to pellet the cells or any residue present. Supernatant of the samples were aliquoted into several microcentrifuge tubes and kept frozen at -70°C until required for biochemical analysis.

The pellet from saliva was rehydrated and resuspended with Tris-EDTA (TE, 10 mM/10 mM) buffer pH 8.0. Pellet suspensions were kept frozen in -20°C until DNA extraction was carried out.

The mouthwash and buccal cytobrush samples were directly frozen in -20°C until DNA extraction was carried out.

Summary on the procedures of sample collection and processing is presented in Figure 3.2.

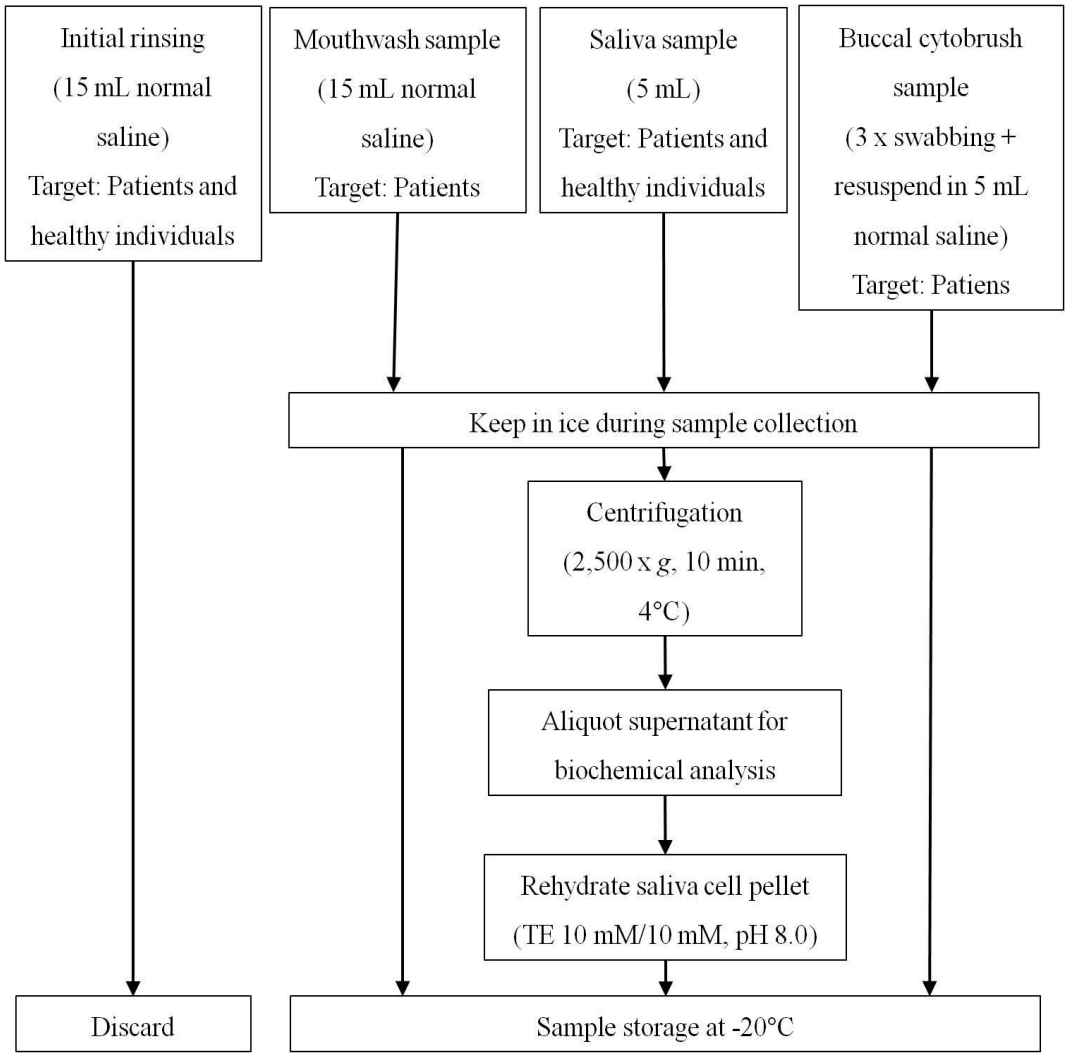

Figure 3.2 Flow chart of sample collection and sample processing procedures for mouthwash, saliva and buccal cytobrush samples

3.3 Preparation of cell pellets for DNA extraction

Mouthwash, saliva cell pellet and buccal cytobrush samples were thawed quickly in a 37°C waterbath (Shel Lab, USA) prior to DNA extraction.

The mouthwash samples were transferred into new 50 mL centrifuge tubes. Cytological brushes from buccal cytobrush samples were agitated for 30 seconds to dislodge the buccal cells from the bristles into the saline solution. The cytological brushes were then discarded.

The mouthwash and buccal cytobrush samples were centrifuged at 3,500 rpm for 15 minutes at 4°C to pellet the cells present. The supernatant was carefully discarded.

The mouthwash and saliva samples pellets were resuspended with 24 mL TE buffer, while the buccal cytobrush samples pellet was resuspended in 10 mL TE buffer. The cell suspensions were divided evenly into two separate tubes. The suspensions from mouthwash and saliva samples were brought to a final volume of 25 mL with TE buffer and the suspension from buccal cytobrush samples were brought to a final volume of 15 mL with TE buffer. All samples were incubated for 10 minutes in ice. Samples were then centrifuged at 3,500 rpm for 15 minutes at 4°C. The supernatant was carefully discarded.

Flow chart for collection of cell pellet for DNA extraction from mouthwash, saliva and buccal cytobrush is summarised in Figure 3.3.

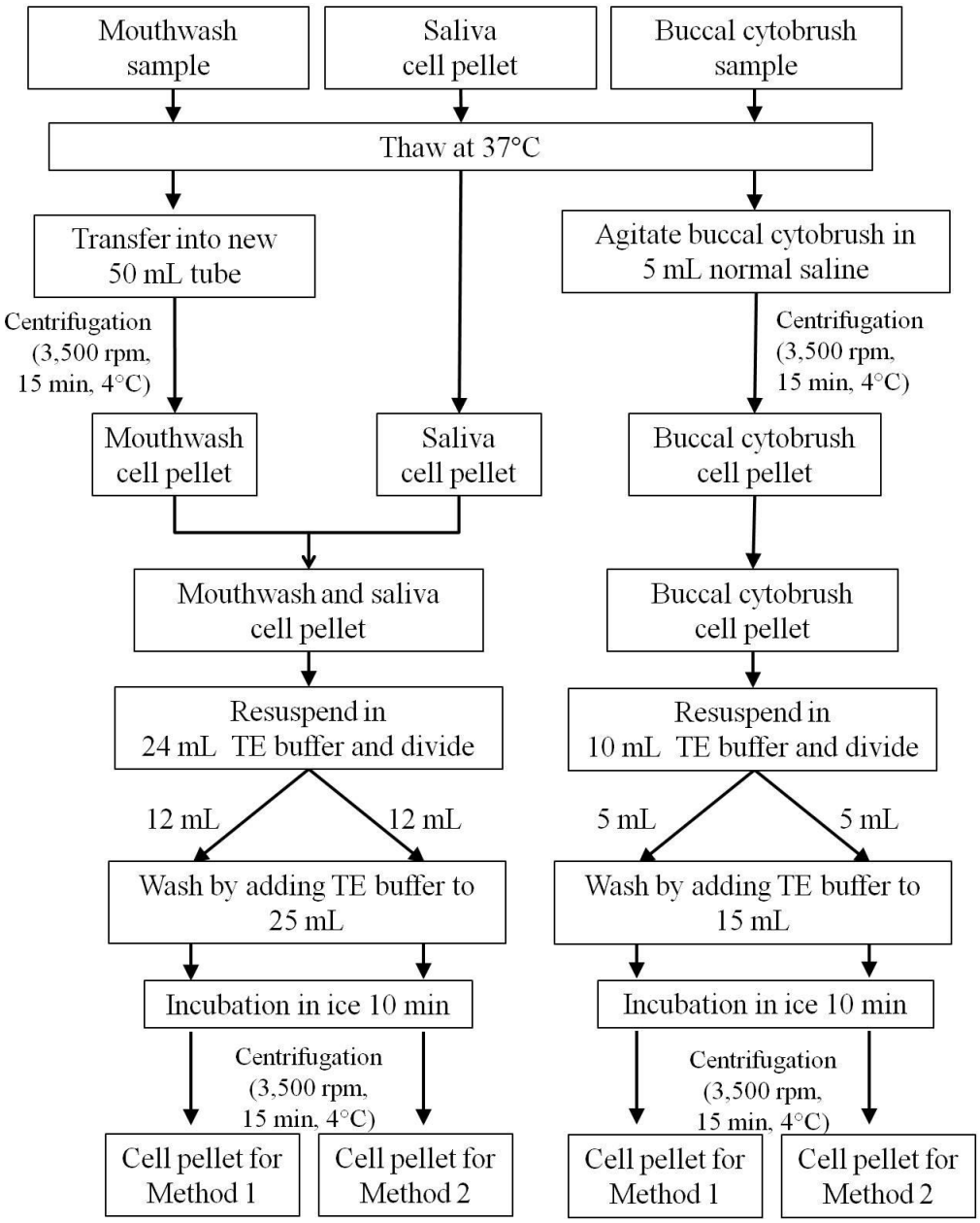

Figure 3.3 Flow chart of cell pellet collection from mouthwash, saliva and buccal cytobrush samples prior to DNA extraction

3.3.1 DNA extraction – Method 1

The pellets obtained were resuspended in 400 µL lysis buffer (0.1M NaCl and 2% w/v SDS in TE buffer) and transferred into 2 ml microcentrifuge tubes (Axygen, USA) containing 70 µL of 10 mg/mL Proteinase K (Invitrogen, USA). The pellets were incubated at 37°C overnight in a shaking incubator.

On Day 2, DNA in solution was purified with equal volumes of phenol:chloroform (ratio 1:1, Merck, Germany). The DNA-phenol-chloroform mixtures were gently inverted and were centrifuged at 14,000 rpm, 25°C for 5 minutes. The lower phenol:chloroform and interphase layers were aspirated using a pipettor. The purification steps were carried out twice.

DNA was precipitated with 1/10 volume of 4 M NaCl (Merck, Germany) and two volumes of ice-cold absolute ethanol and incubated overnight at -70°C. The samples were thawed at 25°C the following day and centrifuged at 14,000 rpm for 5 minutes at 4°C. Purification was carried out using ice-cold 70% ethanol followed by absolute ethanol (Merck, Germany). DNA tubes were centrifuged at 14,000 rpm for 5 minutes at 4°C.

DNA was air-dried in a laminar flow cabinet and solubilised with sterile deionised water. Solubilisation was carried out at 37°C for two hours with shaking. Extracted DNA samples were stored at -20°C.

Summary of Method 1 DNA extraction is presented in Figure 3.4a.

3.3.2 DNA extraction – Method 2

The pellets for mouthwash and buccal cytobrush samples were resuspended in 700 µL lysis buffer and transferred into 2 mL microcentrifuge tubes containing 35 µL of 20 mg/mL Proteinase K.

The pellets for saliva samples were resuspended in 700 µL lysis buffer and transferred into 15 mL centrifuge tubes containing 1 mL of 10 mg/mL dithiotrietol (DTT, Sigma-Aldrich, USA) and 35 µL of 20 mg/mL Proteinase K.

All samples were incubated in a waterbath at 65°C for one hour, followed by 58°C incubation for two hours with shaking. A final incubation at 37°C was carried out overnight in a shaking incubator.

DNA was then purified with an equal volumes of phenol:chloroform (ratio 1:1, Merck, Germany) and the mixtures were inverted gently. The DNA-phenol-chloroform mixtures in 2 mL microcentrifuge tubes were centrifuged at 14,000 rpm, 25°C for 2 minutes. The DNA-phenol-chloroform mixtures in 15 mL centrifuge tubes were centrifuged at 3500 rpm, 25°C for 10 minutes.

The lower phenol:chloroform and interphase layers were aspirated with a pipettor. Purification steps were carried out three times. The top aqueous layer containing DNA was transferred into new 15 mL centrifuge tubes (Labcon, USA).

DNA was precipitated with 1/10 volume of 3 M NaOAc pH 5.6 and two volumes of ice-cold absolute ethanol. The solution was incubated at -70°C overnight. The samples were thawed to 25°C the following day and centrifuged at 14,000 rpm for 5 minutes at 4°C. DNA dehydration was carried out with ice-cold 70% ethanol followed by absolute ethanol. DNA tubes were centrifuged at 14,000 rpm for 5 minutes at 4°C.

DNA was air-dried in a laminar flow cabinet and solubilised in 200 µL of sterile deionised water. Solubilisation was carried out at 37°C for two hours. Extracted DNA samples were stored at -20°C.

Summary of Method 2 DNA extraction is presented in Figure 3.4b.

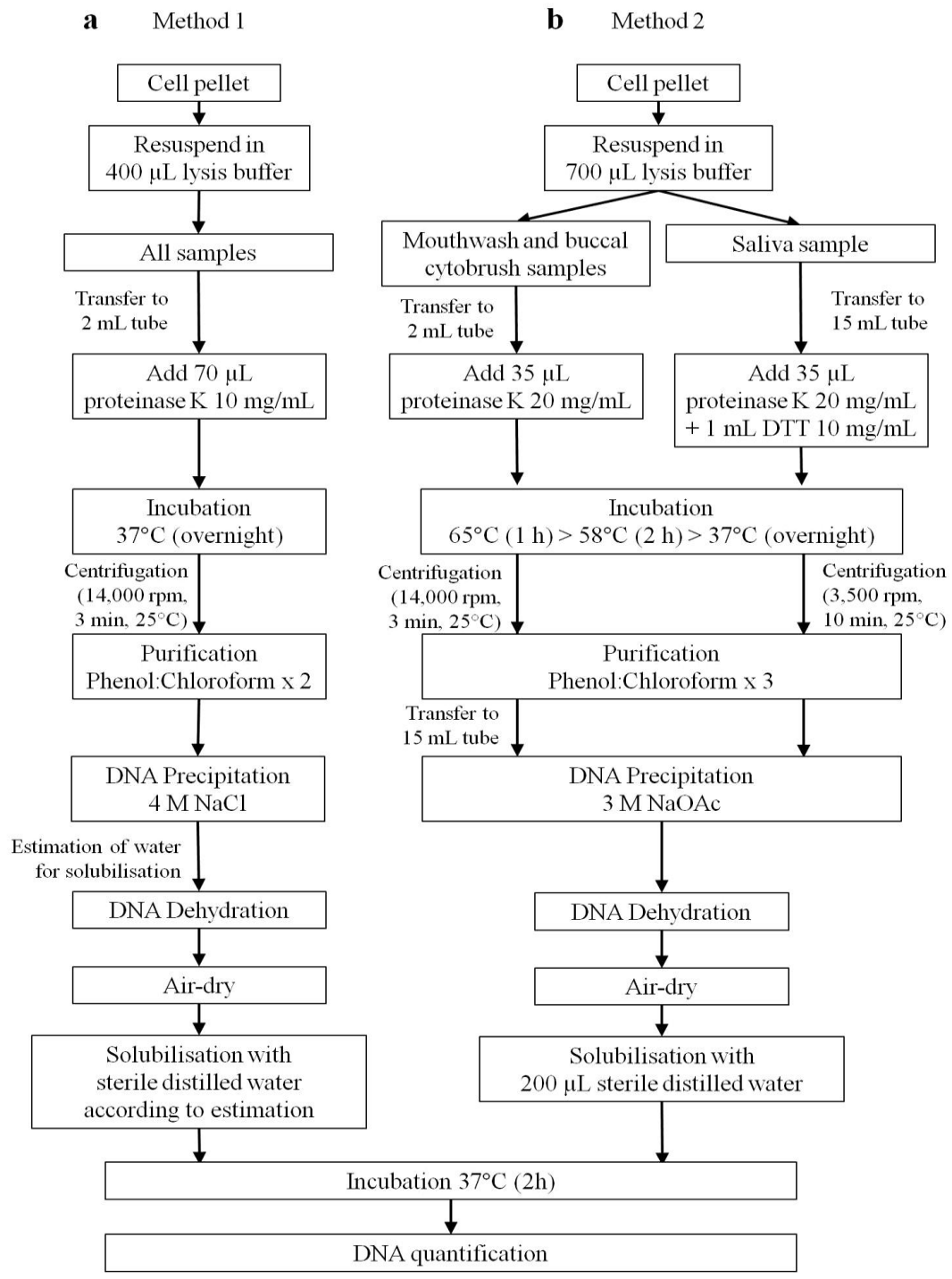

Figure 3.4a & b Flow chart of DNA extraction Methods 1 and 2 for mouthwash, saliva and buccal cytobrush samples

3.3.3 DNA quantification and statistical analysis

DNA quantification was performed in duplicates. Two aliquots of purified DNA were diluted 100X with sterile deionised water. The diluted DNA solution was transferred into a quartz cuvette with 1 cm path length and measured using a UV spectrophotometer (PerkinElmer, USA) at wavelengths of A_{260nm} and A_{280nm}.

The concentration of purified DNA in µg/µL was determined by the formula:

[DNA] (µg/µL) = (A_{260nm} X Dilution Factor X 50 µg/mL) / 1000 µL/mL

Each 1.0 absorbance in 260nm corresponded to 50 µg/mL of double stranded DNA.

Protein contamination was determined by calculating A_{260nm}/A_{280nm}. Purified DNA with A_{260nm}/A_{280nm} ratio in the range of 1.8 – 2.0 is considered as highly pure DNA.

Measurement of DNA concentration and purity was carried out for all sampling methods and DNA extraction techniques.

The data from DNA quantification was used in statistical analysis using SPSS 17 (SPSS Inc., Chicago, IL). The data was tested for normality with Shapiro-Wilk test. Significance of difference in the purified DNA concentration and purity were analysed with Friedman test. The analysis was followed by post hoc test using Wilcoxon signed-rank test for confirmation of the most optimum method for sample collection and DNA extraction (Jaykaran, 2010).

3.4 Archived DNA

Archived DNA extracted from blood samples from normal individuals, β-thalassaemia carriers and β-thalassaemia major patients who were homozygous or compound heterozygous were also used in the study. DNA amplification using Amplification Refractory Mutation System (ARMS), Combine-ARMS (C-ARMS), gap-polymerase chain reaction (gap-PCR) and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was carried out on the archived DNA.

These results were compared with results using DNA from mouthwash, saliva and buccal cytobrush samples. Archived DNA samples were also used as positive controls in the molecular assays. Table 3.4 shows the list of the archived DNA from β-thalassaemia carriers and β-thalassaemia major patients used in this study.

Table 3.4 List of archived DNA from β-thalassaemia carriers and β-thalassaemia major patients used in the study

No	Archived DNA from β-thalassaemia carriers
1	Heterozygous for CAP+1 (A-C)
2	Heterozygous for CD15 (G-A)
3	Heterozygous for CD19 (A-G)
4	Heterozygous for CD26 (G-A)
5	Heterozygous for Poly A (AATAAA-AATAGA)
6	Heterozygous for IVS1-5 (G-C)
7	Heterozygous for IVS1-1 (G-T)
8	Heterozygous for CD8/9 (+G)
9	Heterozygous for IVS2-654 (C-T)
10	Heterozygous for -29 (A-G)
11	Heterozygous for -28 (A-G)
12	Heterozygous for Initiation Codon (T-G)
13	Heterozygous for CD43 (G-T)
14	Heterozygous for CD41/42 (-CTTT)
15	Heterozygous for CD17 (A-T)
16	Heterozygous for CD71/72 (+A)
17	Heterozygous for Filipino β-deletion
18	Heterozygous for 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{o}$-deletion
19	Heterozygous for CD27/28 (+C)

Table 3.4 List of archived DNA from β-thalassaemia carriers and β-thalassaemia major patients used in the study (cont.)

No	. Archived DNA from β-thalassaemia major patients
20	Compound heterozygous for CD41/42/CD17
21	Homozygous for IVS1-5/IVS1-5
22	Homozygous for IVS2-654/IVS2-654
23	Homozygous for CD41/42/CD41/42
24	Homozygous for -28/-28
25	Homozygous for Filipino β-deletion
26	Homozygous for Hb Lepore

3.5 Molecular analysis of β-thalassaemia using non-invasive DNA samples

3.5.1 DNA analysis using the Amplification Refractory Mutation System (ARMS)

DNA analysis using the ARMS was performed using DNA extracted from mouthwash, saliva and buccal cytobrush samples from 49 patients. DNA samples from the remaining one patient was not amplified using ARMS as the patient is homozygous for Haemoglobin Lepore, which was identified using gap-PCR (refer to section 3.5.3.3).

Sixteen common and rare β-globin gene mutations present in the Malay and Chinese ethnic groups were confirmed using the ARMS. The β-globin gene mutations confirmed using ARMS are at the initiation codon for translation (T-G), -29 (A-G), -28 (A-G), CAP+1 (A-C), CD8/9 (+G), CD15 (G-A), CD17 (A-T), CD19 (A-G), CD26 (G-A), IVS1-1 (G-T), IVS1-5 (G-C), CD41/42 (-CTTT), CD71/72 (+A), IVS2-654 (C-T), CD43 (G-T) and Poly A (AATAAA-AATAGA).

The sixteen mutant ARMS primers were matched with different β-globin gene common primers to detect the sixteen β-globin gene mutations (Tan *et al.*, 2004). Internal controls were included in every PCR reaction. Internal control primers A and B amplified an 861 bp product and were used in ARMS for detection of every mutation except for the β-globin gene mutation at IVS2-654. The size of amplified product for IVS2-654 (830 bp) differed from the internal control product (861 bp product) by 31 base pairs. Thus, another set of internal control was used with IVS2-654.

Internal control primers E and F were used for the β-globin gene mutation at IVS2-654 in place of A and B. The amplification of the internal control for IVS2-654 was performed in a separate PCR reaction and produced an amplified product with a size of 323 bp.

Four normal ARMS primers were included to exclude the normal gene sequence for IVS1-5, IVS2-654, CD41/42 and -28 in seven patients who were homozygous for these β-globin gene mutations.

The common ARMS primer sequences are presented in Table 3.5 while the mutant and normal ARMS primer sequences and their amplified product size are listed in Table 3.6.

Table 3.5 Common primer sequences for ARMS for amplification of internal controls and the 16 common and rare β-globin gene mutations

Common primers	Primer direction	Sequence (5' to 3')
A	Sense	CAA TGT ATC ATG CCT CTT TGC ACC
B	Antisense	GAG TCA AGG CTG AGA AGA TGC AGG
C	Sense	ACC TCA CCC TGT GGA GCC AC
D	Antisense	CCC CTT CCT ATG ACA TGA ACT TAA
E	Antisense	CTC TGC ATC ATG GGC AGT GAG CTC
F	Sense	AGT GCT GCA AGA AGA ACA ACT ACC
G	Antisense	TTC GTC TGT TTC CCA TTC TAA ACT

Table 3.6 Mutant and normal primer sequences for ARMS for the detection of 16 β-globin gene mutations, common primers used and molecular weight of amplified product

Primers	Primer direction	Primer type	Sequence (5' to 3')	Common Primer	Amplified product size (bp)
Primer sequences to detect β-globin gene mutations (Malay) at:					
CD26	Sense	Mutant	CGT GGA TGA AGT TGG TGG TA	D	458
IVS1-5	Antisense	Mutant	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG	C	285
	Antisense	Normal	CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC	C	285
IVS1-1	Antisense	Mutant	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA	C	281
Poly A	Sense	Mutant	GGC CTT GAG CAT CTG GAT TCT GCC TAT TAG	B	393
CD15	Sense	Mutant	TGA GGA GAA GTC TGC CGT TAC TGC CCA GTA	D	500
CD19	Sense	Mutant	TGC CGT TAC TGC CCT GTG GGG CAA GGA GAG	D	488
CD8/9	Antisense	Mutant	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	C	215
CAP (+1)	Sense	Mutant	AAA AGT CAG GGC AGA GCC ATC TAT TGG TTC	D	596

Table 3.6 Mutant and normal primer sequences for ARMS for the detection of 16 β-globin gene mutations, common primers used and molecular weight of amplified product (cont.)

Primers	Primer direction	Primer type	Sequence (5' to 3')	Common Primer	Amplified product size (bp)
Primer sequences to detect β-globin gene mutations (Chinese) at:					
IVS2-654	Sense	Mutant	GAA TAA CAG TGA TAA TTT CTG GGT TAA CGT	B	830
	Sense	Normal	GAA TAA CAG TGA TAA TTT CTG GGT TAA CGC	B	830
CD41/42	Antisense	Mutant	GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT	C	439
	Antisense	Normal	GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA	C	443
CD17	Antisense	Mutant	CTC ACC ACC AAC TTC ATC CAC GTT CAG CTA	C	240
-28	Sense	Mutant	AGG GAG GGC AGG AGC CAG GGC TGG GCT TAG	D	624
	Sense	Normal	AGG GAG GGC AGG AGC CAG GGC TGG GCT TAA	D	624
CD43	Antisense	Mutant	ATC AGG AGT GGA CAG ATC CCC AAA GGA GTA	C	448
-29	Sense	Mutant	CAG GGA GGG CAG GAG CCA GGG CTG GGT ATG	D	625
CD71/72	Sense	Mutant	CAA GAA AGT GCT CGG TGC CTC TAA	G	234
Initiation Codon	Sense	Mutant	TGT TCA CTA GCA ACC TCA AAC AGA CAG CAG	D	545

All ARMS reaction mixture utilised 2.5 µL of 10X reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, Invitrogen, USA) and 200 µM of deoxyribonucleotide triphosphate (dNTP, Applied Biosystem, USA).

The reaction mixture prepared for detection of β-globin gene mutation at CD26 contained 1.2 mM magnesium chloride ($MgCl_2$, Invitrogen, USA), 2.5 unit *Taq* DNA polymerase (Invitrogen, USA) and primers (Invitrogen, USA).

For the detection of the other β-globin gene mutations, the concentrations of $MgCl_2$ and *Taq* DNA polymerase were adjusted to 1.5 mM and 0.5 units respectively. However, for mutations at CD15 and CD19, the concentration of $MgCl_2$ was reduced to 1.3 mM and 0.8 mM respectively to reduce non-specific primer hybridisation.

Purified DNA (1 µg) was added into the reaction tubes. Sterile deionised water was added to bring the reaction mixture to a final volume of 25 µL. A non-template control or DNA blank, negative and positive controls for each β-globin gene mutation were included in each assay for validation. Negative control was obtained from DNA of a normal individual who did not posses any β-globin gene mutation. Positive control was obtained from archived DNA of the individuals who were confirmed to carry the specified β-globin gene mutation for the respective ARMS.

Primer pairing, the final concentrations for every mutant and normal primers and their annealing temperatures are listed in Table 3.7 and Table 3.8, respectively. Common primers A and B and common primers E and F serve as internal control. Mutant and normal primers will pair with their respective common primers to produce the amplification product to determine the presence or absence of the respective β-globin gene mutations.

Table 3.7 Final concentrations of mutant primers and annealing temperatures for detection of the 16 β-globin gene mutations using ARMS

β-globin gene mutation	Primer pairs and final concentrations				Annealing temperature (°C)
	Internal control (pmol)		Mutant (pmol)		
CD26	A	: 0.08	CD26	: 0.12	65
	B	: 0.08	D	: 0.12	
IVS1-5	A	: 0.4	IVS1-5	: 0.36	65
	B	: 0.32	C	: 0.4	
IVS1-1	A	: 0.24	IVS1-1	: 0.12	65
	B	: 0.24	C	: 0.12	
Poly A	A	: 0.32	Poly A	: 0.12	65
	B	: 0.32	B	: 0.32	
CD15	A	: 0.08	CD15	: 0.04	60
	B	: 0.12	D	: 0.04	
CD19	A	: 0.16	CD19	: 0.06	60
	B	: 0.16	D	: 0.06	
CD8/9	A	: 0.4	CD8/9	: 0.12	65
	B	: 0.4	C	: 0.32	
CAP (+1)	A	: 0.24	CAP (+1)	: 0.4	65
	B	: 0.4	D	: 0.6	

Table 3.7 Final concentrations of mutant primers and annealing temperatures for detection of the 16 β-globin gene mutations using ARMS (cont.)

β-globin gene mutation	Primer pairs and final concentrations				Annealing temperature (°C)
	Internal control (pmol)		Mutant (pmol)		
IVS2-654*	E	: 0.08	IVS2-654	: 0.08	60
	F	: 0.08	B	: 0.08	
CD41/42	A	: 0.4	CD41/42	: 0.64	65
	B	: 0.24	C	: 0.2	
CD17	A	: 0.24	CD17	: 0.32	65
	B	: 0.32	C	: 0.32	
-28	A	: 0.24	-28	: 0.32	65
	B	: 0.24	D	: 0.64	
CD43	A	: 0.32	CD43	: 0.16	65
	B	: 0.24	C	: 0.2	
-29	A	: 0.4	-29	: 0.32	65
	B	: 0.32	D	: 0.8	
CD71/72	A	: 0.24	CD71/72	: 0.16	60
	B	: 0.32	G	: 0.4	
Initiation codon	A	: 0.4	Initiation codon	: 0.16	65
	B	: 0.32	D	: 0.16	

Note: * - The internal control primers E and F amplified a 323 bp product in a separate reaction

Table 3.8 Final concentrations of normal primers and annealing temperatures for detection of 4 β-globin gene mutations using ARMS

Primer	Primer pairs and final concentrations				Annealing temperature (°C)
	Internal control (pmol)		Mutant (pmol)		
IVS2-654N*	E	: 0.08	IVS2-654N	: 0.08	60
	F	: 0.08	B	: 0.08	
IVS1-5N	A	: 0.4	IVS1-5N	: 0.36	65
	B	: 0.32	C	: 0.4	
CD41/42N	A	: 0.4	CD41/42N	: 0.064	65
	B	: 0.32	C	: 0.064	
-28N	A	: 0.24	-28N	: 0.32	65
	B	: 0.24	D	: 0.64	

Note: * - The internal control primers E and F amplified a 323 bp product size in a separate reaction

PCR was carried out in an Applied Biosystem Veriti thermal cycler. The cycling conditions were 95°C for 5 minutes for complete denaturation of DNA, 93°C for 1 minute (for denaturation of DNA), 60°C or 65°C for 1 minute (for annealing of primers), 72°C for 1.5 minutes (for extension of DNA) followed by 72°C for 3 minutes for final extension. The denaturation-annealing-extension process was performed for 30 cycles.

Using ARMS, DNA from normal individuals will produce an internal control amplified product of 861 bp. For ARMS using common primers E and F, the internal control amplified product will be 323 bp band. DNA of individuals with β-globin gene mutations will produce amplified products with sizes according to the respective β-globin gene mutations. For normal primers, the presence of normal β-globin gene sequence will allow primer annealing and produce the amplified normal β-globin gene sequence.

3.5.2 Combine-ARMS (C-ARMS) for rapid detection of the β-globin gene mutations at CD41/42 (-CTTT) and CD17 (A-T)

For rapid detection of CD41/42 and CD17 in the Chinese patients, C-ARMS for CD41/42/CD17 was performed to detect the two mutations in a single reaction. Thirteen samples which have been identified to carry mutations at CD41/42 or CD17, or compound heterozygous for CD41/42/CD17 through single ARMS were analysed using the C-ARMS.

The reaction mixture for C-ARMS CD41/42/CD17 utilised 2.5 µL of 10X reaction buffer, 200 µM of dNTP, 1.5 mM of $MgCl_2$ and 1.5 units of *Taq* DNA polymerase. Purified DNA (1 µg) was added into the reaction tubes. The reaction mixture was brought to a final volume of 25 µL with sterile deionised water. A non-template control, negative and positive controls for CD41/42, CD17 and CD41/42/CD17 were included in every assay for validation.

The final concentrations of the primers used in the C-ARMS for CD41/42/CD17 are listed in Table 3.9. Common primers A and B serve as internal control. Both CD41/42 and CD17 primers share the same common primer – common C.

Table 3.9 Final concentrations of primers used in C-ARMS for detection of the β-globin gene mutations at CD41/42 and CD17

C-ARMS	**Primer pairs and final concentrations (pmole)**	
CD41/42/CD17	Common A	: 0.4
	Common B	: 0.32
	CD41/42	: 0.08
	CD17	: 0.16
	Common C	: 0.16

The reaction was initiated with complete denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation-annealing-extension (93°C for 1 minute, 65°C for 1 minute and 72°C for 1.5 minutes) and a final extension at 72°C for 3 minutes.

Hybridisation between common primers A and B will produce an internal control amplified product of 861 bp. DNA with β-globin gene mutation at CD41/42 will produce a 439 bp amplified product while DNA with the β-globin gene mutation at CD17 will produce a 240 bp amplified product. DNA from a compound heterozygous CD41/42/CD17 individual will produce both 439 bp and 240 bp amplified products.

3.5.3 Gap-PCR for detection of β-thalassaemia

The β-thalassaemias caused by a large Filipino β-deletion, the Chinese 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{o}$-specific deletion and Hb Lepore were confirmed by DNA amplification across the deleted regions in the β-globin gene complex (gap-PCR) using specific primers that flank the deleted sequences.

3.5.3.1 Detection of the Filipino β-deletion

Mouthwash, saliva and buccal cytobrush DNA samples from one patient was analysed using gap-PCR for detection of the Filipino β-deletion. The PCR mixture was prepared using 2.5 µL of 10X reaction buffer, 200 µM of dNTP, 5.0 µL of Q Solution (Qiagen, USA), 2.5 units of *Taq* DNA polymerase and 0.4 pmole of each of the primers P2, P4 and P5. DNA (1 µg) was added into the reaction tubes. The mixture was brought to a final volume of 25 µL with sterile deionised water. A non-template control, negative, heterozygous positive and homozygous positive controls were included for validation of each assay.

The sequences of the primers are listed in Table 3.10. Primers P2 and P5 will anneal to the normal sequence in the DNA template and was used to detect the presence of the normal β-globin gene sequence. Primers P4 and P5 flank the deleted Filipino β-deletion and will detect the presence of the deletion-specific sequence.

Table 3.10 Primer sequences for gap-PCR to detect the Filipino β-deletion

Primers	Sequence (5' to 3')	Amplified product size (bp)
P2	TCA GAA GCA GAG CTA CTC AG	482
P5	CAT TTA GCT CCC ACA CTC CT	
P4	GTC TAT GCA GGT GTG TAG ACA	376

The amplification process involved 35 cycles of template denaturation at 95°C for 1 minute, primer annealing at 60°C for 1 minute and extension at 72°C for 1 minute.

The presence of the normal β-globin gene sequence allows amplification using primers P2 and P5 and produces a 482 bp amplified product. Absence of the normal β-globin gene sequence will only allow amplification of the primers P4 and P5, producing a 376 bp amplified deletion-specific product. After gel electrophoresis, DNA from a normal individual will show only the 482 bp amplified product. DNA from a homozygous β-thalassaemia individual for the Filipino β-deletion will only yield the 376 bp amplified product. A β-thalassaemia carrier with the Filipino β-deletion will show both the 482 bp and 376 bp amplified products.

3.5.3.2 Detection of the 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion

Mouthwash, saliva and buccal cytobrush DNA samples from one patient was analysed using gap-PCR for detection of the 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{o}$-specific deletion. The reaction mixture contained 2.5 µL of 10X reaction buffer, 200 µM of dNTP, 3 µM of $MgCl_2$, 2.5 units of *Taq* DNA polymerase, 0.2 pmole of primers G1 and G2 and 0.24 pmole of primer G3. Purified DNA (1 µg) was included in the reaction tubes. The total volume of the reaction mixture was 25 µL. For validation of assay, a non-template control, negative and heterozygous positive controls were included in each assay performed.

The sequences of the primers used are listed in Table 3.11. Primers G1 and G2 will anneal to the normal sequence in the DNA template and was used to detect the presence of the normal β-globin gene sequence. Primers G2 and G3 flank the deleted 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$ sequence and will detect the presence of the deletion-specific sequence.

Table 3.11 Primer sequences for gap-PCR to detect the 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion

Primers	Sequence (5' to 3')	Amplified product size (bp)
G1	GGC ATA TAT TGG CTC AGT CA	682
G2	CTT GCA GAA TAA AGC CTA TC	
G3	TCA ACA ATT ATC AAC ATT ACA	508

The DNA amplification reaction was initiated with complete denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation (93°C for 1 minute), annealing (58°C for 1 minute) and extension (72°C for 2 minutes) followed by a final extension at 72°C for 10 minutes.

Amplification of primers G1 and G2 will produce a 682 bp amplified product which shows the presence of the normal β-globin gene sequence in the DNA template. The presence of the $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion will produce a 508 bp deletion-specific amplified product using primers G2 and G3. A normal individual will only amplify the 682 bp normal β-globin gene sequence. DNA from a homozygous individual will only amplify the 508 bp deletion-specific product. The presence of both amplified products after DNA amplification shows that the individual is heterozygous for the $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion.

3.5.3.3 Detection of Haemoglobin Lepore

Mouthwash, saliva and buccal cytobrush DNA samples from one patient was analysed using gap-PCR for detection of Hb Lepore. The reaction mixture for Hb Lepore was made up of 2.5 µL of 10X reaction buffer, 200 µM of dNTP, 1.5 mM of $MgCl_2$, 0.5 units *Taq* DNA polymerase 0.144 pmole primer LA, 0.256 pmole primer LB and 0.8 pmole primer LC. Purified DNA (1 µg) was included in the reaction tubes. The final volume of the reaction mixture was 25 µL. For each assay performed, non-template control, negative and homozygous positive controls were included for validation.

The sequence of primers LA, LB and LC are listed in Table 3.12. Primers LA and LB will anneal to the normal sequence in the DNA template and was used to detect the presence of the normal β-globin gene sequence. Primers LA and LC flank the deleted β-globin sequence and will detect the presence of the deletion-specific sequence.

Table 3.12 Primer sequences for gap-PCR to detect Haemoglobin Lepore

Primers	Sequence (5' to 3')	Amplified product size (bp)
LB	CGA TCT TCA ATA TGC TTA CCA AG	915
LA	CAT TCG TCT GTT TCC CAT TCT A	
LC	GAC ACA CAT GAC GGA ACA GCC AAT	775

The amplification reaction involved complete denaturation at 94°C for 4 minutes, followed by denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and extension at 72°C for 2 minutes. DNA amplification was carried out for 30 cycles before final extension at 72°C for 10 minutes.

The presence of the normal β-globin gene sequence in a normal individual will be amplified as a 915 bp product using primers LA and LB. DNA from an individual homozygous for Hb Lepore will amplify a 775 bp deletion-specific sequence using primers LA and LC. DNA from an individual heterozygous for Hb Lepore will amplify both the 915 bp normal β-globin gene sequence and the 775 bp Hb Lepore deletion-specific gene sequence.

3.5.4 Detection of β-globin gene mutation at CD27/28 (+C) using PCR-RFLP

The detection of the β-globin gene mutation at CD27/28 was performed using PCR-RFLP. DNA from mouthwash, saliva and buccal cytobrush samples from one patient was analysed using the technique. The reaction mixture consisted of 2.5 µL of 10X reaction buffer, 200 µM of dNTP, 1.5 mM of $MgCl_2$, 0.5 units *Taq* DNA polymerase, and 0.32 pmole of each forward (27/28F3) and reverse (27/28R) primer. Purified DNA (1 µg) was added into the reaction tubes. The reaction mixture was brought to a final volume of 25 µL with sterile deionised water. Non-template control, negative and heterozygous positive controls were included in each assay for validation purpose.

The primer sequences are listed in Table 3.13. The primers 27/28F3 and 27/28R will flank the sequence where the β-globin gene mutation at CD27/28 is located. The amplified product will be obtained both from normal individuals and individuals with the β-globin gene mutation. The amplified product was subjected to subsequent restriction enzyme digestion.

Table 3.13 Primer sequences for PCR-RFLP to detect the β-globin gene mutation at CD27/28

Primers	Sequence (5' to 3')	Amplified product size (bp)
27/28F3	CTG GGC ATA AAA GTC AGG G	284
27/28R	GGC AGA GAG AGT CAG TGC CTA	

Reaction was initiated with complete denaturation at 94°C for 5 minutes. DNA amplification was performed at 94°C for 2 minutes, followed by annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes. The denaturation-annealing-extension cycles were repeated 35 times followed by additional incubation at 72°C for 3 minutes. After DNA amplification, the amplification product was electrophoresed in 1.5% w/v agarose gel at 70V for 40 minutes to confirm the presence of the 284 bp amplified product.

For restriction enzyme digestion, the reaction mixture prepared contained 1 unit of NlaIV restriction enzyme (Cat: R0126, New England Biolabs, USA), 2.5 µL of 10X NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT at pH 7.9) and 0.25 µL of 100X bovine serum albumin (BSA, New England Biolabs). Amplification product (15 µL) was added into the reaction tubes. Sterile deionised water was added to bring the reaction mixture to a final volume of 25 µL. Restriction enzyme digestion was carried out at 37°C overnight.

The addition of a single base (+C) at CD27/28 creates a restriction site, which cleaves the DNA amplified product into two fragments with sizes of 170 bp and 114 bp. DNA from a normal individual will only show the 284 bp amplified product after the restriction enzyme treatment and gel electrophoresis. DNA from a heterozygous individual with the β-globin gene mutation at CD27/28 will show the 284 bp undigested amplified product and 170 bp and 114 bp digested products. Individuals homozygous for CD27/28 will only produce the 170 bp and 114 bp digested products.

3.5.5 Gel electrophoresis and visualisation

Amplified DNA (10 µL) from single ARMS, C-ARMS and gap-PCR was electrophoresed in 1.5% w/v agarose gels (SeaKem LE) pre-stained with 2.0 µL ethidium bromide (Sigma-Aldrich, USA). PCR-RFLP products which have smaller molecular weight products was analysed in 2.0% w/v agarose gels. Loading of amplified DNA was carried out using 2 µL 5X loading buffer (3% v/v bromophenol blue, 15% v/v xylene cyanol in 10X Tris-Borate-EDTA (TBE):glycerol:2% w/v SDS solution in ratio of 50:49:1). Agarose gels were electrophoresed for 90 minutes at 70V in 1X TBE (Thermo Scientific) buffer. Electrophoresis of amplified DNA was carried out with molecular weight markers (Thermo Scientific) as reference. Separated fragments were visualised and photographed using the MultiDoc-it Digital Imaging System (UVP, USA).

3.6 Oxidative stress indices and cytokine measurement

The frozen saliva samples were thawed and centrifuged at 10,000 x *g* for 10 minutes in refrigerated microcentrifuge prior to assay to remove mucin. Supernatant was used directly without dilution for the measurement of the oxidative stress indices and cytokine level based on established methods and commercialised kits. All samples were assayed in triplicates.

3.6.1 Ferric reducing antioxidant power

Ferric reducing ability of metabolites in samples was measured using colorimetric method, ferric reducing antioxidant power (FRAP) assay. The FRAP reagent containing Fe^{3+} ion from ferric 2,4,6-tripyridyl-triazine complex (Fe^{3+}-TPTZ) was added into the sample. The concentration of Fe^{2+} produced due to reduction-oxidation reaction between the sample and Fe^{3+}-TPTZ complex was measured (Benzie & Strain, 1996).

The FRAP reagent contains 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloric acid (HCl) and 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$), mixed in the ratio of 10:1:1. The TPTZ reagent is light sensitive and FRAP reagent was protected from light once prepared.

Acetate buffer was prepared by mixing 0.31 g of sodium acetate trihydrate ($NaOAc.3H_2O$) with 1.6 mL of glacial acetic acid and brought up to a final volume of 100 mL with deionised water. Six different concentrations of ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) ranging from 0, 200, 400, 600, 800 and 1000 µM were used as standards.

Reaction was initiated by mixing 10 µL of each sample or standards with 300 µL of FRAP reagent in flat bottom 96-well plate. Absorbance at 593 nm from 0 to 4 minutes was measured and the difference was calculated. The FRAP values were expressed in µM.

3.6.2 Advanced oxidation protein product

Advanced oxidation protein products (AOPP) were measured using colorimetric method. The sample absorbance was compared with the absorbance of chloramine-T after mixing them with potassium iodide (KI) in acidic condition (Witko-Sarsat *et al.*, 1996).

AOPP reagent was prepared by mixing phosphate buffered saline (PBS, Oxoid), 50% acetic acid and 1.16 M KI in 81:15:4 ratios. Prepared reagent was protected from light. Six concentrations of chloramine-T in deionised water (0, 100, 200, 300, 400 and 500 µM) were prepared as standard.

Reaction was initiated by mixing 18 µL of each sample and standards with 200 µL of reagent mixture in flat bottom 96-well plate. Absorbance at 340 nm was measured instantly and results were expressed in µM chloramine unit.

3.6.3 Lipid hydroperoxide

Assessment of lipid peroxidation was performed by measuring the level of lipid hydroperoxide (LOOH) in samples. Method used was based on procedures described by Esterbauer & Cheeseman (1990).

Standards were prepared by diluting 1,1,3,3-tetraethoxypropane (TEP) in deionised water. Six concentrations of TEP (0, 2.5, 5, 10, 15 and 20 µM) were included in each assay to generate a standard curve.

Each sample and standards (150 µL) were mixed with 375 µL of 10.3 mM MPI in acetonitrile and 225 µL of 5 M hydrochloric acid (HCl). Reaction was initiated by incubating the mixture in 45°C waterbath for 40 minutes. The mixture was then centrifuged at 10,000 x *g* for 5 minutes. Supernatant (200 µL) was transferred into flat bottom 96-well plate and absorbance reading at 586 nm was measured. Results were compared with standards to determine the LOOH concentration present in the samples and expressed in µM.

3.6.4 Glutathione peroxidase

Measurement of glutathione peroxidase (GPx) activity was carried out using Glutathione Peroxidase Assay Kit (Cayman Chemical, USA, Cat: 703102). The assay involves measuring the rate of NADPH concentration reduction to determine GPx activity. The assay was carried out in flat bottom 96-well plate according to the procedure supplied with the kit.

The reagents were brought to room temperature and diluted appropriately for each experiment. Blank, samples and control were included in each assay.

Samples and control (20 µL) were added into each well. For blank, 20 µL of Assay buffer were loaded instead. Assay buffer (100 µL) and Co-Substrate mixture (50 µL) were loaded into each well. Reaction was initiated by adding 20 µL of Cumene Hydroperoxide. The plate was agitated to mix the solutions.

Changes of absorbance at 340 nm were monitored for at least 5 minutes at 1 minute interval. Mean of values obtained for each minute were plotted and linear equation is generated. The slope (rate) of the linear curve for each sample and control were subtracted from blank. Enzyme activity was calculated through the formula below.

$$\textbf{GPx activity (nmol/min/mL)} = \frac{\textbf{Rate}}{\textbf{0.00373 µM-1}} \times \frac{\textbf{0.19 mL}}{\textbf{0.02 mL}} \times \textbf{sample dilution}$$

3.6.5 Uric Acid

Assay for measurement of uric acid (UA) level was carried out using Uric Acid Assay Kit (BioVision, Cat: K608-100). The kit was based on enzymatic method to estimate the amount of UA in samples. Preparations and assay were carried out according to the procedures supplied with the kit. The assay was performed in flat bottom 96-well plate.

Blank, standards and samples were included for each assay carried out. Crude standard in dimethyl sulfoxide (DMSO) at concentration of 2 nmol/µL was diluted with assay buffer to produce series of standards with concentrations of 0, 8, 16, 24, 32 and 40 nmol per well. Samples and standards (50 µL) were loaded into the corresponding wells.

Reaction was initiated by adding 50 µL of reaction mix comprising 46 µL of assay buffer, 2 µL of UA probe and 2 µL of UA enzyme mix into each well. Mixtures were incubated at 37°C and protected from light for 30 minutes.

Absorbance reading at 570 nm was carried out after the incubation. The average reading for samples and standards were subtracted from blank. Linear equation was generated from standard curve and the amounts of UA in sample wells were obtained. The UA concentration is calculated using the formula:

$$\textbf{[UA] (nmol/mL)} = \frac{\textbf{Amount of UA in sample well (nmol)}}{\textbf{Sample volume (µL)}} \times 1000$$

3.6.6 Tumor necrosis factor-α and interleukin-6

The levels of cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were estimated using ELISA kits by BioLegend ELISA MAX (Cat: 43204 and 43504, respectively).

Assay was carried out according to the procedures provided with minor alteration. One day prior to performing the assay, Capture Antibody was diluted 200 times in 1X Coating Buffer. Diluted antibody (200 µL) was added into each well of 96-well plate and incubated for 16-18 hours at 4°C.

On the day the assay was performed, each well was washed twice with 300 µL of PBS with 0.05% Tween-20. The wells were further washed twice with 300 µL of PBS. Buffer residue was removed from the wells by tapping the plate upside down on an absorbent paper after each washing.

Antigen blocking was carried out by incubating the wells with 200 µL of 1X Assay Diluent for 1 hour. The plate was sealed and incubated at room temperature on a shaker prior to the washing steps.

Samples and diluted standards (100 µL) were added into the corresponding wells and were incubated for 2 hours which was followed by washing steps. Diluted Detection Antibody (100 µL) was added into all wells and incubation was carried out for 1 hour. The plate was washed again before the addition of 100 µL of Avidin-HRP solution into each well and followed by further 30 minute incubation.

For final washing, the plate was washed twice with 300 µL PBS with 0.05% Tween-20 and thrice with PBS. For each round of washing, the wells were soaked in the buffer for 1 minute. After washing, 100 µL of TMB Substrate solution was added into each well and kept in the dark for 15 minutes to allow colour development.

Reaction was terminated by adding 100 µL of 2 N sulphuric acid (H_2SO_4). Absorbance at 450 nm was measured within 30 minutes. Standard curve was generated to determine the concentration of the cytokine in pg/mL.

3.6.7 Statistical Analysis

Results from biochemical assay were assessed using SPSS 17. The normality in data distribution was first assessed by Shapiro-Wilk test. The alpha level was set to 0.05. Any significance in Shapiro-Wilk value indicates that the data was significantly deviated from normal distribution.

As Shapiro-Wilk test confirmed deviation of data distribution, data was assessed using non-parametric statistical analyses. Significance of difference on the median of parameter levels between β-thalassaemia major patients and healthy individuals groups was also assessd using Mann-Whitney U test.

Correlation between oxidative stress indices and cytokines were estimated using Spearman's correlation.

Analysis for significance of difference between demographic data sub-groups in β-thalassaemia major patients was performed using Kruskal-Wallis H test. Any significant differences observed were further analysed using Mann-Whitney U test to determine the actual groups with significant differences (Jaykaran, 2010).

CHAPTER 4: RESULTS

4.1 Quantity and quality of extracted DNA from non-invasive samples

The results for DNA concentration and purity were presented as median. Comparison of DNA concentration and purity between sampling methods was carried out using Friedman test. Results were presented in chi-square (χ^2) value with degrees of freedom of 2. Friedman test was followed by post-hoc test using Wilcoxon signed-rank test to determine where the differences occurred. The results were reported using the *Z* statistics.

Comparison of DNA concentration and purity between DNA extraction methods 1 and 2 was performed using Wilcoxon signed-rank test. The results were reported using the *Z* statistics.

Difference in DNA concentration and purity between sampling procedures and DNA extraction method was considered as statistically significant if *p* value is less than 0.05 and highly significant if *p* value is less than 0.01.

4.1.1 Concentration and purity of purified DNA extracted using Method 1

Using Method 1, mouthwash samples produced purified DNA with a median concentration of 0.14 µg/µL. The mean DNA concentration of mouthwash samples was 0.17 ± 0.13 µg/µL (mean ± standard deviation (SD)) and the concentration ranged from 0.01 – 0.60 µg/µL. The median concentration of purified DNA from saliva samples was 0.28 µg/µL (mean: 0.38 ± 0.38 µg/µL, range: 0.07 – 2.14 µg/µL) while median concentration of DNA from buccal cytobrush samples was 0.09 µg/µL (mean: 0.19 ± 0.18 µg/µL, range: 0.04 – 0.80 µg/µL). Using Method 1, saliva samples produced the highest median of purified DNA concentration followed by mouthwash and buccal cytobrush samples.

There was a significant difference on DNA concentration, with $\chi^2(2) = 12.98$, $p < 0.05$ depending on the sampling procedure. From the Wilcoxon signed-rank test performed, the median concentration of purified DNA extracted from saliva samples was significantly higher than mouthwash samples ($Z = -3.88$, $p < 0.01$) and buccal cytrobrush samples ($Z = -3.40$, $p < 0.05$).

Figure 4.1 is a graphical representation of the comparison between median purified DNA concentrations for the three sampling procedures using Method 1. Table 4.1 summarise the median, mean, SD, minimum and maximum purified DNA concentration obtained from mouthwash samples.

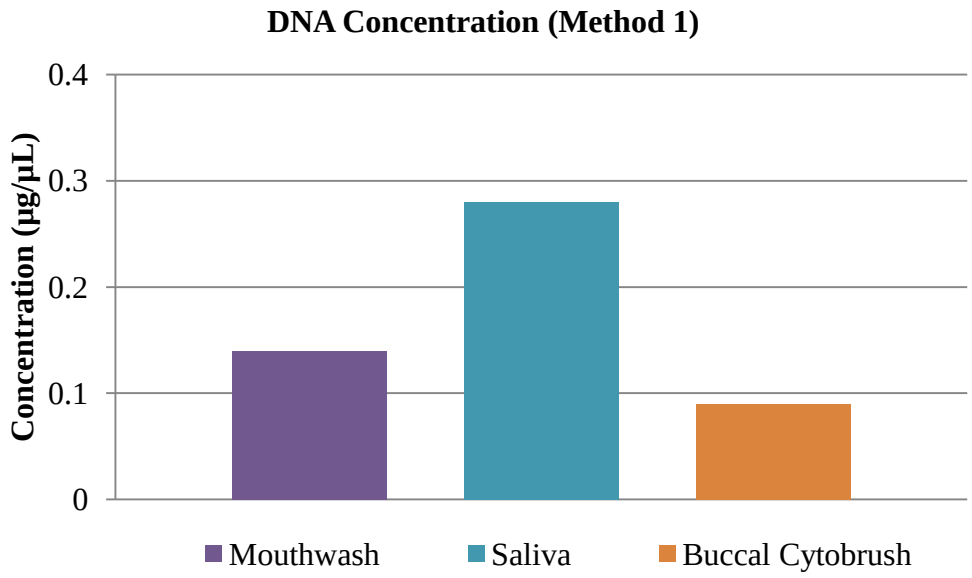

Figure 4.1 Comparison of median of purified DNA concentrations for mouthwash, saliva and buccal cytobrush samples using Method 1

Table 4.1 Comparison of median, mean, SD and range of DNA concentration obtained from mouthwash, saliva and buccal cytobrush samples extracted using Method 1[a]

Concentration (µg/µL)	**Samples**		
	Mouthwash	**Saliva**	**Buccal cytobrush**
Median	0.14	0.28[*,**]	0.09
Mean	0.17	0.38	0.19
SD	0.13	0.38	0.18
Range	0.01 – 0.6	0.07 – 2.14	0.04 – 0.80

[a] Wilcoxon signed-rank test

[*] Significantly higher compared with buccal cytobrush samples ($p < 0.05$)

[**] Significantly higher compared with mouthwash samples ($p < 0.01$)

In aspects of purity, DNA extracted from mouthwash samples has a median purity of 1.64 and mean purity of 1.60 ± 0.17. The DNA purity ranged from 1.15 to 1.90. Saliva samples produced DNA with a median purity of 1.64 (mean: 1.62 ± 0.16, range: 1.32 – 1.91), while the buccal cytobrush samples produced DNA with a median purity of 1.58 (mean: 1.57 ± 0.13, range: 1.27 – 1.87). DNA with the highest purity was obtained from the saliva samples, followed by mouthwash and buccal cytobrush samples using Method 1.

There was a significant difference on DNA purity, with $\chi^2(2) = 6.52$, $p < 0.05$ depending on the sampling procedure. Wilcoxon signed-rank test confirmed that the purity ratio of purified DNA extracted from saliva samples was significantly higher than buccal cytrobrush samples ($Z = -2.29$, $p < 0.05$).

Figure 4.2 summarise of the difference in median purity of purified DNA obtained from the three samples; mouthwash, saliva and buccal cytobrush. The following Table 4.2 present the median, mean, SD and range of DNA purity.

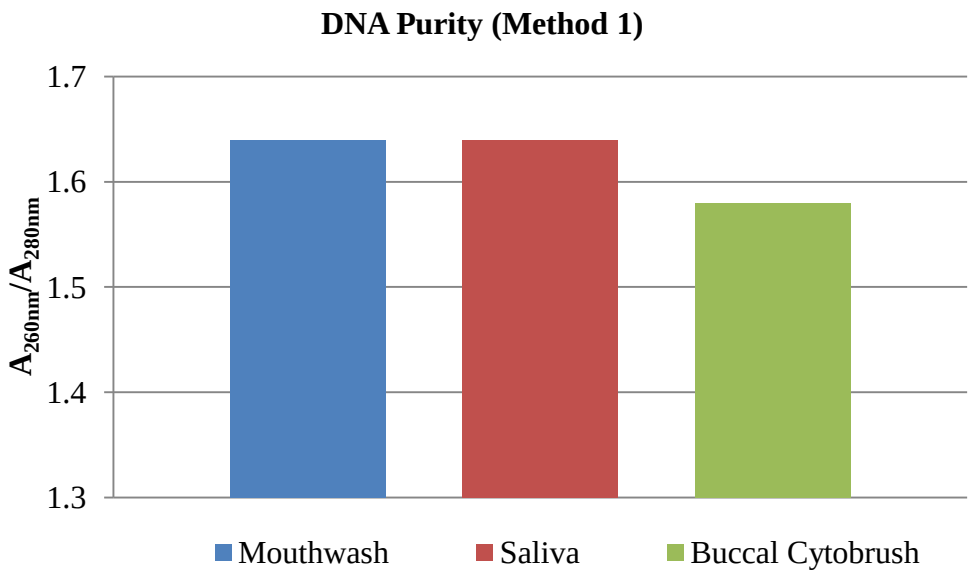

Figure 4.2 Comparison of median of DNA purity between mouthwash, saliva and buccal cytobrush samples using Method 1

Table 4.2 Comparison of median, mean, SD and range of DNA purity obtained from mouthwash, saliva and buccal cytobrush samples extracted using Method 1[a]

Purity	Samples		
	Mouthwash	**Saliva**	**Buccal cytobrush**
Median	1.64	1.64*	1.58
Mean	1.60	1.62	1.57
SD	0.17	0.16	0.13
Range	1.15 – 1.90	1.32 – 1.91	1.27 – 1.87

[a] Wilcoxon signed-rank test

* Significantly higher compared with buccal cytobrush samples ($p < 0.05$)

4.1.2 Concentration and purity of purified DNA extracted using Method 2

For DNA extraction using Method 2, saliva samples also produced purified DNA with the highest median concentration of 0.67 µg/µL (mean: 0.96 ± 0.96 µg/µL, range: 0.01 – 4.7 µg/µL). Mouthwash samples produced the second highest amount of purified DNA with a median concentration of 0.15 µg/µL (mean: 0.26 ± 0.39 µg/µL, range: 0.01 – 4.70). The lowest amount of DNA was obtained from buccal cytobrush samples, with median DNA concentration of 0.08 µg/µL (mean: 0.15 ± 0.33 µg/µL, range <0.01 – 2.14 µg/µL).

Significant difference in DNA concentration was present depending on the sampling procedure, with $\chi^2(2) = 40.44$, $p < 0.01$. The concentration of purified DNA of saliva samples was significantly higher than the DNA concentration of mouthwash ($Z = -5.16$, $p < 0.01$) and buccal cytrobrush samples ($Z = -5.70$, $p < 0.01$).

The DNA concentration from mouthwash samples was also significantly higher than the DNA concentration from buccal cytobrush samples with $Z = -4.03$ ($p < 0.01$).

A graphical representation of the difference in median purified DNA concentration is demonstrated in Figure 4.3. Table 4.3 summarise the median, mean, SD and range of DNA concentration obtained through Friedman test.

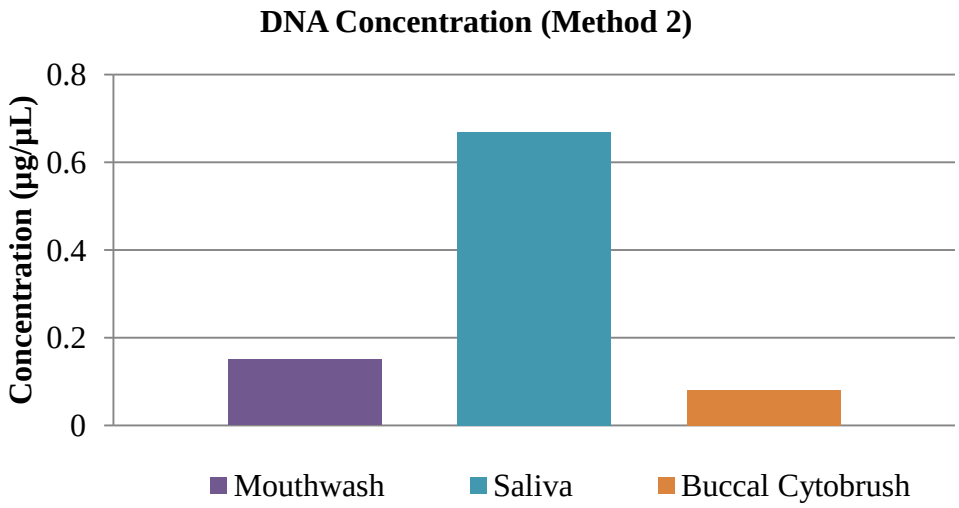

Figure 4.3 Comparison of median of purified DNA concentrations for mouthwash, saliva and buccal cytobrush samples using Method 2

Table 4.3 Comparison of median, mean, SD and range of DNA concentration obtained from mouthwash, saliva and buccal cytobrush samples extracted using Method 2[a]

Concentration (µg/µL)	**Samples**		
	Mouthwash	**Saliva**	**Buccal cytobrush**
Median	0.15[##]	0.67[**]	0.08
Mean	0.26	0.96	0.15
SD	0.39	0.96	0.33
Range	0.01 – 2.6	0.01 – 4.70	< 0.01 – 2.14

[a] Wilcoxon signed-rank test

[##] Significantly higher compared with buccal cytobrush samples ($p < 0.01$)

[**] Significantly higher compared with mouthwash and buccal cytobrush samples ($p < 0.01$)

Using Method 2 DNA extraction, mouthwash samples produced purified DNA with the median purity of 1.75. The mean was 1.74 ± 0.14 with purity ranging from 1.38 – 1.90. On the other hand, DNA extracted from saliva samples has a median purity of 1.70 (mean: 1.65 ± 0.20, range: 1.29 – 2.0). The purified DNA from buccal cytobrush samples have a median purity of 1.68 (mean: 1.62 ± 0.17, range: 1.30 – 1.86). Mouthwash samples produced DNA with the highest purity, followed by saliva and buccal cytobrush samples.

The non-invasive DNA purity was significantly different, with $\chi^2(2) = 20.28$, $p < 0.01$ depending on the sample used for extraction. From the post hoc test performed, the median purity of DNA from mouthwash was significantly higher when compared with purified DNA extracted from saliva ($Z = -2.52$, $p < 0.01$) and buccal cytobrush samples ($Z = -4.11$, $p < 0.01$).

Figure 4.4 shows the summary of the difference in DNA purity based on A_{260nm}/A_{280nm} ratio between mouthwash, saliva and buccal cytobrush samples. Table 4.4 summarise the median, mean, SD and range of DNA purity obtained.

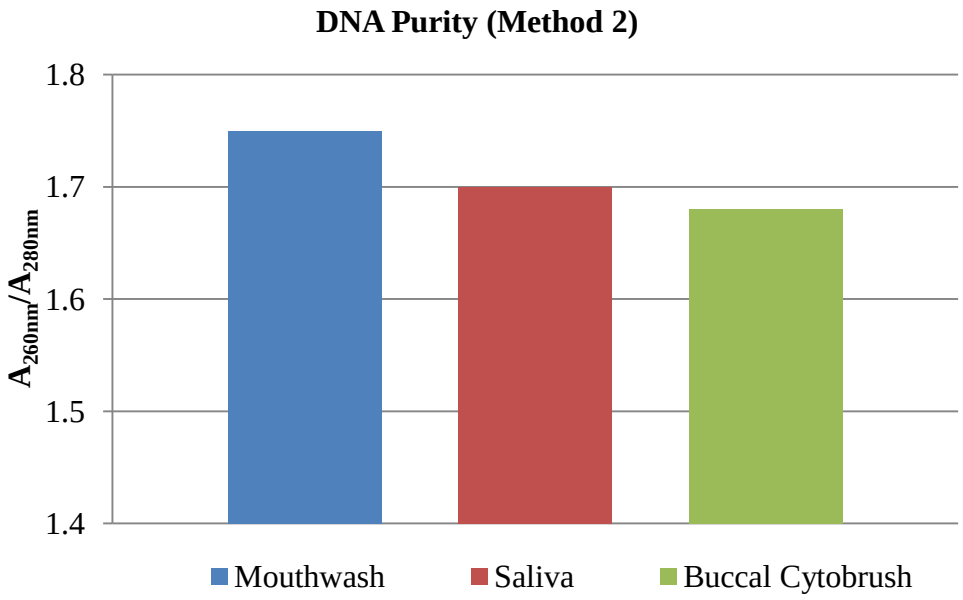

Figure 4.4 Comparison of median of DNA purity between mouthwash, saliva and buccal cytobrush samples using Method 2

Table 4.4 Comparison of median, mean, SD and range of DNA purity obtained from mouthwash, saliva and buccal cytobrush samples extracted using Method 2[a]

Purity	Samples		
	Mouthwash	**Saliva**	**Buccal cytobrush**
Median	1.75**	1.70	1.68
Mean	1.74	1.65	1.62
SD	0.14	0.20	0.17
Range	1.38 – 1.90	1.29 – 2.00	1.30 – 1.86

[a] Wilcoxon signed-rank test

** significantly higher compared with saliva and buccal cytobrush samples ($p < 0.01$)

4.1.3 Comparison of concentration of purified DNA extracted using Methods 1 and 2

The comparison of DNA concentrations between Methods 1 and 2 was performed using Wilcoxon signed-rank test. The comparison indicates that Method 2 was able to extract significantly higher concentration of DNA from saliva samples, with $Z = -3.80$ ($p < 0.01$).

The difference in mouthwash DNA concentration between the methods was not significant ($Z = -1.506$, $p = 0.132$).

However, the median concentration of purified DNA from buccal cytobrush was significantly reduced when the samples were extracted using Method 2 compared with Method 1 ($Z = -2.00$, $p < 0.05$).

Figure 4.5 summarise the comparison in median purified DNA concentration obtained from mouthwash, saliva and buccal cytobrush samples when extracted using Method 1 and 2.

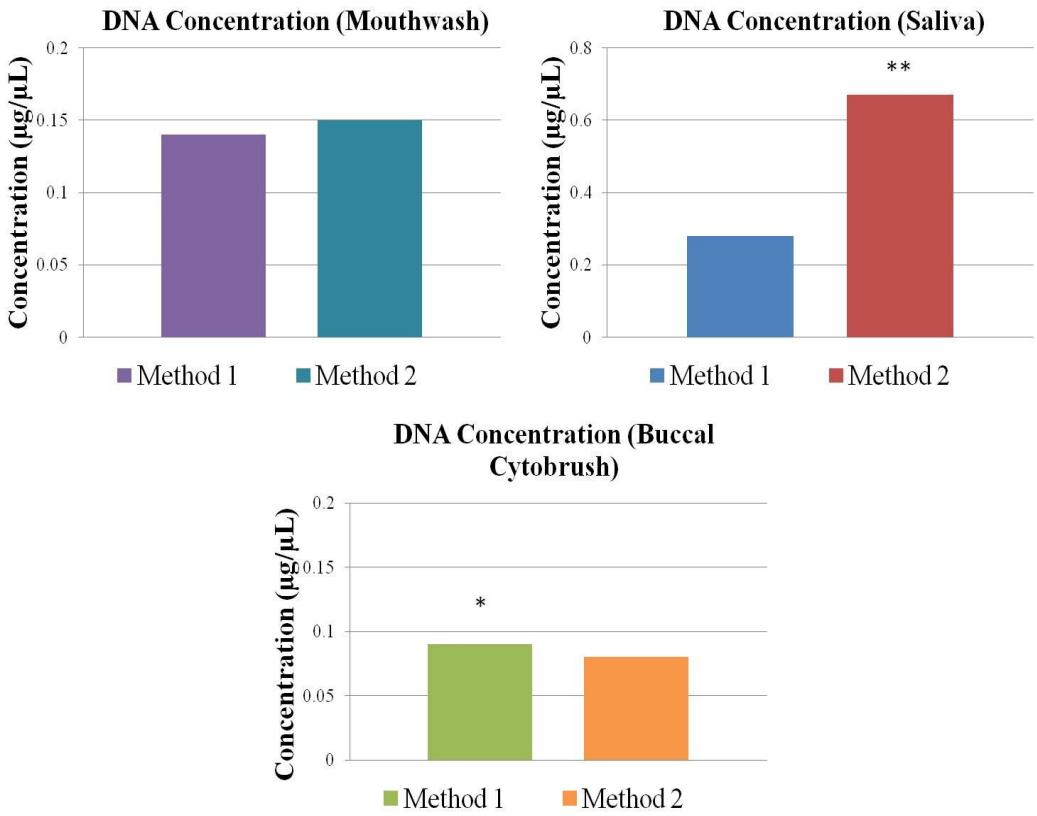

Figure 4.5 Comparison of median purified DNA concentrations between extraction Methods 1 and 2 for mouthwash, saliva and buccal cytobrush samples

* $p < 0.05$ and ** $p < 0.01$ when compared between methods by Wilcoxon signed-rank test

4.1.4 Comparison of purity of purified DNA extracted using Methods 1 and 2

With regards to DNA purity, Method 2 was able to produce DNA with a higher median A_{260nm}/A_{280nm} ratio for all sampling procedures.

Using Method 2, significant increase in DNA purity was observed in mouthwash (Z = -2.52, $p < 0.05$) and saliva samples (Z = 4.11, $p < 0.01$).

However, no significant difference was observed in buccal cytobrush DNA samples purity (Z = -1.38, p = 0. 17).

Figure 4.6 presents the comparison in median of DNA purity between the two extraction methods for mouthwash, saliva and buccal cytobrush samplings.

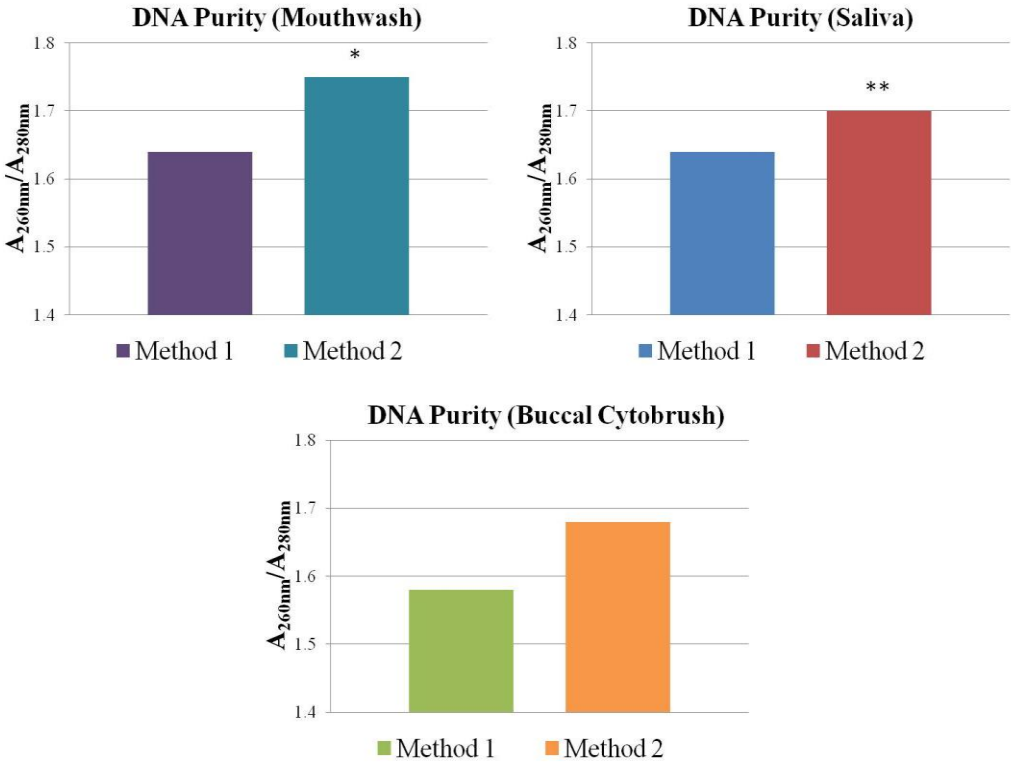

Figure 4.6 Comparison of median DNA purity between extraction Methods 1 and 2 for mouthwash, saliva and buccal cytobrush samples

* $p < 0.05$ and ** $p < 0.01$ when compared between methods by Wilcoxon signed-rank test

4.2 Amplification of purified DNA for molecular analysis of β-thalassaemia

The results for molecular analysis utilising purified non-invasive DNA from mouthwash, saliva and buccal cytobrush samples are presented as the percentage of successful DNA amplifications using the different molecular techniques – Amplification Refractory Mutation System (ARMS), Combine-ARMS, gap-PCR and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

4.2.1 Amplification of purified DNA from archived and non-invasive samples using Amplification Refractory Mutation System (ARMS)

Forty-nine purified DNA samples from each mouthwash, saliva and buccal cytobrush sample collection were analysed using ARMS. Using Method 1, successful DNA amplification was obtained in 55% of mouthwash samples, 65% of saliva samples and 81% of buccal cytobrush samples.

Using Method 2, the successful DNA amplification was increased to 87% for mouthwash samples, 83% for saliva samples and 86% for buccal cytobrush samples. The results show that Method 2 produced purified DNA that produced more successful amplifications compared with Method 1. Archived DNA produced 100% successful amplifications for ARMS.

Results for archived DNA amplification using ARMS are shown in Figure 4.7, 4.8 and 4.9. Results for mouthwash, saliva and buccal cytobrush DNA amplification using ARMS for the β-globin gene mutation at CD26 are shown in Figure 4.10.

Figure 4.7 shows the archived DNA amplified for β-globin gene mutations common in the Malays (lanes 2-9). Lane 1 is the internal control amplified as an 861 bp band in DNA from a normal individual by using common primers A and B. The 861 bp internal control band can be observed in all the lanes 1-9 which contain amplified DNA from archived samples of heterozygous β-thalassaemia carriers.

Using ARMS, the DNA with β-globin gene mutation at CAP+1 was amplified as a 596 bp band (lane 2), CD15 as a 500 bp band (lane 3), CD19 as a 488 bp band (lane 4), CD26 as a 458 bp band (lane 5), Poly A as a 393 bp band (lane 6), IVS1-5 as a 285 bp band (lane 7), IVS1-1 as a 281 bp band (lane 8) and CD8/9 as a 215 bp band (lane 9)

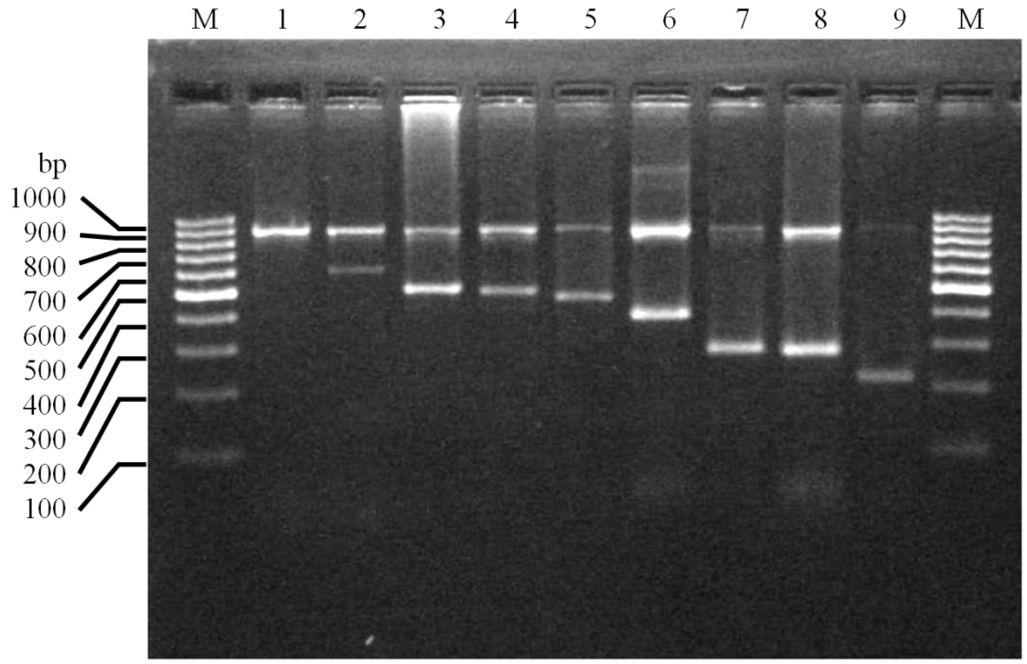

Figure 4.7 Gel electrophoresis after ARMS amplification for confirmation of β-globin gene mutations common in the Malays

Lane M: 100 bp molecular weight marker

Lane 1: Common A and B internal control, 861 bp

Lane 2: CAP+1, 596 bp

Lane 3: CD15, 500 bp

Lane 4: CD19, 488 bp

Lane 5: CD26, 458 bp

Lane 6: PolyA, 393 bp

Lane 7: IVS1-5, 285 bp

Lane 8: IVS1-1, 281 bp

Lane 9: CD8/9, 215 bp

Figure 4.8 shows the archived DNA amplified for β-globin gene mutations common in the Chinese (lanes 2-7, 9 and 10). Lane 1 is the internal control amplified as an 861 bp band in DNA from a normal individual by using common primers A and B. The 861 bp internal control band can be observed in all the lanes 3-7, 9 and 10 which contain amplified DNA from archived DNA samples of heterozygous β-thalassaemia carriers.

Using ARMS, the β-globin gene mutation for IVS2-654 was amplified as a 830 bp band (lane 2), -29 as a 625 bp band (lane 3), -28 as a 624 bp band (lane 4), Initiation codon as a 545 bp band (lane 5), CD43 as a 448 bp band (lane 6), CD41/42 as a 439 bp band (lane 7), CD17 as a 240 bp band (lane 9) and CD71/72 as a 234 bp band (lane 10).

In lane 8, DNA from a normal individual was amplified using common primers E and F producing a 323 bp internal control band. Since the DNA of β-thalassaemia carriers with the β-globin gene mutation at IVS2-654 amplified an 830 bp band, the common primers A and B which produce the 861 bp internal control was not used for this mutation due to the small difference in size of amplified products. Thus, the internal control for IVS2-654 mutation was amplified in a separate reaction using common primers E and F.

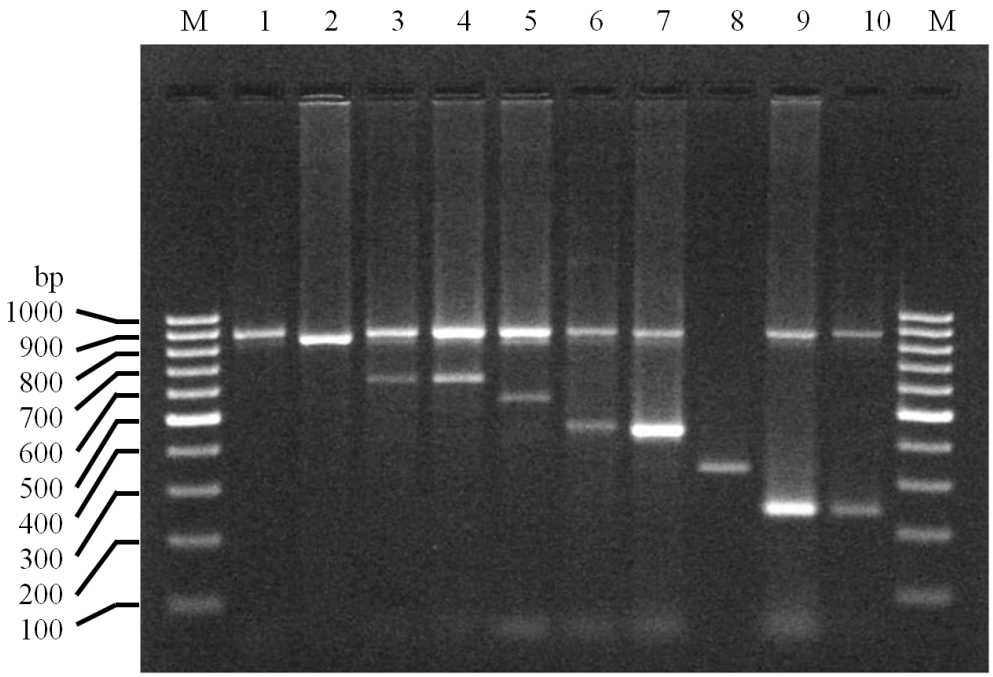

Figure 4.8 Gel electrophoresis after ARMS amplification for confirmation of β-globin gene mutations common in the Chinese

Lane M: 100 bp molecular weight marker

Lane 1: Common primers A and B internal control, 861 bp

Lane 2: IVS2-654, 830 bp

Lane 3: -29, 625 bp

Lane 4: -28, 624 bp

Lane 5: Initiation Codon, 545 bp

Lane 6: CD43, 448 bp

Lane 7: CD41/42, 439 bp

Lane 8: Common primers E and F internal control, 323 bp

Lane 9: CD17, 240 bp

Lane 10: CD71/72, 234 bp

Homozygosity for β-thalassaemia mutations (IVS2-654, -28, CD41/42 and IVS1-5) in 7 patients was confirmed by amplification of the normal β-globin gene sequences. For example, using ARMS, a patient who is homozygous for the IVS2-654 mutation will not amplify the normal IVS2-654 gene sequence. DNA from normal individuals or compound heterozygous β-thalassaemia major individuals will amplify the normal gene sequence, producing an 830 bp amplified product band using a normal primer.

Figure 4.9 shows the ARMS products after DNA amplification using normal primers for normal sequences at IVS2-654, -28, CD41/42 and IVS1-5. Lane 1 is the common A and B internal control amplified as 861 bp band in DNA from a β-thalassaemia major individual who is homozygous for CD41/42/CD41/42. The 861 bp internal control band can be observed in lanes 3-5. The 861 bp internal control was not used for amplification of normal gene sequence for IVS2-654 due to small difference in amplified product size.

Using ARMS, normal gene sequence for IVS2-654 was amplified as a 830 bp band (lane 2), normal gene sequence for -28 was amplified as 624 bp band (lane 3), normal gene sequence for CD41/42 was amplified as 443 bp band (lane 4) and normal gene sequence for IVS1-5 was amplified as 285 bp band (lane 5).

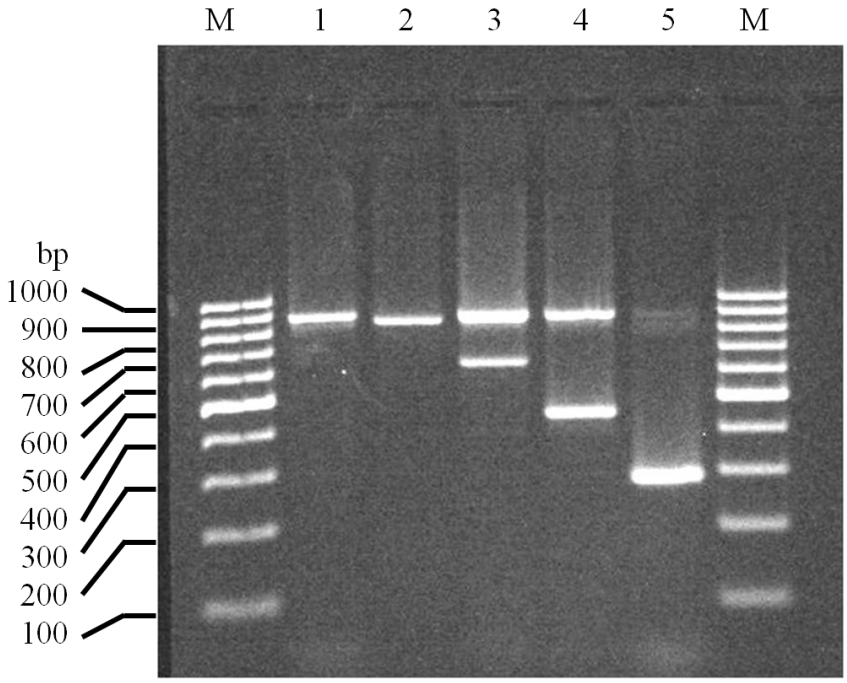

Figure 4.9 Gel electrophoresis after ARMS amplification for the normal gene sequences for detection of homozygous patients

Lane M: 100 bp molecular weight marker

Lane 1: Common primers A and B internal control, 861 bp

Lane 2: IVS2-654 normal sequence, 830 bp

Lane 3: -28 normal sequence, 624 bp

Lane 4: CD41/42 normal sequence, 443 bp

Lane 5: IVS1-5 normal sequence, 285 bp

Figure 4.10 shows DNA amplification for ARMS for detection of the β-globin gene mutation at CD26. Lane 1 is the non-template control where DNA was not included in the reaction mixture. Lane 2 is the negative control which amplified the 861 bp internal control band using the DNA from a normal individual. Lane 3 contains the amplified product of a positive control which is a β-thalassaemia carrier with a β-globin gene mutation at CD26.

Lanes 4-6 show amplification of purified DNA extracted using Method 1 while lanes 7-9 are amplified products of DNA extracted using Method 2. DNA from both methods was well amplified, but the intensity of the 458 bp CD26 band is slightly more distinct with DNA extracted using Method 2.

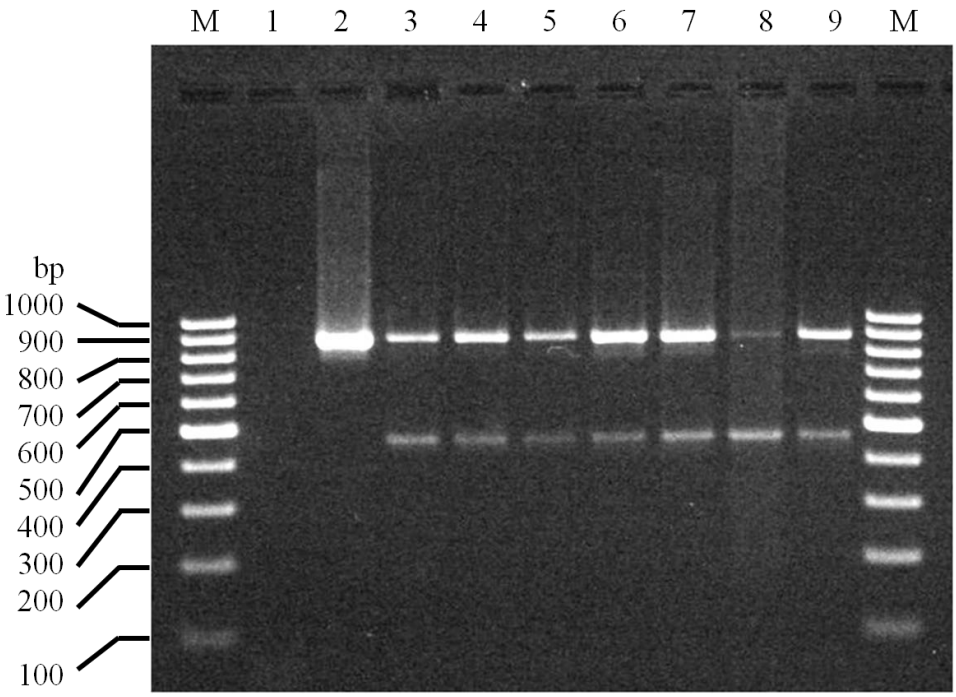

Figure 4.10 Comparison of ARMS amplification products from DNA extracted from mouthwash, saliva and buccal cytobrush samples using both Methods 1 and 2

The β-globin gene mutation amplified is CD26

Lane M: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; amplification of 861 bp product using DNA from a normal individual

Lane 3: Positive control for CD26; amplification of the 861 bp and 458 bp products

Lanes 4-6: DNA extracted using Method 1 from mouthwash, saliva and buccal cytobrush samples; amplification of 861 bp and 458 bp products

Lanes 7-9: DNA extracted using Method 2 from mouthwash, saliva and buccal cytobrush samples; amplification of 861 bp and 458 bp products

4.2.2 Molecular analysis using Combine-ARMS for CD41/42/CD17

Thirteen samples from β-thalassaemia major patients confirmed to carry mutations at CD41/42 or CD17 or compound heterozygous for CD41/42/CD17 were analysed using Combine-ARMS (C-ARMS).

The C-ARMS specifically detects both β-globin gene mutations at CD41/42 and CD17 in a single reaction. Using Method 1, successful DNA amplification was obtained in 77% of mouthwash samples, 85% of saliva samples and 77% of buccal cytobrush samples. The same results were also obtained from mouthwash and saliva samples extracted using Method 2. Purified DNA from all buccal cytobrush samples extracted using Method 2 were successfully amplified.

Figure 4.11 shows the amplified products from C-ARMS for CD41/42/CD17. Lane 1 is the non-template control where DNA was not included in the reaction. Lane 2 is the negative control where DNA of a normal individual was added into the reaction mixture. The DNA only amplified the 861 bp internal control band.

Three positive controls were utilised in the reaction. In lane 3, amplified product of DNA from an individual with the β-globin gene mutation at CD41/42 amplified both the 861 bp internal control band and the 439 bp band. Lane 4 is positive control for β-globin gene mutation at CD17 which amplified both the 861 bp internal control band and the 240 bp band. In lane 5, the positive control for compound heterozygous CD41/42/CD17 amplified the 861 bp internal control band, 439 bp band for CD41/42 and 240 bp band for CD17.

Lanes 6-8 show amplification of purified DNA extracted using Method 1 while lanes 9-11 are amplified product of DNA extracted using Method 2. All samples were successfully amplified.

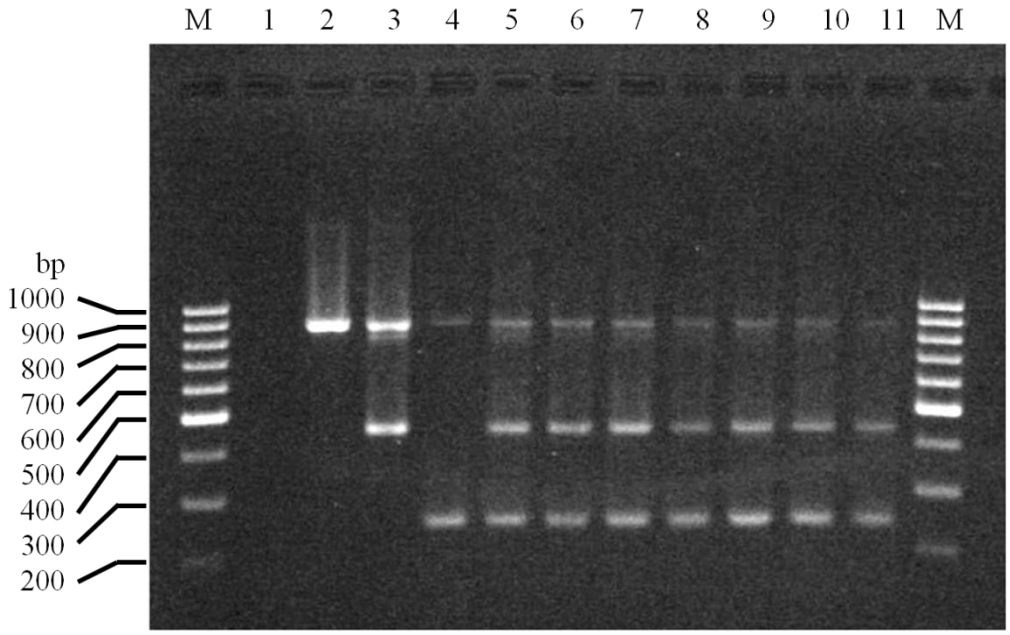

Figure 4.11 Amplification products of C-ARMS for CD41/42/CD17 for a Chinese patient using DNA samples extracted with Method 1 and Method 2

Lane M: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; amplification of 861 bp product of DNA from a normal individual

Lane 3: Positive control for CD41/42; amplification of 861 bp and 439 bp products

Lane 4: Positive control for CD17; amplification of 861 bp and 240 bp products

Lane 5: Positive control for compound heterozygous CD41/42/CD17 individual; amplification of 861 bp, 439 bp and 240 bp products

Lanes 6-8: DNA extracted from compound heterozygous using Method 1 from mouthwash, saliva and buccal cytobrush samples

Lanes 9-11: DNA extracted from compound heterozygous individual using Method 2 from mouthwash, saliva and buccal cytobrush samples

4.2.3 Characterisation of β-thalassaemia using gap-PCR

4.2.3.1 Detection of the Filipino β-deletion

Purified DNA from mouthwash, saliva and buccal cytobrush samples from one patient previously confirmed with the Filipino β-deletion was used in the gap-PCR along with negative, positive heterozygous and positive homozygous controls. The β-thalassaemia major patient was previously confirmed with the Filipino β-deletion and CD26 using DNA extracted from blood sample. The results of gel electrophoresis performed on the amplified samples are presented in Figure 4.12.

Lane 1 is the DNA blank which will show no DNA amplification. Lane 2 is the DNA from a normal individual (negative control) which will only amplify the 482 bp normal sequence. Lane 3 is the heterozygous positive control from a Filipino β-deletion carrier. This sample amplified both the 482 bp normal and 376 bp deletion-specific sequences. Lane 4 shows the amplified product of a homozygous individual which amplified only the 376 bp deletion-specific sequence. The purified DNA from mouthwash, saliva and buccal cytobrush samples are shown in lanes 5 - 10.

Purified patient DNA from mouthwash and buccal cytobrush samples isolated using both extraction methods amplified both the 482 bp normal and 376 bp deletion-specific sequence at the same product band intensity, as observed in lanes 5, 7, 8 and 10.

However, DNA from saliva extracted using both methods (lane 6 and 9) did not amplify the specified products. Smearing can be observed in lane 6 (saliva sample extracted with Method 1), suggesting that DNA is present in the sample, but the DNA may have been sheared during processing.

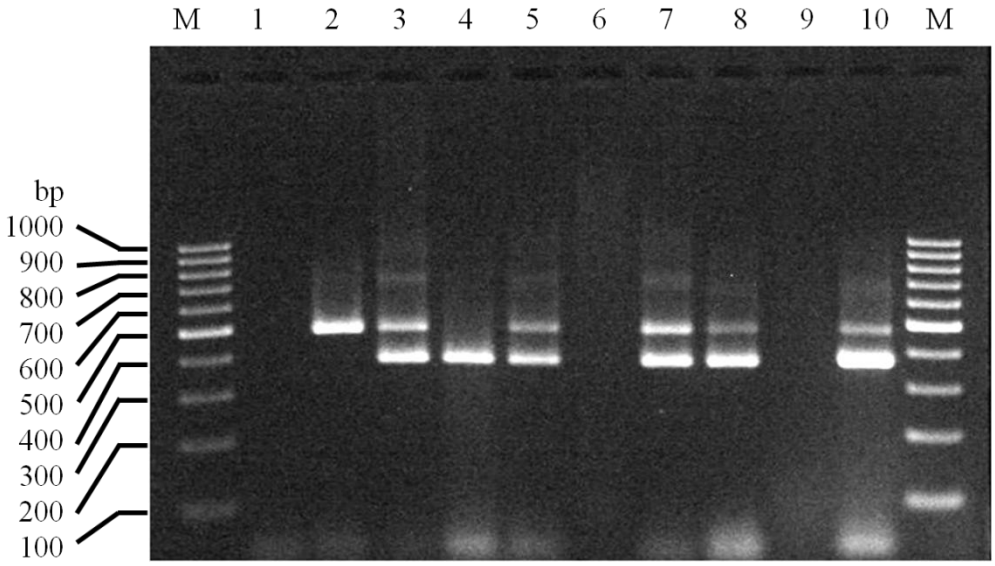

Figure 4.12 Gel electrophoresis after gap-PCR amplification for the detection of the Filipino β-deletion

Lane M: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; 482 bp normal band (DNA from a normal individual)

Lane 3: Heterozygous positive control; 482 bp and 376 bp bands (DNA from a 118 kb Filipino β-deletion carrier)

Lane 4: Homozygous positive control; 376 bp deletion-specific band (DNA from a homozygous patient with the Filipino β-deletion)

Lanes 5-7: DNA extracted using Method 1 from mouthwash, saliva and buccal cytobrush samples

Lanes 8-10: DNA extracted using Method 2 from mouthwash, saliva and buccal cytobrush samples

4.2.3.2 Detection of the 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion

Purified DNA from one β-thalassaemia major patient previously characterised with CD41/42/$^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion was used in the gap-PCR. Gap-PCR was performed with negative (normal individual) and heterozygous positive controls. Results for the gap-PCR are shown in Figure 4.13.

Lane 1 is the DNA blank. Lane 2 contains DNA from a normal individual which amplified only the 682 bp normal sequence. Lane 3 is DNA from a heterozygous individual (positive control) which amplified the 682 bp normal and 508 bp deletion-specific sequence. Lanes 4 - 9 are amplified products of purified DNA from mouthwash, saliva and buccal cytobrush samples.

Amplification was successful for all samples except for the buccal cytobrush sample extracted using Method 1 (lane 6). Inadequate amount of DNA in the sample or reduced DNA purity may be the possible causes of the amplification failure. Amplification of DNA from mouthwash extracted with Method 1 (Lane 4) produced bands with better intensity when compared with the mouthwash sample extracted with Method 2 (lane 7).

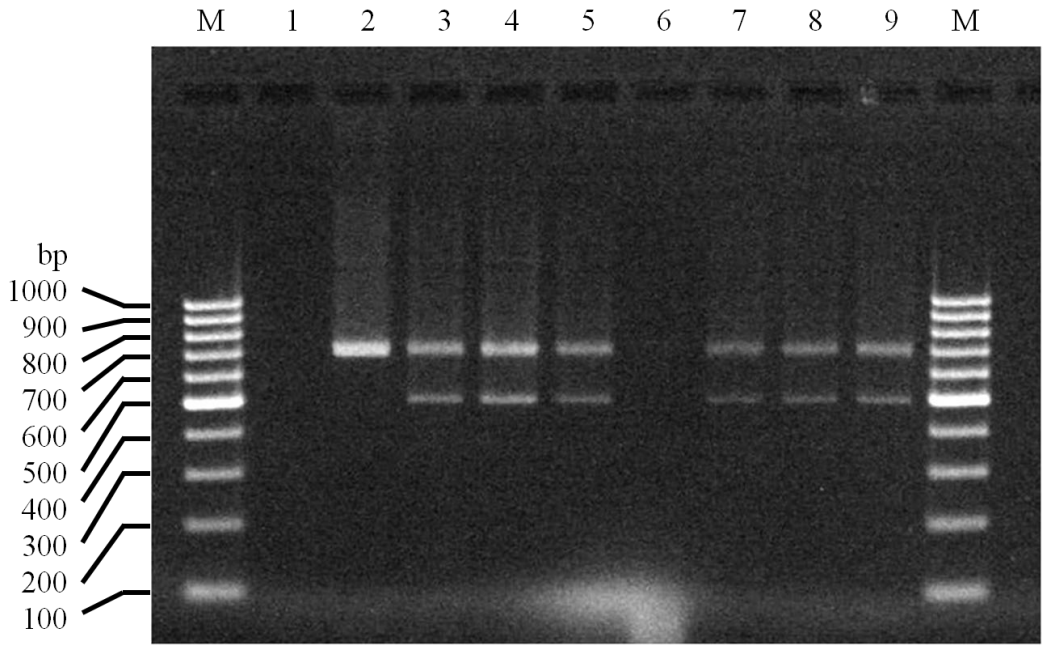

Figure 4.13 Gel electrophoresis of PCR products after gap-PCR amplification for the detection of the 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion

Lane M: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; 682 bp band from DNA from normal individual

Lane 3: Heterozygous positive control; 682 bp and 508 bp bands from DNA from 100 kb $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-deletion carrier

Lanes 4-6: PCR products from DNA extracted using Method 1 from mouthwash, saliva and buccal cytobrush samples; 682 bp and 508 bp bands

Lanes 7-9: PCR products from DNA extracted using Method 2 from mouthwash, saliva and buccal cytobrush samples; 682 bp and 508 bp bands

4.2.3.3 Detection of Hb Lepore

One β-thalassaemia major patient was previously confirmed with homozygous Hb Lepore. Purified DNA from mouthwash, saliva and buccal cytobrush samples was used for gap-PCR to detect Hb Lepore.

As shown in Figure 4.14, lane 1 is the DNA blank. Lane 2 is the negative control using DNA from a normal individual which only amplify the 915 bp normal band. Lane 3 is the homozygous positive control using DNA from individual with homozygous Hb Lepore. This control only amplifies the 775 bp deletion-specific sequence. Lanes 4 – 9 are the amplification products of DNA from mouthwash, saliva and buccal cytobrush extracted using Methods 1 and 2.

Purified DNA extracted with Method 1 from mouthwash and saliva samples (lanes 4 and 5) did not show any amplification. Only DNA from buccal cytobrush extracted with the same method amplified the targeted DNA sequence as observed in lane 6. On the other hand, all purified DNA extracted with Method 2 (lanes 7 – 9) amplified the 775 bp sequence, confirming that DNA extracted from mouthwash, saliva and buccal cytobrush can be used for molecular studies.

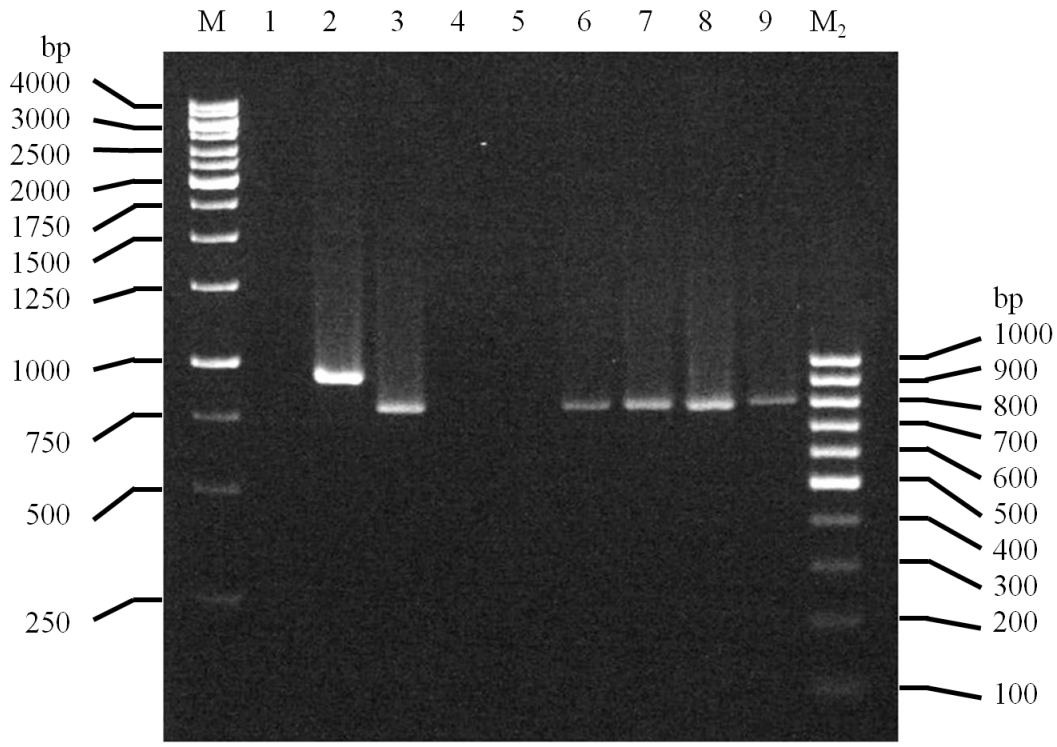

Figure 4.14 Gel electrophoresis of PCR products after gap-PCR for detection of Hb Lepore

Lane M: 1000 bp molecular weight marker

Lane M_2: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; 915 bp band using DNA from normal individual

Lane 3: Positive control; 775 bp deletion-specific band using DNA from homozygous Hb Lepore individual

Lane 4-6: DNA extracted using Method 1 from mouthwash, saliva and buccal cytobrush samples; 775 bp deletion-specific band

Lane 7-9: DNA extracted using Method 2 from mouthwash, saliva and buccal cytobrush samples; 775 bp deletion-specific band

4.2.4 Detection of mutation at CD27/28 using PCR-RFLP

Purified DNA from one patient with compound heterozygosity for CD41/42/CD27/28 was analysed using PCR-RFLP to detect the β-globin gene mutation at CD27/28. Gel electrophoresis after treatment of amplification products with restriction endonuclease NlaIV is shown in Figure 4.15.

Lane 1 is the DNA blank with no added DNA template. Lane 2 shows the products after restriction endonuclease treatment in DNA from a normal individual. The 284 bp amplified band is not digested as the restriction site is not present in normal individuals. Lane 3 shows the digestion products of positive control DNA from a heterozygous individual who carries the β-globin gene mutation at CD27/28. Restriction enzyme digestion produced the 170 bp and 114 bp digested fragments.

Lanes 4 – 6 show restriction endonuclease digestion of amplified DNA from mouthwash, saliva and buccal cytobrush samples extracted using Method 1. Lanes 7 – 9 show restriction endonuclease digestion of amplified DNA from mouthwash, saliva and buccal cytobrush using Method 2. All samples showed the 170 bp and 114 bp digested products. The undigested 284 bp band was also present after restriction enzyme digestion as the patient was compound heterozygous for CD41/42/CD27/28.

DNA from buccal cytobrush extracted with Method 2 (lane 9) produced a less distinct 284 bp undigested band and fainter 170 bp and 114 bp digested bands compared with the sample extracted with Method 1. This might be due to the lower DNA concentration of the sample extracted using Method 2.

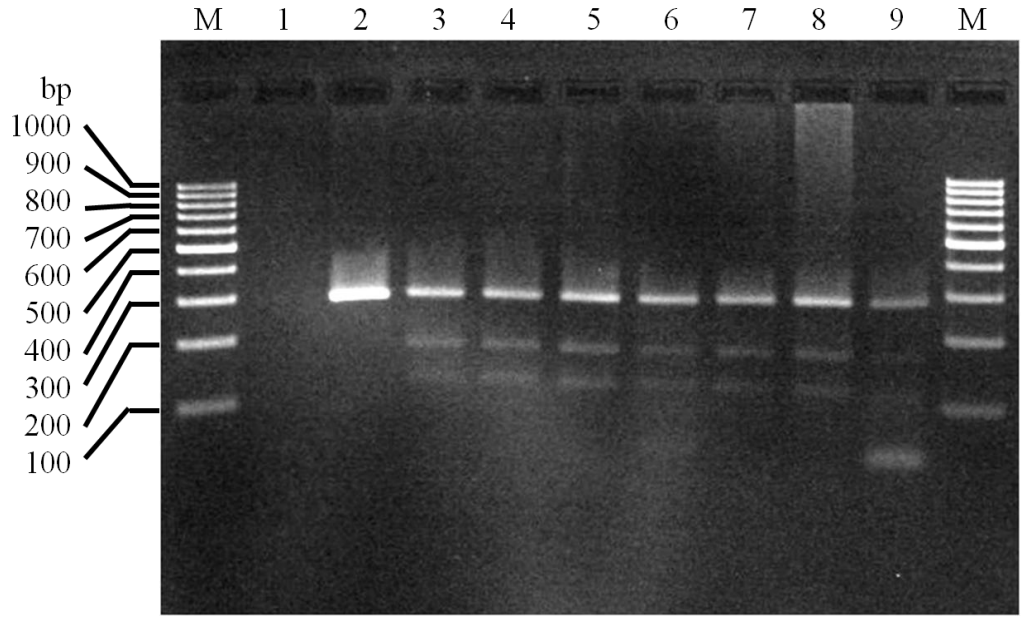

Figure 4.15 Gel electrophoresis after treatment of amplified DNA with restriction endonuclease NlaIV

Lane M: 100 bp molecular weight marker

Lane 1: DNA blank

Lane 2: Negative control; DNA from a normal individual (284 bp band) Lane 3: Positive control; DNA from an individual who is heterozygous for CD27/28 (284 bp, 170 bp and 114 bp bands)

Lane 4-6: DNA extracted from mouthwash, saliva and buccal cytobrush samples of a compound heterozygous patient for CD41/42/CD27/28 using Method 1 (284 bp, 170 bp and 114 bp bands)

Lane 7-9: DNA extracted from mouthwash, saliva and buccal cytobrush samples of a compound heterozygous patient for CD41/42/CD27/28 using Method 2 (284 bp, 170 bp and 114 bp bands)

4.3 Statistical analysis of salivary biochemical parameters

Assessment by Shapiro-Wilk test indicated that the data for all biochemical parameters were not normally distributed. Thus, the non-parametric statistical analyses were performed on the data to determine the significance of difference between groups.

Biochemical analysis results were expressed in median. Significance in difference were evaluated by Mann-Whitney U test. The difference is considered as statistically significant if the p value is less than 0.05 and highly significant if p value is less than 0.01.

Correlation analysis was carried out using Spearman's rank order correlation and presented as Spearman's rho (r_s). Comparison of parameters according to demographic data of β-thalassaemia major patients was performed using Kruskal-Wallis H test followed by Mann-Whitney U test as post-hoc test.

4.3.1 Measurement of salivary oxidative stress indices and cytokine levels

When expressed in median, the LOOH, GPx, UA and IL-6 levels were higher in the β-thalassaemia major patients group. On the other hand, FRAP value, AOPP and TNF-α levels were higher in the healthy controls group.

From Mann-Whitney test performed, only GPx activity was observed to be significantly increased in the β-thalassaemia major patients group (U = 781.50, $p < 0.01$). The activity was increased by 35% in the patients group compared with the healthy controls.

Table 4.5 summarises the median level of oxidative stress indices and cytokine levels analysed in the saliva samples.

Table 4.5 Comparison of median of oxidative stress indices and cytokine levels between β-thalassaemia major patients and healthy controls[a]

Parameters	Unit	Median	
		Beta-thalassaemia major patients	**Healthy controls**
FRAP	μM	184.76	197.50
AOPP	μM	71.51	76.67
LOOH	μM	0.26	0.19
GPx	nmol/min/mL	11.72[**]	8.66
UA	nmol/mL	97.26	82.72
TNF-α	pg/mL	7.13	7.98
IL-6	pg/mL	18.24	13.89

[a] Mann-Whitney U test

[**] $p < 0.01$ when compared with healthy controls group

4.3.2 Correlation analysis between salivary oxidative stress indices and cytokines in patient samples

Five significant correlations were observed after correlation analysis was performed on the oxidative stress indices and cytokine levels in β-thalassaemia major patients group.

Salivary AOPP shows moderate and highly significant correlation with FRAP value ($r_s(63) = 0.43$, $p < 0.01$). On the other hand, UA show strong and highly significant correlation with FRAP value ($r_s(63) = 0.72$, $p < 0.01$). No other significant correlation was obtained between FRAP value and other biochemical parameters.

Weak positive and statistically significant correlation was observed between salivary AOPP level and UA level ($r_s(63) = 0.25$, $p < 0.05$).

For salivary LOOH, a weak negative and significant correlation was observed between LOOH and IL-6 ($r_s(63) = -0.25$, $p < 0.05$).

Another weak negative and statistically significant correlation was observed between UA and TNF-α which was statistically significant ($r_s(63) = -0.25$, $p < 0.05$).

Table 4.6 summarises the correlation between the salivary oxidative stress indices and salivary cytokines observed in this study.

Table 4.6 Spearman's rho (r_s) value for correlation analysis between salivary oxidative stress indices and cytokines in β-thalassaemia patients group[a]

Parameters	FRAP	AOPP	LOOH	GPx	UA	TNF-α
AOPP	0.43**					
LOOH	0.17	-0.05				
GPx	0.14	0.19	0.13			
UA	0.72**	0.25*	0.19	0.14		
TNF-α	-0.19	0.09	-0.21	-0.17	-0.25*	
IL-6	0.06	0.22	-0.25*	0.01	0.10	0.13

[a] Spearman's rank order correlation

* Correlation is significant at the 0.05 level (2-tailed) by Spearman's rank order correlation

** Correlation is significant at the 0.01 level (2-tailed) by Spearman's rank order correlation

4.3.3 Comparison of salivary oxidative stress indices and cytokine level according to demographic data of β-thalassaemia major patients

No significant differences were observed in the oxidative stress indices and cytokine levels between the two genders in the β-thalassaemia major patients group.

Significant difference was observed in AOPP level depending on the genetic classification of β-thalassaemia, $\chi 2(2) = 6.38$, $p < 0.05$ with mean ranks of 46.67 for β^+-thalassaemia homozygotes, 29.21 for β^+/β^0-compound heterozygotes and 34.47 for β^0-thalassaemia homozygotes.

Mann-Whitney U test was performed and confirmed that the AOPP level in β^+-thalassaemia homozygotes patients was significantly higher than β^+/β^0-compound heterozygotes patients ($U = 90.00$, $p < 0.05$) and β^0-thalassaemia homozygotes patients ($U = 39.00$, $p < 0.05$)

TNF-α level was significantly different between the age groups, $\chi^2(2) = 7.74$, $p < 0.05$ with mean ranks of 34.88 for patients below 10-years, 36.54 for patients between 11 – 20-years and 18.64 for patients aged between 21 – 30-years.

Mann-Whitney U test performed confirmed that the TNF-α levels were significantly lower in patients aged between 21 – 30-years compared with patients below 10-years ($U = 52.00$, $p < 0.05$). The level of TNF-α in patients aged between 21 – 30-years was also significantly lower than patients aged between 11 – 20-years ($U = 87.00$, $p < 0.01$).

Statistically significant difference in TNF-α level was observed depending on the ethnic group of β-thalassaemia major patients, $\chi^2(2) = 6.27$, $p < 0.05$ with mean ranks of 28.08 for Malay ethnicity, 37.98 for Chinese ethnicity and 43.31 for Other ethnicity.

From the Mann-Whitney U test, the TNF-α level in β-thalassaemia major patients from Malay ethnicity is significantly higher from β-thalassaemia major patients from Other ethnicity ($U = 81.50$, $p < 0.05$).

CHAPTER 5: DISCUSSIONS

5.1 Selection of methods and materials in sample collection

Mouthwash, saliva and buccal cytobrush were successfully collected from β-thalassaemia major patients.

5.1.1 Normal saline as mouthwash solution

The present study used normal saline as the mouthwash solution as this allows the participation of younger toddlers. Normal saline is the most suitable mouthwash solution as it is simple and non-toxic, thus safe for the use in toddlers and young children (Lum & Marchand, 1998).

Young children are not advised to use commercialised mouthwash. Alcohol poisoning by ingestion in children is more common in mouthwash compared with other solutions with higher alcohol content (Riordan *et al.*, 2002). Although small amount of alcohol ingestion may not be fatal for children, it may result in severe intoxication due to their low body weight (Shulman & Wells, 1997).

The restriction also applies to alcohol free mouthwash. Phenolic compounds present in the mouthwash may intoxicate a person if ingested in large amount (Hoo *et al.*, 2003). Previous observation by Kolahi *et al.* (2006) also reported that ingestion of antimicrobial agents such as chlorhexidine in large quantity may produce inflammation (Cole *et al.*, 2013). As reported by Whitford (1992), over consumption of sodium fluoride present in mouthwash solutions may also cause severe problems such as muscular cramps and cardiac arrest (Ozsvath, 2009).

In addition, commercialised mouthwash solution containing alcohol produces burning sensation in the oral cavity and may produce unpleasant taste (Garcia-Closas *et al.*, 2001; Mulot *et al.*, 2005). Thus, additional rinsing is needed. Published report states that brushing teeth prior to sample collection reduce the amount of cells collected (Feigelson *et al.*, 2001). Additional rinsing may also reduce the number of cells collected on the subsequent sample collection method.

Previous reports by other investigators used other solutions such as sterile water and sucrose as mouthwash solution in place of commercialised mouthwash solution (Aidar & Line, 2007; Mulot *et al.*, 2005). Commercialised mouthwash solution is only used for collection of buccal cells for participants with age above 20 years-old (Garcia-Closas *et al.*, 2001; King *et al.*, 2002; Lum & Marchand, 1998).

Trials on mouthwash samples were tried out to prevent the co-purification of commercialised mouthwash dyes as it may disturb the DNA performance for molecular analysis (Heath *et al.*, 2001). In the preliminary tests conducted prior to this study, mouthwash dye was observed in the extracted DNA solution without affecting the DNA amplification performance. However, the result may not be significant due to the small sample size. Hence, the use of commercialised mouthwash was not recommended for large scale studies.

5.1.2 Collection of saliva by direct expectoration

The present study aims to obtain unstimulated saliva for molecular analysis and estimation of biochemical parameters. However, the presence of the salty taste from the mouthwash is considered as a stimulus for saliva flow. Thus, direct expectoration was selected as the means of sample collection to prevent additional stimulation of saliva flow as previous reports have observed a difference in analyte concentration due to induction of saliva flow (Dizgah & Hosseini, 2011; Moore *et al.*, 2001; Sculley & Langley-Evans, 2002).

The use of external materials such as chewing gum and cotton roll should be avoided to prevent buccal cell lost due to the attachment of the cells onto the materials. Other researchers also used direct expectoration as the initial method for collection of whole saliva samples before using additional materials such as antibiotic- or stabiliser-treated cards (Harty *et al.*, 2000; Mulot *et al.*, 2005). The extraction of chewing gum and cotton roll can be difficult during DNA extraction. Contaminant of the materials may be expected, thus affecting the concentration and purity of the sample. In addition, the amount of salivary fluid collected cannot be properly determined before the materials are extracted.

5.1.3 Direct buccal cell collection with buccal cytobrush

The use of sterile buccal cytobrush or cotton swabs was seen as the most economical method to collect buccal epithelial cells compared with the mouthwash sampling method (Cheng *et al.*, 2010; Cozier *et al.*, 2004; King *et al.*, 2002). The method is easily performed and involves minimal amount of liquid, thus making it preferable for mailing.

The use of cytobrush was preferred over cotton swabs or other forms of swabs in the present study as it allowed maximising the amount of buccal cells during collection and reduced cost. When compared with swabs, brushes appear to be less dense and the collected cells will not be retained in the bristles. The use of other swabs such as foam swabs can be costly and may require more collection units to achieve a good DNA yield (Rogers *et al.*, 2007).

The suspension of cells in normal saline solution enabled the same cytobrush to be used again for second and third collection attempts for the same patient. Previous published investigations have reported that more than one collection is needed to obtain sufficient DNA using buccal cytobrush or swabs (Cozier *et al.*, 2004; Rogers *et al.*, 2007). In addition, previous investigation showed that delayed DNA extraction and keeping the swabs air-dried will reduce the amount of DNA recovered from the samples (Meulenbelt *et al.*, 1995).

5.2 Concentration and purity of purified DNA from non-invasive samples

High concentration and purity DNA samples were successfully extracted from mouthwash, saliva and buccal cytobrush samples collected from β-thalassaemia major patients.

5.2.1 Comparison of the purified DNA concentration and purity between sampling and extraction methods

Other published investigations mainly use DNA yield in their reports. Thus, direct comparison between the results of present study and published reports could not be performed. However, there was a similar trend that was observed in all reported investigations, which is the comparison in DNA yield and purity between sampling methods used by the investigators. Thus, this basis was used in the present study to determine which sampling method could provide extracted DNA with the most optimum quantity and quality.

5.2.1.1 Mouthwash samples

Comparison between Methods 1 and 2 showed no notable differences in concentration of DNA extracted from mouthwash samples. The DNA purity was slightly increased using Method 2 compared with Method 1.

When compared between sampling procedures, mouthwash sample provided second highest concentration of DNA. In aspect of purity, highest DNA purity was obtained from mouthwash samples.

The same observation has been reported where DNA yield and purity was compared between saliva and buccal swab samples and DNA from mouthwash was the second highest in amount and highest in purity (Rogers *et al.*, 2007). In addition, the purity varied greatly between DNA from mouthwash and buccal swab samples (1.78 vs 1.10, respectively).

Other investigations also obtained higher DNA yield and purity from mouthwash samples ranging from 4.0 – 88.4 µg of DNA compared with buccal swab samples which was 3.5 – 15.8 µg (Garcia-Closas *et al.*, 2001; King *et al.*, 2002; Mulot *et al.*, 2005).

5.2.1.2 Saliva samples

In the present study, saliva samples DNA concentration was almost 2-times greater than mouthwash DNA and almost 3-times greater then buccal cytobrush DNA when extracted with Method 1. The concentration was improved using Method 2, where the concentration was more than 4-times of mouthwash DNA and more than 8-times of buccal cytobrush DNA.

In return, the purity was compromised compared with mouthwash samples. Using Method 1, similar median purity was obtained from both mouthwash and saliva samples. The DNA purity was improved using Method 2. In both occasions, the purity values were higher than buccal cytobrush.

There are limited investigations on direct comparison in DNA yield and purity involving saliva samples and other non-invasive samples. A report by a group of investigators shows that the similar pattern of DNA amount and purity was obtained in their investigation (Rogers *et al.*, 2007). DNA yield of saliva samples obtained which was 154.9 µg was almost 3-fold higher compared with mouthwash samples (54.7 µg). When compared with buccal swab samples DNA yield (12.7 µg), the saliva samples produced 12-times more DNA.

In aspects of purity, the purity of DNA from mouthwash and saliva from Rogers and team investigation did not differ greatly (1.78 vs 1.74, respectively). However, as observed in the present study, saliva samples DNA purity managed to surpass DNA purity of buccal swab samples.

5.2.1.3 Buccal cytobrush samples

Buccal cytobrush samples provided the lowest concentration of DNA compared with other procedures. Median concentration of buccal cytobrush samples were slightly reduced when extracted with Method 2. The buccal cytobrush DNA purity was the lowest among the three sampling method – mouthwash, saliva and buccal cytobrush. In addition, the purity did not differ much when extracted using Method 2.

Previous reports have also observed that buccal swabs could only provide small amount of DNA ranging from 3.5 – 15.8 µg (Garcia-Closas *et al.*, 2001; King *et al.*, 2002; Mulot *et al.*, 2005; Rogers *et al.*, 2007). In all instances, buccal swab DNA yields were lower than mouthwash DNA yield. In addition and in accordance to the present study finding, the means of DNA purity from published reports were slightly lower by 0.1 than mouthwash DNA. There was one situation where the purity was on par with mouthwash samples DNA purity (Garcia-Closas *et al.*, 2001). In two other instances, the means of buccal swab DNA purity were 1.15 and 1.10, which was lower than the means of saliva samples DNA purity, which were 1.63 and 1.74, respectively (Hansen *et al.*, 2007; Rogers *et al.*, 2007).

Observation from previous published works support that saliva sample may produce highest amount of DNA, followed by mouthwash and buccal cytobrush samples. When observed in the aspects of purity, mouthwash samples provide DNA with highest purity, followed by saliva and buccal cytobrush samples.

5.2.2 Factors affecting the quantity of the purified DNA

A number of factors may have affected the concentration of DNA extracted from non-invasive samples. The factors are not specific to one type of sampling procedure, but present in all the samples at different level.

5.2.2.1 Preparations and actions performed during sample collection

The presence of microorganism – normal oral cavity flora – can reduce the amount of extracted DNA (Saftlas *et al.*, 2004). Microbial metabolism can degrade nucleic acids, thus reducing the nucleic acid quantity. The degree of degradation depends on the storage conditions such as temperature and the presence of stabilising agents in the storage solution (Aidar & Line, 2007; Hansen *et al.*, 2007; Ng *et al.*, 2006). The use of mouthwash solutions or addition of buffers containing ethanol and storage at low temperatures can inhibit bacterial growth and reduce nucleic acid degradation.

In this study, additional rinsing prior to sample collection was performed to reduce the bacteria load in the samples. However, this step may reduce the number of buccal cells (Feigelson *et al.*, 2001). But prior rinsing was still performed to remove as much contaminants as possible including food debris. To further reduce nucleic acid degradation, all samples were stored directly in -20°C after collection until DNA extraction was performed. Published reports also confirmed that samples in normal saline are stable for up to 6 months when stored in -20°C (Lum & Marchand, 1998).

The amount of brush strokes and timing can also contribute to the DNA yield (King *et al.*, 2002; London *et al.*, 2001; Mulot *et al.*, 2005). Although the brushing time was fixed in this study, the amount of strokes performed by the participants differs and thus may result in different quantities of buccal cells obtained. Increasing the number of brushes used or brush strokes will improve the purified DNA yield as reported by Cozier *et al.* (2004). Thus, in this study the sampling procedure for buccal cytobrush collection was repeated three times to increase the quantity of epithelial cells recovered.

Motions in the oral cavity and cheek rubbing may also increased the number of exfoliating cells during the mouthwash sample collection (Aidar & Line, 2007). However, Shao *et al.* (2007) reported that cheek rubbing against the teeth prior to swishing of mouthwash solution did not produce any increase in DNA yield. Thus, cheek rubbing may not provide much different in the DNA quantity in this study.

5.2.2.2 Surface area and level of desquamation of the sampling site

When comparison was made between sampling procedures, the involvement of a larger surface area during mouthwash collection may contribute to the increased amount of buccal cells collected during the procedure.

Swishing of mouthwash solution involves contact with the palate and tongue, including the gutter area, which is located between the gums and lips. The buccal cells collected from the gutter area can provide a 2-fold higher amount of purified DNA compared wtih rinsing only on the cheek area alone (Saftlas *et al.*, 2004). Thus, the involvement of gutter area during mouth rinsing can increase the DNA concentration.

Collection of saliva samples involves drooling which drives the fluid in contact with the tongue, part of cheek including the gutter area. Thus, the cells from gutter area were also collected indirectly in the saliva samples.

5.2.2.3 Sequence in sample collection procedures

In this study, the sampling procedures were carried out consecutively, beginning with mouthwash, saliva to buccal cytobrush sampling. As a result, the numbers of cells suitable for DNA extraction may have reduced after each procedure. Two situations was observed previously by other researchers. Decrease of DNA yield was observed when immediate repeat sampling was carried out on the same area where previous sampling was performed (Burger *et al.*, 2005). However, other investigators did not observed any decrease in recovered DNA after repeat sampling was performed on the same cheek (Meulenbelt *et al.*, 1995).

The results of this study finding were consistent with that of Burger *et al.* (2005). Buccal cytobrush sampling was the last to be performed after mouthwash and saliva sampling. The number of collectable epithelial cells may have been reduced by the time buccal cytobrush sampling was carried out. Even though the procedure was performed three times to achieve a better yield of purified DNA, the probable reduced number of epithelial cells has resulted in lower DNA concentrations.

5.2.2.4 Presence of leukocytes

Leukocytes are present in the salivary fluid and previous reports have confirmed the presence of leukocytes in whole saliva (Kaufman & Lamster, 2000; Kumar *et al.*, 2014; Pandeshwar & Das, 2014; Schiott & Loe, 1970). The cells migrate from the blood circulation through the gingival crevices between the teeth into the salivary fluid. This may be the main factor to the high yield of purified DNA in saliva samples. Collection of whole salivary fluid directly collects the leukocytes present between the teeth during the fluid pooling process.

The function of salivary fluid is to maintain the moisture of the oral mucosa. Therefore, leukocytes can also be present on the surface of the oral cavity. As the mouthwash solution was in contact with the oral mucosa, leukocytes can be collected during swishing. In addition, salivary fluid was observed to be incorporated into the samples when the mouthwash solution was expectorated out by the participants. Thus, leukocytes from saliva can be introduced into the mouthwash sample. This factor may have increased the amount of purified DNA recovered after the extraction procedure.

Leukocytes are also present in the buccal cytobrush samples as the brush is in contact with the oral mucosa. Trace amounts of salivary fluid will also be collected during the brushing procedure. However, buccal cytobrush sampling only relies on a limited surface area. The amount of leukocytes collected will be lower than the mouthwash samples. The salivary fluid collected on the brush bristles is also lower in amount. As a result, the additional cellular components do not provide much difference in increasing the DNA concentration in buccal cytobrush samples.

5.2.2.5 Sample and reagent components

Mucin is rich with cysteine and readily forms disulphide bonds which thicken the substance (Villar, 2007). The presence of mucin may hinder the action of lysis buffer and proteinase K during the process of DNA extraction. To overcome the problem, buffer containing EDTA was added prior to storage and EDTA was also incorporated into the lysis buffer (Aidar & Line, 2007). EDTA chelates calcium ions which prevent interchain links between the cysteine molecules (Villar, 2007).

The chelationg action of EDTA also aids in DNA preservation which prevents exonuclease activity in the sample (Cowan, 1998). This may also be a factor of increased amount of DNA in saliva samples compared with the mouthwash and buccal cytobrush samples, since the samples were stored in buffer containing EDTA.

DTT have been used as mucolytic agent to liquefy sputum samples prior to the analysis (Hammerschlag *et al.*, 1980; Loppow *et al.*, 2000; Tockman *et al.*, 1995). DTT reduce the disulfide bonds and maintained the sulphide groups in reduced state (Cleland, 1963). This results in reduction of mucin thickness. The addition of DTT in Method 2 during the extraction process may have enhanced the dilution of mucin and improved the action of lysis buffer and proteinase K (Dimitrakakis *et al.*, 2010).

5.2.2.6 Repetitive sample transfer

When compared with saliva and buccal cytobrush samples, extraction of DNA from mouthwash samples involves transferring the mouthwash samples into fresh tubes. Although the initial containers were rinsed thoroughly during the transferring process, there are possibilities that the buccal cells collected were strongly adhered to the inner surface of the container. This may result in the reduction of the number of cells available for the extraction process. However, this may not be the major factor, since the DNA yield for mouthwash samples were significantly higher when compared with buccal cytobrush for both methods.

5.2.2.7 Standardisation of solubilising medium volume

When comparisons were made between extraction methods, the increase in DNA concentration in Method 2 may be due to the standardisation of the amount of sterile deionised water used during DNA solubilisation.

Compared with Method 2 where all DNA was solubilised in 200 µL of sterile deionised water, the amount of water used to solubilise the DNA in Method 1 was based on visual observation of the amount of precipitated DNA. Visual error may have occurred during observation which resulted overestimation of precipitated DNA. In the end, more sterile deionised water was added into for solubilisation and the DNA samples become diluted. This may have affected the average concentration of the purified DNA.

5.2.2.8 Overestimation by spectrophotometry

Despite the high yield of DNA from mouthwash and saliva samples, measurement by spectrophotometry at 260 nm may have overestimated the actual DNA yield. Spectrophotometry is not able to distinguish between human DNA and microbial DNA (Moore *et al.*, 2001; Mulot *et al.*, 2005). Thus, the final DNA concentration may encompass the two DNA.

Measurement of DNA by using fluorochrome such as PicoGreen may also produce more accurate results for DNA amplifications (Rylander-Rudqvist *et al.*, 2006). PicoGreen specifically binds to double stranded DNA. The actual quantity of double stranded DNA usable for downstream application can thus be more accurately determined. However, since bacterial DNA is also double stranded DNA, PicoGreen is not able to discriminate the non-human DNA present in the sample. It was suggested that better quantification can be achieved by using specific probes to hybridise human DNA in collected samples (Feigelson *et al.*, 2001). Nevertheless, hybridisation using specific probes can be expensive and labour intensive (Garcia-Closas *et al.*, 2001).

5.2.3 Factors affecting the quality of purified DNA

5.2.3.1 Mucin content in samples

In aspects of purity, the presence of glycoprotein such as of mucin may have reduced the DNA purity (Navazesh *et al.*, 1992; Sanchez *et al.*, 2011). Since the mucin content is higher in saliva, the purity of salivary DNA was reduced when compared with mouthwash DNA samples. Although the number of purification cycles was increased in Method 2, there was no enhancement in DNA purity.

5.2.3.2 External factors

Buccal cytobrush samples are the least contaminated with saliva. Hence, it should be expected that buccal cytobrush samples will have higher DNA purity when compared with the other sampling methods. In contrast, the results showed that the extracted DNA with the lowest purity was obtained from buccal cytobrush, which is in agreement with published data (Garcia-Closas *et al.*, 2001; Hansen *et al.*, 2007; King *et al.*, 2002; Mulot *et al.*, 2005; Rogers *et al.*, 2007).

Food debris present in the oral cavity include carbohydrates and protein. These substances will be digested and solubilised into the solution during extraction process. Unlike hydrophobic proteins which are readily removed by organic purification process, the hydrophilic proteins, polysaccharides, aromatic substances, buffer salt and even trace amount of phenol can be co-purified and present in the purified DNA (Hansen *et al.*, 2007; Moreira, 1998; Philibert *et al.*, 2008).

Improper rinsing prior to sample collection can result in improper removal of food debris. Although mouthwash and saliva sample collections were performed earlier than buccal cytobrush sampling, the collection of smaller sized food debris is more likely to happen during cheek brushing. Compared with larger sized debris, small sized food debris can appear similar to cell collected after centrifugation.

5.2.4 Factors affecting the variation of purified DNA concentration and purity

5.2.4.1 Improper procedure execution

Improper mouth rinsing prior to sample collection by some participants may have affected the efficacy of the mouthwash to remove food debris. Large food particle prior to extraction process were removed but small particles may remain as they cannot be distinguished from the buccal cell pellet. These particles may have been digested and incorporated into the DNA solution, thus lowering the DNA purity.

It was also observed in this study that improper brushing during buccal cytobrush process may have increased the amount of contaminants. A number of participants were observed to have their cytobrush in contact with their teeth more than with their cheeks especially in the younger participants. More food particles and microorganisms were collected compared with the buccal cells.

5.2.4.2 Desquamation level of epithelial cells

The variation in purified DNA yield may be due to differences in buccal cell desquamation levels between individuals (King *et al.*, 2002; Zayats *et al.*, 2009). Epithelial cells in certain individuals may detach more easily than others. Even though the force and amount of time used to swab or rub the cheeks are almost identical, the amount of cells collected may be substantially different. As a result, the final purified DNA yield may vary.

5.2.4.3 Intensity of actions during sample collection

Instead of relying on cheek rubbing prior to mouthwash sample collection, rubbing while swishing may increase the amount of purified DNA in mouthwash samples (Shao *et al.*, 2007). The force of rubbing and swishing may be different between individuals, and this factor may affect the final amount of purified DNA. The force of rubbing may also affect the amount of purified DNA collected from buccal cytobrush (Meulenbelt *et al.*, 1995; Mulot *et al.*, 2005; Nedel *et al.*, 2009).

5.3 Molecular analysis of DNA extracted from non-invasive samples

Purified DNA from non-invasive samples mouthwash, saliva and buccal cytobrush were successfully amplified using various methods for molecular analysis for β-thalassaemia.

The successful amplification of extracted DNA samples in this study using ARMS increased when the samples were extracted using Method 2. DNA amplification of purified DNA from mouthwash samples increased from 55% (Method 1) to 87% (Method 2), while saliva samples amplification increased from 65% to 83% and buccal cytobrush samples amplification increased from 81% to 86%.

In previous experiments, failures of DNA amplification and genotyping were mainly due to the degradation of the DNA samples which was confirmed by gel electrophoresis. High amount of high molecular weight DNA was detected in mouthwash compared with buccal cytobrush samples. This contributes to the ability of buccal cytobrush DNA to amplify large-sized sequence (Cheng *et al.*, 2010; Garcia-Closas *et al.*, 2001; Rogers *et al.*, 2007). The targeted sequences in the present study are small and DNA amplification was observed in the three non-invasive samples.

Failure in the DNA amplifications without degradation may be due to the presence of PCR inhibitors in the samples (Burger *et al.*, 2005; Zayats *et al.*, 2009). Inhibitors such as salts co-purified during purification process may not be detectable using spectrophotometry at 260 nm and 280 nm. Trace amount of salts and EDTA may still be present in the samples and affected the sample ability to amplify in PCR. This could be the main factor to the amplification failures occurred.

Other than the presence of contaminants, successful amplification could be affected by the amount purified DNA template used in the PCR preparation (Altshuler, 2006). The excessive amount of DNA sequence not targeted by the primer may lead to false priming and halt the DNA amplification process.

For purified DNA obtained from Method 1, the DNA concentration in the saliva samples was higher compared with mouthwash samples. Thus, lower volume of saliva DNA sample was used in the reaction preparation. DNA template and the contaminant were readily diluted for the final mixture. False priming and reaction disturbance by contaminant were reduced.

DNA extraction and purification using Method 2 have improved the DNA concentrations and purity for both mouthwash and saliva samples. As the DNA concentration and purity for both samples was improved, low volume of solubilised DNA was required for reaction preparation. Better DNA and contaminant dilution in reaction preparation was achieved for both sampling procedures. This was observed in the increase of amplification success percentage for both mouthwash (from 55% to 87%) and saliva samples (from 65% to 83%).

On the other hand, the actual DNA concentration used from buccal cytobrush samples could be too low from the beginning for both Methods 1 and 2. Thus, inhibition of PCR by non-targetted DNA template was not prominent although the volume of solubilised DNA added into the reaction mixture was higher compared with saliva and mouthwash samples.

The DNA concentration was reduced when buccal cytobrush samples were extracted with Method 2. This may reduce the amplification success due to excessive reduction of DNA template. However, the increase in DNA purity may have contributed in maintaining the successful DNA amplification percentage (81% for Method 1 compared with 86% for Method 2).

5.4 Cost comparison between DNA extraction methods

DNA extraction cost comparison was made between Method 2 DNA extraction procedure and 4 other DNA extraction kits readily available in Malaysia. Table 5.1 presents the cost comparison of one preparation of DNA extraction.

Table 5.1 Cost comparison between Method 2 and DNA extraction kits readily available in Malaysia (per preparation)

DNA extraction protocol	Cost per preparation (RM)
Method 2	11.00
Brand E	9.20
Brand Q	13.00
Brand T	15.60
Brand O	100.00

Method 2 costs RM 11.00, which is the second lowest after Brand E (RM 9.20/preparation). The other brands cost more for one preparation, up to RM 100.00/preparation. Although Method 2 cost more compared with Brand E, Method 2 can be used for DNA extraction of samples collected from mouthwash, saliva and buccal cytobrush.

Even though the amount of sample fluid used in commercialised DNA extraction kits is usually small in volume, the entire cells collected may be resuspended in the buffer provided in the kit. However, the amount of lysis reagents may not be able to lyse and digest the cells properly. This will later cause clogging in the spin column in the later process during the extraction procedures as observed in the preliminary testing. Thus, it will be necessary for the sample to be separated into a number of aliquots prior to extraction process. This needs more than one preparation for a single sample for extraction. Thus, even though the cost of Brand E per preparation may be lower than Method 2 in this study, more than one preparation was required for DNA extraction.

Other preliminary tests were carried out using preparations from Brand Q on aliquots of mouthwash, saliva and buccal cytobrush samples. The DNA samples collected were smaller in amount compared with Method 2. During quantification process, the measurement could not be obtained suggesting that the amount of DNA present in the sample was too low. Thus, extracted DNA quantity and quality could not be determined. More preparations may be needed to achieve the desirable amount of DNA from one sample.

5.5 Measurement of oxidative stress indices and cytokines in saliva samples

Oxidative stress markers and cytokine levels were successfully measured using saliva samples from β-thalassaemia major patients and healthy controls. Concentrations of analyte of interest were within detectable limit.

5.5.1 Comparison of parameters between β-thalassaemia major patients and healthy controls

As presented in the previous chapter, when analysis was performed by considering the data distribution, increase in GPx activity was still observed in the patients group.

The enzyme GPx is one of the enzymatic antioxidant present in blood circulation other than catalase and superoxide dismutase (Ghone *et al.*, 2008; Sclafani *et al.*, 2013). The enzyme prevents erythrocytes breakdown caused by hydroxyl radicals. The hydroxyl radicals abstract hydrogen atom from polyunsaturated lipid molecules and form LOOH (Gutteridge, 1995). The example of polyunsaturated lipid molecules includes the phospholipid layer of the cell membrane. The increase in median GPx activity suggests the increase in enzymatic antioxidant activity to inactivate the presence of free radicals.

Earlier published investigation on patients with renal failure has reported that the GPx activity could readily affects the level of AOPP and TNF-α in the patient system. Reduction in GPx activity could cause the increase in protein oxidation in patients (Witko-Sarsat *et al.*, 1998).

The level of GPx activity is affected by the level of reduced glutathione (GSH). Depletion in GSH commonly occurred in patients with iron-overload such as transfusion dependent thalassaemia major patients. Due to the decrease in enzymatic antioxidant activity, non-enzymatic antioxidant activity may play a more significant role to reduce oxidative stress (Kuppusamy & Tan, 2011).

FRAP assay measures the total non-enzymatic antioxidant activity involving electron transfer between sample and the reagent (Muller *et al.*, 2011; Witko-Sarsat *et al.*, 1998). In the present study, the non-enzymatic antioxidant activity did not differ much between β-thalassaemia major patients and healthy controls. This suggests that in the patients, the free radicals that may be inactivated by non-enzymatic antioxidant were not significantly different from the healthy controls. Nevertheless, the presence of oxidants such as H_2O_2 and lipid hydroperoxide may drive the enzymatic antioxidant, GPx.

5.5.2 Correlation between biochemical parameters in patients

As presented in the Result section, there was a strong positive correlation between patients salivary FRAP and UA level. Although UA is usually associated with oxidative damage, it could also exhibit antioxidant role (Soukup *et al.*, 2012). As reported by Benzie & Strain (1996), UA is one of the non-enzymatic antioxidant which could react readily with the FRAP reagent. Thus, it was expected that the increase in salivary UA level will give a strong effect on the salivary FRAP value.

Using non-parametric analysis, there was a weak positive correlation between salivary UA and AOPP level and moderate correlation between salivary FRAP and AOPP level. The increase in AOPP was reported to be associated with the increase in monocyte activation and inflammation (Witko-Sarsat *et al.*, 1998). The increase in monocyte activation could also lead to cellular damage and increased amount of purines and UA due to purine metabolism.

As UA also exhibit antioxidant properties, the increase in UA may have helped in decelerating the speed of protein oxidation. Furthermore, urate, which is the physiological form of UA have been reported to have the ability to prevent protein nitration (Ames *et al.*, 1981).

Reported investigations show that excess plasma UA is able to induce inflammatory cytokines including TNF-α in medical conditions (di Giovine *et al.*, 1991 ; Zhou *et al.*, 2012). However, the observation in the present study using both parametric and non-parametric analysis shows that the increase in salivary UA provide minimal contribution in reducing salivary TNF-α production. As the rate of protein oxidation may have been decreased by UA, the pro-inflammatory cytokine production can be reduced to prevent more cellular damage.

According to various reports, increase in lipid peroxidation may activate the cascade of IL-6 secretion (Davì *et al.*, 2003; Everekliogluu *et al.*, 2002). In contrast, the non-parametric statistical analysis in the present study shows that reduction of LOOH provided a weak induction to increase the IL-6 secretion. IL-6 is also reported to exhibit anti-inflammatory properties, by preventing further destruction of epithelial cells (Scheller *et al.*, 2011). This is possibly due to the IL-6 ability to induce cells to enter mitosis (Kishimoto, 2003). The reduction of cellular damage which was indicated by decreased in LOOH level may have triggered IL-6 to induce cell proliferation, especially for erythrocytes.

5.5.3 Comparison of parameters between demographic data of β-thalassaemia major patients

There are notable difference in TNF-α level between Other ethnicity and Malay ethnicity where the median level was higher in the Malay ethnicity compared with Other ethnicity.

There is a possibility that the level of oxidative stress indices differ depending on the ethnicity as presented by other investigation (Fisher *et al.*, 2012; Kuppusamy *et al.*, 2005). In the report by Fisher *et al.* (2012), the level of oxidative stress indices can be affected by difference in muscle mitochondrial function and also food intake. In comparison with this study, there can be minor differences in the food intake preferences between the ethnicity in Malaysia. This may indirectly affect the level of antioxidants especially in patients.

However, the number of samples may have played a major role in the observation. The number of samples for Other ethnicity was lower compared to Malay ethnicity (8 vs 37). Any irregularities may results in contradictory findings, since both parametric and non-parametric statistical analysis are sensitive to large difference in sample size (Moran & Solomon, 2002).

When analysed according to age groups, the level of TNF-α was also higher in the patients below 20-years old. As observed in this study, all but 1 patient by this age have started chelation therapy. However, the total duration of therapy for these patients was not as long as the older patients. The extent of iron removal may not be as adequate as the patients from the older age group.

There are also possibilities that a number of these patients might not be compliant to the treatment. Poor compliance to chelation treatment will not remove the accumulated iron adequately and cause complications in patients (Galanello *et al.*, 2010). Due to inadequate removal of excess iron, redox imbalance still occurs in the patients leading to increase in oxidative stress markers.

As presented in Chapter 2, β^0-thalassaemia homozygotes have zero production of β-globin chain, which totally nullifies HbA production. In comparison, with β^0-thalassaemia homozygotes, β^+/β^0 compound heterozygotes and β^+ homozygotes have different level of β-globin chain production (Telen & Kaufman, 1999). Thus, β^0-thalassaemia homozygotes have higher amount of free α-globin chain which can precipitate and disrupt the erythrocyte membrane integrity and was expected to have higher oxidative stress indices levels.

However, in the present study, AOPP level were higher in β^+-thalassaemia homozygotes compared with β^+/β^0 compound heterozygotes and β^0 homozygotes. Perhaps this is because AOPP is considered as more sensitive oxidative stress marker especially when compared with lipid peroxidation markers such as LOOH (Kuppusamy & Tan, 2011; Witko-Sarsat *et al.*, 1998).

Other than that, external factors which are not assessed such as co-inheritance of α-globin gene mutations may have ameliorate the oxidative stress severity by reducing the free α-globin chains production (Camaschella *et al.*, 1995; Nadkarni *et al.*, 2001). Polymorphism such as *Xmn*I could also reduce the severity of the condition by promoting the production of γ-globin chains which bind with free α-globin chains in place of β-globin and reducing the α-globin chain toxicity (Chinelato *et al.*, 2011; Thein, 2004).

5.5.4 Factors affecting the analytes concentration

5.5.4.1 Dilution of analytes in saliva

The oxidative stress markers and cytokines measured in saliva originated from gingival crevicular fluid (GCF) and tissue metabolites. Other than GCF, the fluid component of the whole saliva was made of fluids from salivary glands and nasal and bronchial secretions (Kaufman & Lamster, 2000; Sculley & Langley-Evans, 2002). Due to this factor, it was expected that the concentration of analytes measured in saliva samples were lower compared with the analyte levels reported in other studies using plasma samples (Kuppusamy & Tan, 2011; Morabito *et al.*, 2007).

5.5.4.2 Combination of chelating agent and compliance towards chelation therapy

A study on oxidative stress in β-thalassaemia major patients attending monthly blood transfusion in UMMC has been reported (Kuppusamy & Tan, 2011). The study showed that despite undergoing iron chelation therapy, iron overload was still observed in the patients. Plasma oxidative stress indices of chelated and unchelated β-thalassaemia major patients measured in the study were compared with healthy controls. The results showed that chelated patients were also under oxidative stress and their oxidative stress markers were significantly elevated compared with healthy controls.

However, the chelated β-thalassaemia major patients recruited in the study were on single iron chelation therapy using deferoxamine (DFO). Compared with the published report, during β-thalassaemia major patient recruitment for the present study, 23% of the patients had undergone combination iron chelation treatment. The treatment involved combination of DFO and deferiprone (DFP), DFO and deferasirox (DFX) or DFP and DFX.

Published data has reported better rate of iron secretion from the patients' circulation due to combination of targeted iron pool by different iron chelators. The patient under combination therapy managed to discontinue hormone therapy after improvement of endocrine glands and have better cardiac function (Galanello *et al.*, 2010). In addition, recent clinical observation has reported that DFX alone may reduce oxidative stress in transfusion dependent patients (Saigo *et al.*, 2013). These factors could be the contributors to the improvement of the oxidative stress status in β-thalassaemia major patients.

Although the patients below 20-years old appeared to have higher level of inflammatory cytokines, the overall oxidative stress status may suggest that the compliance towards chelation therapy is improving as the patients grow older. In addition, the knowledge about the importance of chelation therapy in patients and support from responsible bodies in encouraging proper treatment for patients could have improved.

5.5.4.3 Supplementary prescription

Compared to healthy controls, almost 50% of the β-thalassaemia major patients were prescribed with additional supplementary vitamins including vitamins C, D and E other than iron chelators. As described by Benzie & Strain (1996), ascorbic acid and α-tocopherol could react effectively with FRAP assay reagent. It is possible that additional supplement may have boosted up their antioxidant level up to a level almost similar to healthy controls and reduced or attenuated oxidative stress indices levels and cytokine production in their system.

5.5.4.4 Involuntary stimulation

Mouth rinsing with normal saline prior to sample collection may stimulate whole saliva flow the same way as citric acid due to the taste (Sculley & Langley-Evans, 2002). Participants were reminded about minimising chewing and tongue movement to prevent additional stimulation. Even though participants were allowed to drink, this was only to prevent them from expectorating forced thick saliva due to dehydrated mouth. However, it was still possible that the participants performed such actions during the sample collection resulting in excessive stimulation of salivary fluid flow.

Reports have confirmed that stimulated saliva samples have reduced amount of analyte due to increased secretion of salivary gland fluid (Miller *et al.*, 2010). This factor further diluted the analyte of interest in the whole saliva. This could be further corrected by taking saliva flow rate into consideration (Moore *et al.*, 2001; Sculley & Langley-Evans, 2002).

5.6 Limitations

Malaysia has a multi-ethnic population with many different languages. Differences in language proficiency, especially in understanding the terminology used with paediatric patients may play a major role in differences in sample collection. Although laymen terms were used during recruitment of participants, the extent of understanding the terms explained may have lead to variations in sample collection.

Non-invasive samples are painless and simple to collect. However, public perception towards samples originating from expectorates such as saliva are unavoidable. Participants tend to feel uneasy when handling the samples, probably due to unpleasant odour and appearances. This factor also resulted in rejection towards requests to participate in the research which limited the number of samples collected.

Physiological factors may reduce and alter the appearance and the amount of sample collected. Dryness of mouth due to reduced water consumption can contribute to increased viscosity of saliva samples. Participants tend to refuse to drink although they were allowed to do so during sample collection. Increase in sample viscosity may limit the usable amount of sample and increase the amount of contaminants. In addition, the sample will be hard to handle especially during separation of the salivary fluid during sample processing and increase the amount of mucin in the sample.

Better precision in analyte measurement could be achieved if saliva flow rate was considered during sample collection. However, the procedure could be time consuming especially in achieving the desired amount of sample to accommodate the analysis requirements. In addition, strict observation may be required during sample collection to obtain the precise time to determine the saliva flow rate. Throughout the investigation, direct observation on the participants made them tend to force the saliva flow. This resulted in thick and mucinous saliva which was hard to be aliquoted during sample processing.

Similar to collection of samples for molecular analysis, bacterial contamination may result in degradation of oxidative stress indices and the cytokines. Despite of the attempt to remove oral cavity flora by initial rinsing and freezing, there may still be possibility that trace amounts of bacteria present in the sample. The bacteria metabolic activities may affect the level of biochemical parameters in the samples.

5.7 Future recommendations

Further improvement for DNA extraction and purification can be performed to increase successful amplification. DNA extraction using other materials such as agarose gel may reduce the amount of inhibitors present in the sample for better DNA amplification (Moreira, 1998). On the other hand, the use of a protein precipitation method may reduce the need to use organic solvent for non-invasive DNA sample purification (Heath *et al.*, 2001). With better DNA extraction and purification methods, isolation of DNA from samples such as urine can be included as an option for non-invasive DNA samples.

DNA from non-invasive samples can be further quantified using other methods to determine the human DNA yield and purity. This includes the use of fluorochromes, hybridisation technique and measurement at A_{230nm} (Feigelson *et al.*, 2001; Hansen *et al.*, 2007; Philibert *et al.*, 2008; Rylander-Rudqvist *et al.*, 2006). These methods have been reported to provide better results for DNA quantification.

Biomarkers concentration in saliva samples is easily affected by participant's actions during sample collection. For future studies, better procedures in whole saliva sample collection are required. Written sample collection procedures can be used for participant's reference to prevent any confusion and variation in sample collection.

Saliva flow rate can be estimated to provide better information on analyte concentration (Moore *et al.*, 2001; Sculley & Langley-Evans, 2002). Sample collection procedures can be modified to include determination of saliva volume collected within a specified time. Perhaps this way more samples could be collected without the risk of diluting the analyte concentrations due to over stimulating the saliva. Samples can also be used to analyse more antioxidants and oxidative stress markers.

Other non-invasive sample such as urine can also be used for measurement of oxidative stress markers (Kirschbaum, 2001; Schwemmer *et al.*, 2000). Although some of the oxidative stress indices and cytokines analysed in this study could not be analysed using urine samples, comparison and correlation between salivary and urinary FRAP, AOPP, LOOH and UA can be performed. In addition, other biomarkers like 8-iso-$PGF_{2\alpha}$, hepcidin and 8-hydroxydeoxyguanosine can be analysed using urine samples (Lin *et al.*, 2015; Nemeth, 2010; Wu *et al.*, 2004).

CHAPTER 6: CONCLUSION

In summary, mouthwash, saliva and buccal cytobrush samples can provide reasonable amounts of DNA with acceptable purity. The sample collection procedures are convenient and easy to perform by researchers and participants. The DNA extraction and purification procedures using Method 2 provided purified amplifiable DNA. The purified DNA is sufficient in amounts and can be used for molecular characterisation of β-globin gene mutations in β-thalassaemia major patients with reproducible results. Mouthwash and saliva samples may serve as the most suitable sampling procedures to be used to obtain high quantity and quality non-invasive DNA for molecular characterisation of disorders like thalasseamia.

Saliva can also be used in biochemical analysis to estimate the levels of oxidative stress in β-thalassaemia major patients. The oxidative stress indices and cytokine levels in the saliva samples were detectable and can be measured using in-house methods and commercialised assay kits. However, only selected oxidative stress indices and cytokines are sensitive and readily detected in saliva samples.

Through the current study, the oxidative stress status in the β-thalassaemia major patients has improved. This observation is mainly due to better compliance towards treatment regimes, combination iron chelation therapies, education, additional supplementation and better patient management.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. DNA genotyping and antioxidant status in β-thalassaemia major patients using non-invasive sampling techniques. International Anatomical Sciences and Cell Biology Conference, 26th -29th May 2010. National University of Singapore, Singapore (Poster presentation).

2. Assessment of DNA extracted from non-invasive samples – mouthwash, saliva and buccal cells – for molecular analysis of β-thalsssaemia. National Postgraduate Conference in Molecular Medicine, 13th -14th April 2011. Universiti Sains Malaysia, Kota Bahru, Kelantan, Malaysia (Poster presentation).

3. Evaluation of concentration and purity of DNA extracted from mouthwash, saliva and buccal cytobrush samples. 1st International Conference on Molecular Diagnostics and Biomarker Discovery, 23rd -25th October 2013. Universiti Sains Malaysia, Penang, Malaysia (Poster presentation).

4. Rahim, MRA, Kho, SL, Tan, JAMA and Kuppusamy, UR. Non-invasive DNA sampling for molecular analysis of beta-thalassemia: Amiable alternative sampling methods with accurate results for pediatric patients. *Clin Lab.* (Accepted).

5. Rahim, MRA, Kuppusamy, UR and Tan, JAMA. Salivary oxidative stress indices and cytokine levels in beta-thalassaemia major patients. (Manuscript in preparation).

APPENDIX

Appendix A

Figure A1: Patient information sheet (Bahasa Malaysia)

RISALAH MAKLUMAT

Kajian Penanda Biokimia Tidak Invasif dalam Pesakit Beta-Talassemia Major dan Intermedia

Sila baca dengan teliti maklumat yang disediakan. Sila dapatkan bantuan dan rujukan dari doktor anda jika terdapat sebarang persoalan.

Tajuk kajian:
Kajian Penanda Biokimia Tidak Invasif dalam Pesakit Beta-Talassemia Major dan Intermedia

Pengenalan

Beta-talasemia adalah penyakit darah yang boleh diwarisi. Penyakit ini menurunkan kandungan hemoglobin yang terhasil dalam darah anda untuk mengangkut oksigen. Oleh itu, ia akan menyebabkan anemia, yakni penyakit kekurangan darah. Dianggarkan 4.5% penduduk Malaysia berbangsa Cina dan Melayu adalah pembawa β-thalassaemia, di mana mereka tidak menunjukkan sebarang tanda penyakit kekurangan darah.

Namun, β-talasemia major akan menyebabkan penghidapnya mengalami masalah kekurangan darah yang teruk, sehinggakan mereka memerlukan pemindahan darah setiap bulan. Walaupun pemindahan darah mampu melanjutkan tempoh hayat seseorang pesakit, ia boleh menyebabkan lebihan zat besi terkumpul dalam tubuh pesakit. Kesihatan pesakit yang mendapat pemindahan darah tetapi tidak mendapat rawatan penyingkiran lebihan zat besi yang mencukupi akan merosot. Tanpa rawatan ini, lebihan zat besi akan menyebabkan pembentukan radikal bebas yang akan menurunkan kandungan antioksidan yang mampu melindungi tubuh dari kerosakan. Tekanan oksidatif akan menyebabkan kerosakan organ dalaman, termasuk hati, kelenjar pituitari, pankreas dan jantung.

Apakah tujuan kajian ini?

Kajian ini bertujuan memerhatikan keberkesanan teknik baru mengumpul sampel yang tidak invasif dalam penentuan genetik dan memerhatikan tahap tekanan oksidatif pada pesakit talasemia major.

Apakah langkah-langkah yang perlu diikuti?

Pesakit akan diberi larutan salin steril untuk berkumur. Kemudian, sampel air liur akan dikumpul di dalam tiub steril, diikuti dengan pengumpulan sel pipi yang di ambil dari dalam mulut.

Parameter dalam kerosakan makromolekul and tahap antioksidan keseluruhan akan dianalisa dari air liur pesakit.

Kajian molecular untuk memastikan mutasi pada β-globin pada pesakit β-talasemia akan dilakukan dengan menggunakan teknik amplifikasi DNA.

Keputusan untuk kedua-dua ujian akan dimaklumkan kepada pesakit setelah analisa selesai dan disahkan benar.

Siapakah yang patut menyertai kajian ini?

Pesakit β-talasemia major berumur dalam lingkungan 4-15 tahun yang mengambil atau tidak mengambil ubatan desferrioxamine/ deferiprone.

Apakah manfaat yang boleh didapati dari kajian ini?

a) Kepada peserta

Keputusan kajian ini akan memberitahu pesakit tentang status genetik dan tekanan oksidatif mereka.

b) Kepada pengkaji

Kajian ini memberikan maklumat tentang tahap tekanan oksidatif pada pesakit dan pendekatan baru dalam analisis molekular untuk β-talasemia.

Adakah sebarang kesan sampingan?

Tiada

Bolehkah saya menolak untuk melibatkan diri dalam kajian ini?

YA. Penglibatan adalah secara sukarela.

Siapakah yang saya boleh hubungi sekiranya saya mempunyai soalan tentang kajian ini?

Profesor Madya Umah Rani Kuppusamy / Profesor Mary Anne Tan Jin Ai
Jabatan Perubatan Molekul
Fakulti Perubatan
Universiti Malaya
Tel: 03-7967 4903 / 7967 4900

Figure A2: Patient information sheet (English)

PATIENT INFORMATION SHEET

Study of Non-invasive Biochemical Markers in Beta-Thalassaemia Major and Intermediate Patients

Please read the following information carefully, do not hesitate to discuss any questions you may have with your doctor.

Study title:
Salivary DNA genotyping and oxidative stress markers in beta thalassaemia major patients

Introduction

Beta (β)-thalassaemia is an inherited disease of the blood. The disease reduces the amount of haemoglobin (to carry oxygen) your body produces, therefore, it can cause anaemia (shortage of blood). About 4.5%of Malaysian Chinese and Malays are β-thalassaemia carriers who show little signs of anaemia.

β-thalassaemia major, however, results in severe anaemia where monthly blood transfusions are necessary. While blood transfusion has the advantage of prolonging the patient's life, they cause iron over-load. Patients show progressive deterioration clinically when given blood transfusions without adequate iron-chelation therapy. Without chelation therapy, the accumulating iron results in free radical formation that depletes cellular antioxidants that protect our body. The body is under oxidative stress that will induce progressive damage to organs including liver, pituitary gland, pancreas and the heart.

What is the purpose of this study?

This study would evaluate a new non-invasive approach to genotype and assess oxidative stress status in thalassaemia major patients and compare it using usual approach.

What is the procedure to be followed?

Blood will be drawn from the patient before transfusion started. Patient will be given sterile saline solution to gargle. Then, saliva sample will be collected into sterile tube, followed by cheek cells collection from oral mucosa.

Parameters of macromolecular damage and total antioxidant levels will be analyzed using collected saliva.

Molecular studies to confirm β-globin mutations in β-thalassaemia patients will be carried out using DNA amplification techniques.

Results from both molecular and biochemical studies will be made available to the patients when data has been analyzed and confirmed.

Who should enter the study?

β-thalassaemia major patients aged between 4-15 years who have been undergoing desferrioxamine/ deferiprone chelation therapy and those without.

What will be the advantages / benefits of this study?

a) To you as the subject

The result from this study will inform β-thalassaemia major patients about their oxidative stress and antioxidant status and their genetic status.

b) To the investigators

Provide valuable data on oxidative stress levels in β-thalassaemia major patients and new approach for molecular analysis on β-thalassaemia.

What are the possible drawbacks?

None

Can I refuse to take part in this study?

YES, this study is on a voluntary basis.

Who should I contact if I have additional questions during the course of this study?

Assoc. Professor Umah Rani Kuppusamy /
Professor Mary Anne Tan Jin Ai
Department of Molecular Medicine
Faculty of Medicine
University of Malaya
Tel: 03-7967 4903 / 7967 4900

Figure A3: Informed consent form (Bahasa Malaysia)

BORANG PERSETUJUAN UNTUK KAJIAN

Kami yang menandatangani borang ini bersetuju untuk mengambil bahagian dalam kajian klinikal bertajuk
Kajian Penanda Biokimia Tidak Invasif dalam Pesakit Beta-Talassemia Major dan Intermedia
Kami telahpun memahami bahawa sampel darah, kumuran, air liur, sel pipi air kencing akan diambil dari pesakit.

Kami memahami bahawa:

Tujuan dan perincian kajian klinikal ini dari segi metodologi, kesan sampingan dan kelebihan/manfaat kajian ini (seperti termaktub di dalam risalah maklumat).

Selepas mengetahui dan memahami segala manfaat dan kelemahan yang mungkin yang mungkin ada dalam kajian ini, kami secara sukarela membenarkan pesakit mengambil bahagian dalam kajian dengan tajuk seperti yang tercatat di atas.

Kami memahami bahawa pessakit boleh menarik diri dari kajian ini pada bila-bila masa tanpa menyatakan sebarang sebab musabab atau dalam situasi di mana doktor yang merawat mendapati tiada faedah dari rawatan biasa.

Ditandatangani oleh:

.....

Pesakit (jika pesakit berumur lebih 18 tahun): .....

Ibubapa (jika pesakit berumur kurang 18 tahun): .....

No KP : .....

Saya mengakui bahawa saya telah menerangkan dengan teliti kepada pesakit tentang perincian dan tujuan kajian yang telah tercatat di atas.

Ditandatangani oleh:

.....

Saksi / Doktor / Jururawat / Juruteknologi Makmal Perubatan

Nama : .....

No KP : .....

Figure A4: Informed consent form (English)

INFORMED CONSENT FORM FOR STUDY

We, the undersigned, hereby agree to participate in the clinical research titled
Study of Non-invasive Biochemical Markers in Beta-Thalassaemia Major and Intermediate Patients
We understand that samples of blood, mouthwash, saliva, cheek cells and urine will be taken from patient.

We understand that:

The purpose and nature of the clinical research in terms of methodology, possible drawbacks and advantage/benefits (as per patient information sheet).

After knowing and understanding all the possible advantages and disadvantages in this clinical research, we voluntarily consent with our own free will to let the patient to participate in the clinical research specified above.

We understand that the patient can withdraw from this clinical research at any time without assigning any reason whatsoever and in such a situation shall not be denied the benefits of usual treatment by the attending doctors.

Signed:

.....

Patient (if patient is above 18 years old): .....

Parents (if patient is below 18 years old): .....

NRIC : .....

I have explained to the patient the nature and the purpose of the above-mentioned clinical research.

Signed:

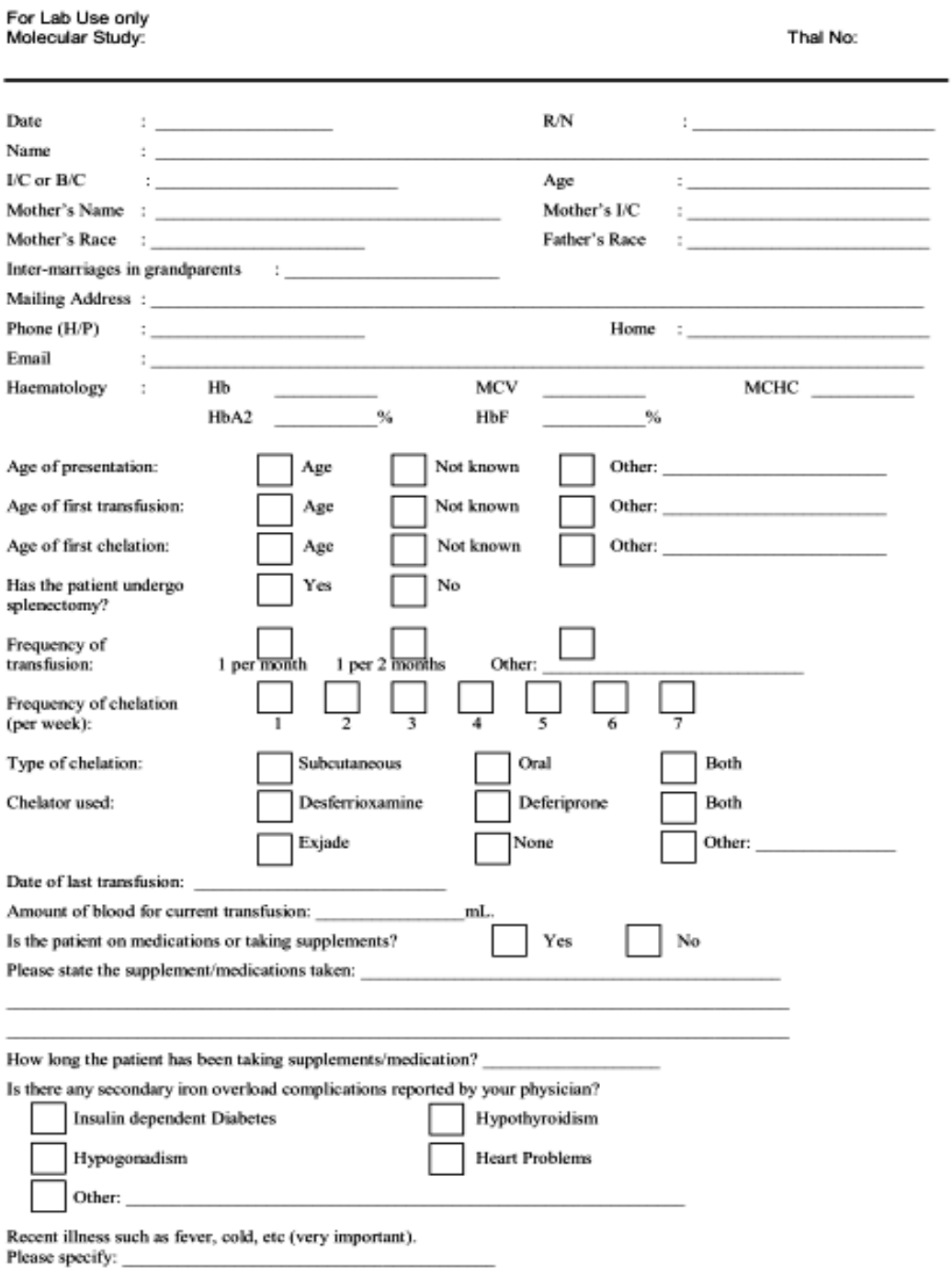
.....

Witness / Doctor / Nurse / Medical Laboratory Technologist

Name : .....

NRIC : .....

Figure A5: Questionnaire form



For Lab Use only
Molecular Study:

Thal No:

Date : _____ R/N : _____

Name : _____

I/C or B/C : _____ Age : _____

Mother's Name : _____ Mother's I/C : _____

Mother's Race : _____ Father's Race : _____

Inter-marriages in grandparents : _____

Mailing Address : _____

Phone (H/P) : _____ Home : _____

Email : _____

Haematology : Hb _____ MCV _____ MCHC _____

HbA2 _____% HbF _____%

Age of presentation: ☐ Age ☐ Not known ☐ Other: _____

Age of first transfusion: ☐ Age ☐ Not known ☐ Other: _____

Age of first chelation: ☐ Age ☐ Not known ☐ Other: _____

Has the patient undergo splenectomy? ☐ Yes ☐ No

Frequency of transfusion: ☐ 1 per month ☐ 1 per 2 months ☐ Other: _____

Frequency of chelation (per week): ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7

Type of chelation: ☐ Subcutaneous ☐ Oral ☐ Both

Chelator used: ☐ Desferrioxamine ☐ Deferiprone ☐ Both

☐ Exjade ☐ None ☐ Other: _____

Date of last transfusion: _____

Amount of blood for current transfusion: _____mL.

Is the patient on medications or taking supplements? ☐ Yes ☐ No

Please state the supplement/medications taken: _____

_____

_____

How long the patient has been taking supplements/medication? _____

Is there any secondary iron overload complications reported by your physician?

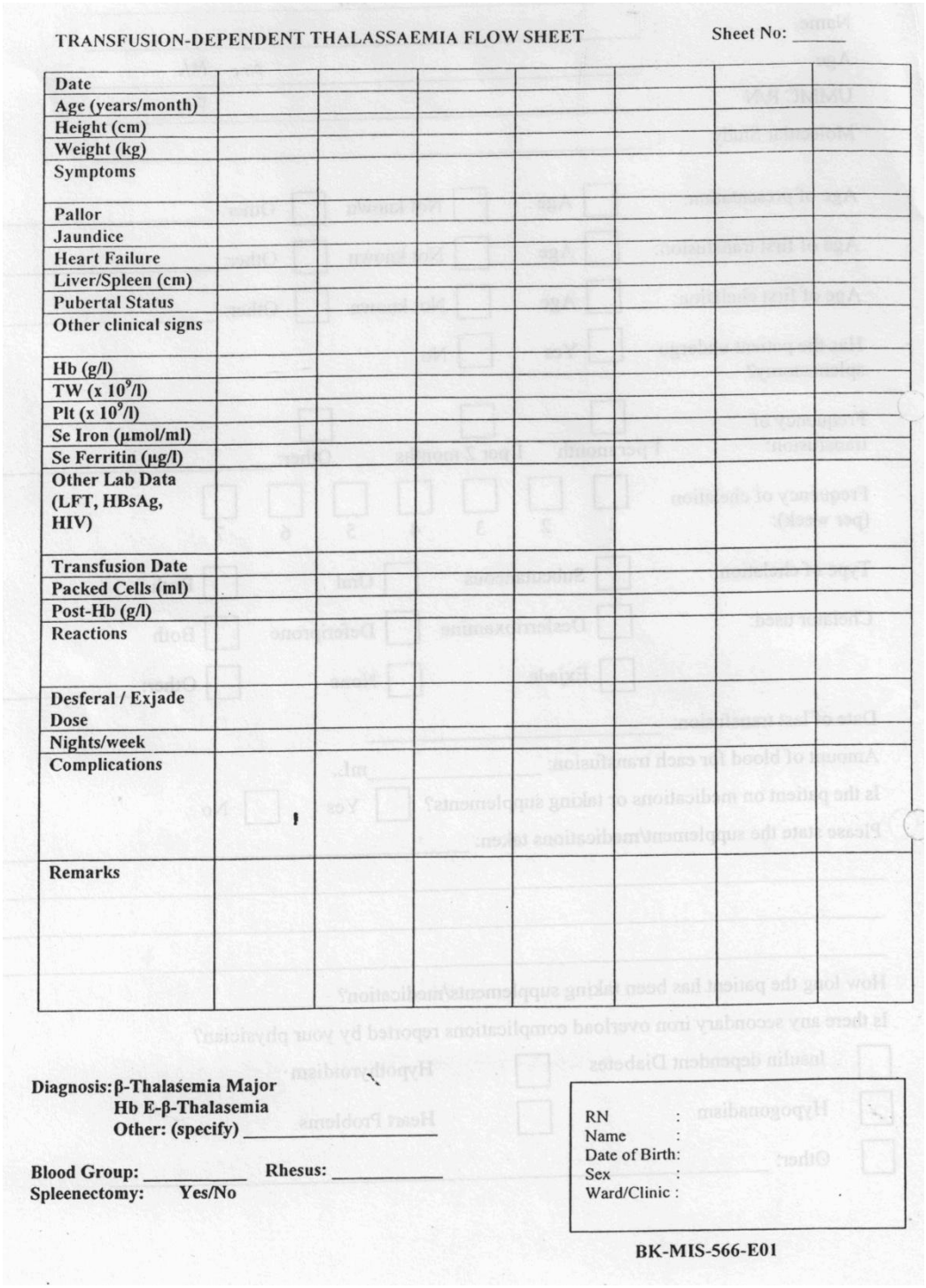
☐ Insulin dependent Diabetes ☐ Hypothyroidism

☐ Hypogonadism ☐ Heart Problems

☐ Other: _____

Recent illness such as fever, cold, etc (very important).
Please specify: _____

Figure A6: Transfusion dependent thalassaemia flow sheet



TRANSFUSION-DEPENDENT THALASSAEMIA FLOW SHEET

Sheet No: _____

Date							
Age (years/month)							
Height (cm)							
Weight (kg)							
Symptoms							
Pallor							
Jaundice							
Heart Failure							
Liver/Spleen (cm)							
Pubertal Status							
Other clinical signs							
Hb (g/l)							
TW (x 10^9/l)							
Plt (x 10^9/l)							
Se Iron (µmol/ml)							
Se Ferritin (µg/l)							
Other Lab Data (LFT, HBsAg, HIV)							
Transfusion Date							
Packed Cells (ml)							
Post-Hb (g/l)							
Reactions							
Desferal / Exjade Dose							
Nights/week							
Complications							
Remarks							

Diagnosis: β-Thalasemia Major
Hb E-β-Thalasemia
Other: (specify) _____

Blood Group: _____ Rhesus: _____
Spleenectomy: Yes/No

RN :
Name :
Date of Birth:
Sex :
Ward/Clinic :

BK-MIS-566-E01

Appendix B

1) Reagent for sample collection

a. Normal saline (0.85% NaCl)

- 8.5 g NaCl (MW: 58.44 g/mol)
- Filtered water

NaCl powder was dissolved in 1000 mL of water. The solution was autoclaved.

2) Reagents for DNA extraction

a. 1 M Tris(hydroxymethyl)aminomethane (Tris, pH 8.0)

- 121.1 g Tris (MW: 121.1 g/mol)
- Sterile deionised water
- Concentrated hydrochloric acid (HCl)

Tris powder was dissolved in 800 mL of water. Concentrated HCl was added to adjust the pH to 8.0. Water was added to make up the volume to 1000 mL. The solution was filtered and autoclaved.

b. 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0)

- 186.1 g sodium EDTA ($Na_2EDTA.2H_2O$,MW: 372.24 g/mol)
- Sterile deionised water
- 10 M sodium hydroxide (NaOH)

EDTA powder was dissolved in 800 mL of water. NaOH was added to adjust the pH to 8.0. Water was added to make up the volume to 1000 mL. The solution was filtered and autoclaved.

c. 10 M sodium hydroxide (NaOH)

- 40 g NaOH (MW: 40 g/mol)
- Sterile deionised water

NaOH pellets were left to dissolve in 100 mL of water. Additional precaution was needed as the reaction was exothermic.

d. Tris-EDTA buffer (TE 10 mM/10 mM, pH 8.0)

- 10 mL 1 M Tris
- 20 mL 0.5 M EDTA
- Sterile deionised water

Both solution were mixed and diluted with deionised water up to 1000 mL. The solution was autoclaved.

e. 4 M sodium chloride (NaCl)

- 23.38 g NaCl (MW: 58.44 g/mol)
- Sterile deionised water

NaCl powder was dissolved in 100 mL of water. The solution was filtered and autoclaved.

f. 10% sodium dodecyl sulphate (SDS)

- 10 g SDS (MW: 288.37 g/mol)
- Sterile deionised water

SDS powder was dissolved in 100 mL of water. The solution was filter sterilised.

g. Lysis buffer

- 1.25 mL of 4 M NaCl
- 10 mL of 10% SDS
- TE buffer

NaCl and SDS were mixed with TE buffer and the solution was made up to 50 mL.

h. 10 mg/mL proteinase K

- 100 mg of lyophilised proteinase K (fungal) (Invitrogen, Cat: 25530-015)
- 10 mL ultrapure water

The enzyme was reconstituted with the ultrapure water. The solution was aliquoted into several tubes and stored at -20°C until needed.

i. 20 mg/mL proteinase K

- 100 mg of lyophilised proteinase K
- 5 mL ultrapure water

The enzyme was reconstituted with the ultrapure water. The solution was aliquoted into several tubes and stored at -20°C until needed.

j. 10 mg/mL dithiothreitol (DTT)

- 500 mg of DTT
- 50 mL sterile deionised water

The powder was reconstituted with the sterile water and stored at 4°C until needed.

k. Tris buffered phenol

- 1 kg phenol (crystal form)
- 0.5 M Tris (pH 8.0)
- 1 g 8-hydroxyquinolin

Phenol crystals were allowed to liquefy in its amber bottle at 68°C waterbath. 8-hydroxyquinolin was added into the phenol liquid. Phenol was mixed with equal volume of 0.5 M Tris and the bottle was agitated vigorously. The solution mixture was allowed to separate overnight.

The aqueous layer was removed on the following day. New 0.5 M Tris was added and the mixture was agitated vigorously and allowed to separate.

The mixing step was repeated until the phenol has reached the pH of 7.6 – 8.0.

A portion of the aqueous layer was left on top of the phenol liquid to prevent phenol oxidation. The solution was stored at 4°C

l. Phenol: chloroform (1:1)

- 500 mL Tris buffered phenol
- 500 mL chloroform

Both solution was mixed in a amber bottle and stored at 4°C.

m. 3 M sodium acetate (NaOAc, pH 5.6)

- 24.61 g NaOAc (MW: 82.03 g/mol)
- Sterile deionised water
- Glacial acetic acid

NaOAc powder was dissolved in 50 mL of water. Glacial acetic acid was added to adjust the pH to 5.6. The solution was filtered and autoclaved.

3) Reagents for gel electrophoresis

a. 10X Tris-Borate-EDTA buffer (TBE, pH 8.3)

- 108 g Tris
- 55 g boric acid (MW: 61.8 g/mol)
- 40 mL 0.5 M EDTA
- Sterile deionised water

All powders and solution were mixed and diluted with deionised water up to 1000 mL. The solution was filtered and autoclaved.

b. 5X TBE loading buffer

- 10 mL 10X TBE
- 9.8 mL glycerol
- 0.2 mL of 10% SDS
- 6 mg bromophenol blue
- 30 mg xylene cyanol

All components were mixed in sterile reagent bottle and stored protected from light.

4) Reagents for FRAP assay

a. 1000 µM ferrous sulphate ($FeSO_4$, standard stock solution)

- 0.0028 g $FeSO_4.7H_2O$
- 10 mL deionised water

The powder was dissolved in water. The stock solution was used to produce 200, 400, 600 and 800 µM solutions.

b. 300 mM acetate buffer (pH 3.6)

- 0.31 g NaOAc
- 1.6 mL acetic acid
- Deionised water

NaOAc was dissolved in 90 mL of water. Acetic acid was added and the solution was brought to final volume of 100 mL

c. 10 mM TPTZ in 40 mM HCl

- 0.0156 g TPTZ
- 0.2 mL of 1 M HCl
- Deionised water

TPTZ was added into HCl and brought up to final volume of 5 mL with water. The solution was protected from light.

d. 20 mM ferric chloride ($FeCl_3.6H_2O$)

- 0.0541 g $FeCl_3.6H_2O$
- 10 mL deionised water

The powder was dissolved in water.

e. FRAP reagent

- 50 mL acetate buffer
- 5 mL TPTZ
- 5 mL $FeCl_3$

All solutions were combined together. The final solution was protected from light.

5) Reagents for AOPP assay

a. 500 µM chloramine-T (standard stock solution)

- 0.0028 g chloramine-T
- 25 mL deionised water

Chloramine-T was dissolved in water and further used to produce 100, 200, 300 and 400 µM solutions. The solution was protected from light.

b. Phosphate buffered saline (PBS)

- 1 PBS tablet
- 100 mL deionised water

The tablet was dissolved in water and stored at 4°C until needed.

c. 50% acetic acid

- 50 mL glacial acetic acid
- 50 mL deionised water

The acid was diluted with water. The solution can be kept for later use at room temperature.

d. 1.16 M potassium iodide (KI)

- 0.9628 g KI
- 5 mL deionised water

The KI powder was dissolved in water. The solution was protected from light.

e. AOPP reagent

- 81% of PBS
- 15% of 50% acetic acid
- 4% of KI

All solutions were combined together. The final solution was protected from light.

6) Reagents for LOOH assay

a. 1 mM 1,1,3,3-tetraethoxypropane (TEP, standard stock solution)

- 12.5 µL TEP
- 50 mL deionised water

Water (12.5 µL) was removed from the tube. TEP was added and vortexed to mix. The stock solution was further used to produce 20, 15, 10, 5 and 2.5 µM solutions. The solution was protected from light.

b. 10.3 mM 1-methyl-2-phenylindole (MPI) in acetonitrile

- 0.0213 g MPI
- 10 mL acetonitrile

The powder was dissolved in the solvent and protected from light.

c. 5 M HCl

- 50 mL of stock HCl (37%)
- 50 mL deionised water

The acid was diluted with water. The solution can be kept for later use at room temperature and protected from light.

7) Reagents for ELISA

a. Phosphate buffered saline (PBS)

- 10 PBS tablet
- 1000 mL deionised water

The tablet was dissolved in water and stored at 4°C until needed.

b. Washing buffer

- 50 mL PBS
- 25 µL Tween-20

PBS (25 µL) was removed and Tween-20 was added. The solution was mixed gently.

Appendix C

Figure C1: Standard curve for FRAP assay

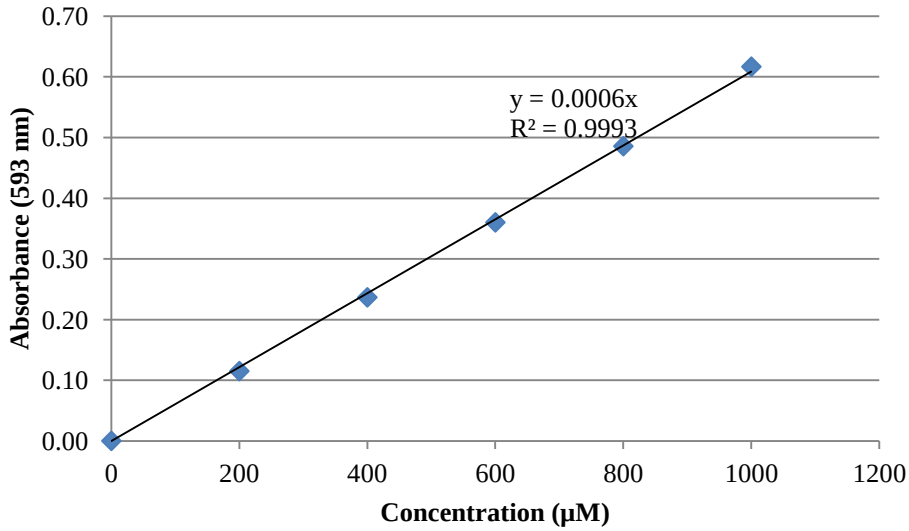


Figure C2: Standard curve for AOPP assay

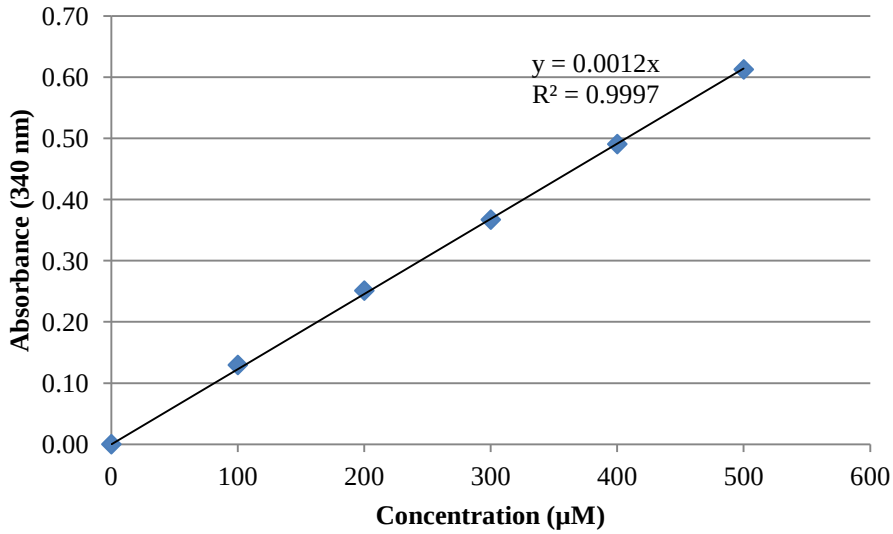


Figure C3: Standard curve for LOOH assay

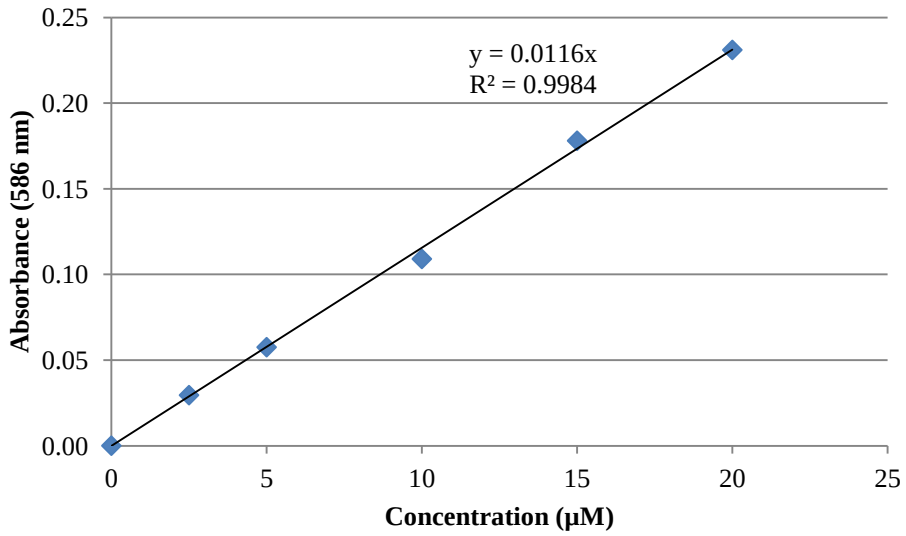


Figure C4: Activity curve for control in GPx assay

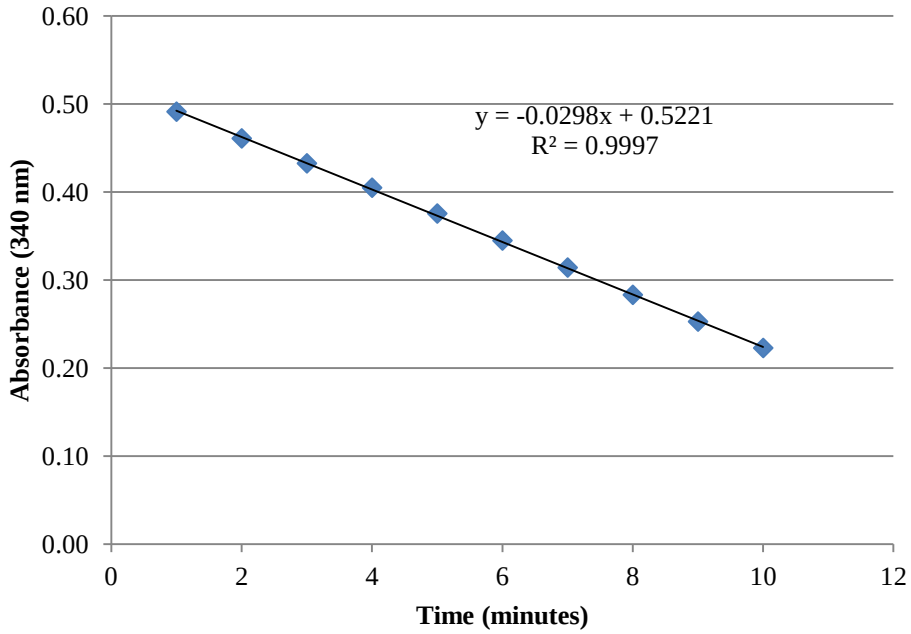


Figure C5: Standard curve for UA assay

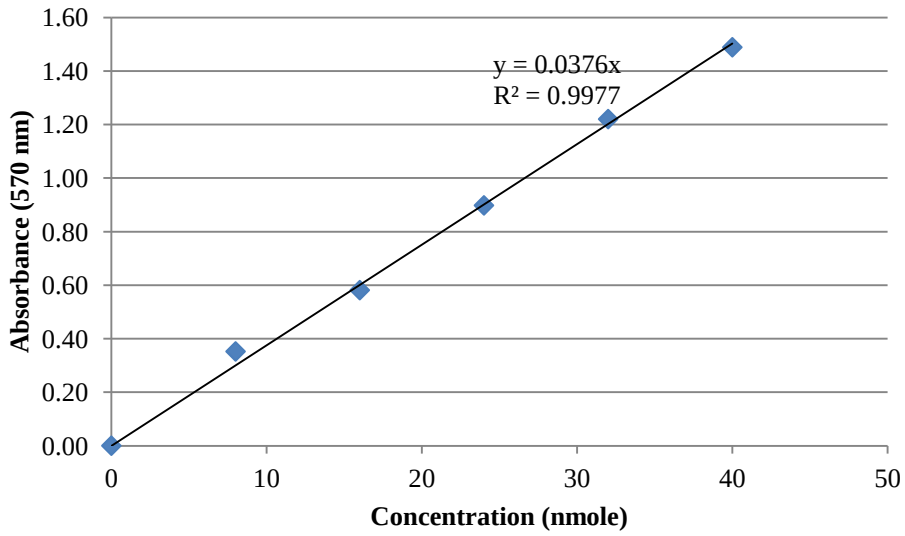


Figure C6: Standard curve for ELISA