CONFIRMATORY DIAGNOSIS OF HEMOGLOBIN VARIANTS IN THAILAND BY MULTIPLEX ARMS-PCR

NOPPAWAN TANGBUBPHA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(CLINICAL PATHOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2011

Thesis
entitled

CONFIRMATORY DIAGNOSIS OF HEMOGLOBIN VARIANTS IN THAILAND BY MULTIPLEX ARMS-PCR

Noppawan Tangbubpha
.....
Miss Noppawan Tangbubpha
Candidate

Sumalee Jindadamrongwech
.....
Assist. Prof. Sumalee Jindadamrongwech, Ph.D.
Major-Advisor

.....
Assoc. Prof. Suporn Chuncharunee, M.D., Board of internal medicine, and hematology, Clinical and research fellow in Hematology
Co-Advisor

Punnee Butthep
.....
Assoc. Prof. Punnee Butthep, Ph.D.
Co-Advisor

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya, M.D., Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

.....
Assist.Prof. Pitak Santanirand, Ph.D.
Program Director
Master of Science Program in Clinical Pathology
Faculty of Medicine Ramathibodi Hospital, Mahidol University

Thesis
entitled

CONFIRMATORY DIAGNOSIS OF HEMOGLOBIN VARIANTS IN THAILAND BY MULTIPLEX ARMS-PCR

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Clinical Pathology)
on
September 21, 2011

Noppawan Tangbubpha
.....
Miss Noppawan Tangbubpha
Candidate

Khaimuk Ch.
.....
Lect. Khaimuk Changsri
Ph.D.
Chair

Sumalee Jindadamrongwech
.....
Assist. Prof. Sumalee Jindadamrongwech,
Ph.D.
Member

.....
Assoc. Prof. Suporn Chuncharunee,
M.D., Board of internal medicine, and
hematology, Clinical and research fellow in
Hematology
Member

Punnee Butthep
.....
Assoc.Prof. Punnee Butthep,
Ph.D.
Member

B. Mahaisavariya
.....
Prof. Banchong Mahaisavariya,
M.D., Dip. Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Rajata Rajatanavin,
M.D., F.A.C.E.
Dean
Faculty of Medicine Ramathibodi Hospital,
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my honest gratitude and deepest appreciate and sincere gratitude to my advisor and co-advisor, Assist. Prof. Sumalee Jindadamrongwech, Assoc. Prof. Suporn Chuncharunee and Assoc. Prof. Punnee Butthep for their guidance, valuable advise supervision and encouragement throughout the course of this thesis. They were never lacking in kindness and support. Without their valuable advice, this work has never been accomplished successfully.

I am very grateful to the member of the supervisory committee, Dr. Khaimuk Changsri for her helpful comments, guidance and valuable advice about this thesis defense.

My special thanks are directed to Miss Kobkorn Pongjantharasatien and all members of Blood Disease Diagnostic Center for her kindness in training me for laboratory techniques and constructive suggestion.

My appreciations are extended to Dr. Raewadee Wisedpanichkij and all members of the Division of Hematology, Department of Pathology, Ramathibodi Hospital for their useful technical guidance, support and cheerfulness.

I wish to thanks for all friends in the Master Degree Program in Clinical Pathology and all friends in Ramathibodi Hospital for helpful, cheerfulness and warm atmosphere.

Most of all, I am deeply indebted to patients and subjects who donated blood specimens for investigation and Grant from the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand for project support.

Finally, I would like to express my deepest appreciation to my dearest family for their infinite love, kindness, understanding and cheering throughout this study. This would never have been possible without all of you.

Noppawan Tangbubpha

CONFIRMATORY DIAGNOSIS OF HEMOGLOBIN VARIANTS IN THAILAND BY MULTIPLEX ARMS-PCR

NOPPAWAN TANGBUBPHA 5037450 RACP/M

M.Sc. (CLINICAL PATHOLOGY)

THESIS ADVISORY COMMITTEE: SUMALEE JINDADAMRONGWECH, Ph.D., PUNNEE BUTTHEP, Ph.D., SUPORN CHUNCHARUNEE, MD

ABSTRACT

Hemoglobin (Hb) variant is an abnormal structure Hb due to globin genes mutation. The effect of Hb variants are varied, from no clinical implication to clinical significance if combines either with thalassemia or with their own. The Hb variants commonly found in Thailand are Hb E and Hb Constant Spring. They are clinically significant when associate with thalassemia.

This study was aimed at some rare Hb variants in Thailand. Based on Hb typing, a Hb variant was screened to present as an abnormal peak or sometimes in the same peak of normal Hb fraction such as Hb F and Hb A_2. Moreover, different Hb variants may separate in the same fraction. Two automated Hb typing machines were used in comparative, the Variant II HPLC and the Capillarys-2 CZE. Three multiplex ARMS-PCR test were set up to identify 12 Hb variants.

The first group of Hb variants were those eluted before Hb A on HPLC. The second and the third groups were those eluted after Hb A on HPLC, but the latter was for the Middle East population. The variants positive by PCR were confirmed by direct DNA sequencing. The results showed the successful of the multiplex ARMS-PCR in detection of rare 7 Hb variants in Thai and 2 variants in the Middle East samples.

KEY WORDS: HB VARIANT / MULTIPLEX / ARMS-PCR

99 pages

การตรวจยืนยันชนิดของฮีโมโกลบินผิดปกติที่พบในประเทศไทย โดยวิธี MULTIPLEX ARMS-PCR

CONFIRMATORY DIAGNOSIS OF HEMOGLOBIN VARIANTS IN THAILAND BY MULTIPLEX ARMS-PCR

นพวรรณ ตั้งบุบผา 5037450 RACP/M

วท.ม. (พยาธิวิทยาคลินิก)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : สุมาลี จินดาดำรงเวช, Ph.D. (อณูพันธุศาสตร์และพันธุวิศวกรรมศาสตร์), พรรณี บุตรเทพ, Ph.D. (วิทยาศาสตร์การแพทย์), สุภร จันท์จารุณี, M.D.

บทคัดย่อ

ฮีโมโกลบินผิดปกติเป็นความผิดปกติของการสังเคราะห์สายโกลบินที่มีโครงสร้างของสายฮีโมโกลบินผิดปกติเนื่องจากเกิดการมิวเตชั่นของโกลบินยีน อาการของผู้ป่วยที่มีภาวะฮีโมโกลบินผิดปกติมีความหลากหลายตั้งแต่ไม่มีอาการทางคลินิกจนถึงก่อให้เกิดอาการทางคลินิกเมื่อเกิดร่วมกับภาวะธาลัสซีเมียหรือเกิดร่วมกับฮีโมโกลบินผิดปกติเอง ฮีโมโกลบินผิดปกติที่พบบ่อยในประเทศไทยได้แก่ ฮีโมโกลบินอี และฮีโมโกลบินคอนสแตนสปริงซึ่งฮีโมโกลบินผิดปกติทั้งสองชนิดนี้ก่อให้เกิดภาวะทางคลินิกที่สำคัญเมื่อเกิดร่วมกับธาลัสซีเมีย

วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อศึกษาชนิดของฮีโมโกลบินผิดปกติที่พบได้ไม่บ่อยในประเทศไทย จากการตรวจแยกชนิดฮีโมโกลบินเบื้องต้นจะพบลักษณะ peak ของฮีโมโกลบินผิดปกติ หรือบางชนิดพบรวมอยู่กับ peak ที่พบในคนปกติด้วยเช่นที่พบตรงตำแหน่งเดียวกับ Hb F และ Hb A_2 เป็นต้น นอกจากนั้นยังพบว่ามีฮีโมโกลบินผิดปกติหลายชนิดที่ถูกแยกออกมาในตำแหน่งเดียวกันอีกด้วย การศึกษาครั้งนี้ได้ทำการแยกชนิดฮีโมโกลบินโดยใช้เครื่องวิเคราะห์อัตโนมัติ Variant II HPLC และ Capillarys-2 CE และได้พัฒนา Multiplex AMRS-PCR พัฒนาขึ้นมา 3 ชุดเพื่อใช้ตรวจยืนยันชนิดของฮีโมโกลบินผิดปกติทั้งหมด 12 ชนิด โดยกลุ่มที่หนึ่งใช้ตรวจยืนยันฮีโมโกลบินผิดปกติที่พบ peak ถูกแยกออกมาก่อนตำแหน่ง Hb A ด้วยเครื่อง HPLC ในกลุ่มที่สองและสามใช้ตรวจยืนยันฮีโมโกลบินผิดปกติที่พบ peak ถูกแยกออกมาหลังตำแหน่ง Hb A ด้วยเครื่อง HPLC แต่ในกลุ่มที่สามเป็นชนิดที่พบได้ในชาวตะวันออกกลาง และได้ทำการตรวจยืนยันผลที่ได้จากวิธี Multiplex AMRS-PCR ที่พัฒนาขึ้น ด้วยวิธี DNA sequencing การศึกษาครั้งนี้ประสบความสำเร็จในการพัฒนาเทคนิค Multiplex AMRS-PCR โดยสามารถตรวจยืนยันฮีโมโกลบินผิดปกติได้ 7 ชนิดในคนไทยและ 2 ชนิดในชาวตะวันออกกลาง

99 หน้า

CONTENTS

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF ABBREVIATIONS

α chain	=	alpha chain
A	=	Alanine
ARMS	=	amplification refractory mutation system
Arg	=	Arginine
Asn	=	Asparagine
Asp	=	Aspartate
β chain	=	beta chain
BIOTECH	=	biotechnology
bp	=	Base pair
C	=	Cytosine
CD	=	Cluster designation
°C	=	Degree of Celsius
CZE	=	Capillary Zone Electrophoresis
dNTP	=	Deoxynucleotide triphosphate
DNA	=	Deoxyribonucleic acid
ε	=	epsilon
EDTA	=	Ethylene diamine tetracetate
et al	=	et alii (Latin), and other
fl	=	femtoliters
γ	=	gamma
g	=	Gram
G	=	Guanine
Gly	=	Glycine
Gln	=	Glutamine
Glu	=	Glutamate
Hb	=	hemoglobin
Hct	=	hematocrit

LIST OF ABBREVIATIONS (cont.)

Hb A	=	hemoglobin A
Hb Anantharaj	=	hemoglobin Anantharaj; J-Wenchang-Wuming
Hb A_2	=	hemoglobin A_2
Hb C	=	hemoglobin C
Hb CS	=	hemoglobin Constant Spring
Hb D-Punjab	=	hemoglobin D-Punjab; D-North; D-Portugal; Oak Ridge; D-Chicago; D-Los Angeles
Hb E	=	hemoglobin E
Hb F	=	hemoglobin F
Hb Hope	=	hemoglobin Hope
Hb J-Bangkok	=	hemoglobin J-Bangkok; J-Mienung; J-Korat; J-Manado
HbQ-Thailand	=	hemoglobin Q-Thailand; G-Taichung; Mahidol; Kurashiki
Hb Queens	=	hemoglobin Queens; Ogi
Hb S	=	hemoglobin S
Hb Siam	=	hemoglobin Siam; Ottawa
Hb Siriraj	=	hemoglobin Siriraj; G-Siriraj
Hb Tak	=	hemoglobin Tak
HPLC	=	high performance liquid chromatography
KCl	=	Potassium chloride
His	=	Histidine
hr	=	Hour (s)
L	=	Litre
Leu	=	Leucine
Lys	=	Lysine
m	=	Milli (10^{-3})
M	=	Molar
MARMS	=	Multiplex Amplification Refractory Mutation System

LIST OF ABBREVIATIONS (cont.)

mg	=	milligram
$MgCl_2$	=	Magnesium chloride
MCV	=	mean corpuscular volume
MCH	=	mean corpuscular hemoglobin
MCHC	=	mean corpuscular hemoglobin concentration
min	=	Minute
µl	=	microlitre
µM	=	micromolar
mM	=	millimolar
ml	=	millilitre
n	=	Nano (10^{-9})
NSTDA	=	National Science and Technology Development Agency
PCR	=	Polymerase chain reaction
Pg	=	picogram
Pos	=	Positive
Rbc	=	red blood cell
RDW	=	red cell distribution width
SD	=	standard deviation
sec	=	Second (s)
T	=	Thymine
TBE	=	Tris-borate-ethylene diamine tetraacetic acid
Tm	=	Melting temperature
U	=	Unit (s)
UV	=	Ultraviolet
V	=	Volt; voltage
Val	=	Valine
µ	=	Micro (10^{-6})
WBC	=	White blood cell

CHAPTER I
INTRODUCTION

Background and problems

More than 900 hemoglobin (Hb) variants have now been found worldwide, most of which are single point mutations, including α globin chains, β globin chains, γ globin chains, δ globin chains, C-terminal elongations and δ-β hybrid hemoglobin resulting from crossing-over. Several hemoglobinopathies are found in Thailand, most are rare and do not cause clinical symptoms. However, some of high prevalent Hb variants have clinical implications such as Hb E, Hb constant Spring or Hb Tak, either on their own or in association with thalassemia e.g. Hb E/β^o-thalassemia. Alkaline and acid hemoglobin electrophoresis are the conventional used methods for investigation hemoglobin variants. Hemoglobin fraction analysis by high performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) have the advantage of quantifying Hb F, A, A_2, E and other Hb variants. However, the definite identification of Hb variants can be achieved only by DNA analysis or amino acid sequencing. Because of in Hb variants screening methods (HPLC and CZE), many different Hb variants may be co-eluted in the same window (HPLC) or the same zone (CZE). In this study the results from both methods were compared with the databases of each method to predict the possible Hb variants in each case for further identification by DNA analysis. The standard procedure for molecular characterization of known mutation is southern blot analysis and amplification refractory mutation system (ARMS). Southern-blot hybridization of particular restriction enzyme digests to labeled complementary gene probes is typically used for the diagnosis of deletion/mutation causing Hb variants, however this technique is laborious, observer-dependent, and reported to have poor sensitivity. The amplification refractory mutation system (ARMS) is assay to detect point mutations in the present or absence of allele-specific primers, which is faster, safer (no radioactivity involved), and easier

to interpret than Southern blot analysis. But it usually detects one mutation per reaction, resulting of laborious and expensive for characterizing Hb variant in each location of variant chromatogram. Thus the MARMS-PCR (multiplex amplification refractory mutation system) assay have been developed to detection of Hb variants in each location of variant chromatogram. This technique could be amplified multiple DNA targets in one reaction by using more than one pair of primers that make the less expense of reagents and preparation time is less than others systems. The method is rapid and simple and can be performed in routine laboratory.

CHAPTER II
OBJECTIVE

Develop multiplex ARMS-PCR assays for identification and confirmation of hemoglobin variants found in Thailand

CHAPTER III
LITERATURE REVIEW

Human hemoglobin (Hb) is the iron containing oxygen-transport in red blood cells. Hb molecule is a tetrameric polypeptide comprised four subunits that are two alpha (α or ζ) and two non-alpha (ε, γ, δ or β) globin chain. (1, 2). In normal human adults the major hemoglobin component is Hb A ($\alpha_2\beta_2$) comprising about 97 %. A minor fraction accounting for about 2 % is Hb A_2 ($\alpha_2\delta_2$) Trace of Hb F ($\alpha 2\gamma 2$) or fetal hemoglobin (approximately 1 %) has still been found despite its predominance during fetal period (3). The genes controlling globin production are on chromosome 16 (alpha; α globin genes) and chromosome 11 (beta; β, gamma; γ, and delta; δ globin genes) (1-4). As seen in the Figure 3.1 (5), the alpha globin molecule concentration is rather stable and expressed until birth and after birth, because it is needed for both fetal and adult hemoglobin production. The beta globin molecule appears early in fetal life at low levels and begins to rapidly increase after 30 weeks gestational age and complete about 30 weeks postnatally. The transition from gamma globin synthesis to beta globin synthesis begins before birth. By approximately six months of age. The delta globin appears at a low level at about 30 weeks gestational age and maintains a low profile throughout life. In the thalassemia patient, a mutation or deletion of the genes that control globin production occurs. This leads to a decreased production of the corresponding globin chains and an abnormal hemoglobin ratio (α: non-α) (6-8). This abnormal ratio leads to decreased synthesis of hemoglobin and the expression of thalassemia. The globin that is produced in normal amounts winds up in excess and forms red cell aggregates or inclusions. These aggregates become oxidized and damage the cell membrane, leading either to hemolysis, ineffective erythropoiesis, or both (9, 10).

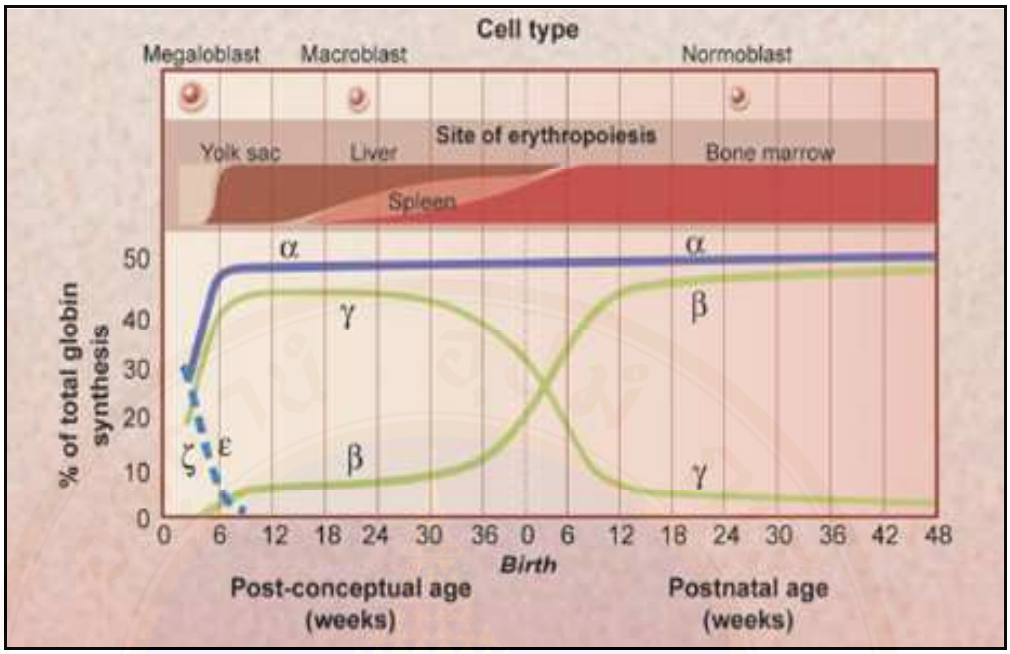

Figure 3.1 Gene expression of hemoglobin before and after birth. Also identifies the types of cells and organs in which the gene expression (5)

3.1 Hemoglobinopathy

Hemoglobinopathies or inherited disorders of Hb synthesis could involve either heme or globin parts of the molecule. However, very little is known about the genetic defects of heme synthesis (2, 11). Subsequently, the term hemoglobinopathy is usually restricted to the inherited disorders of the structure synthesis of globin (11). Hemoglobinopathy can be classified into two main groups as thalassemia which are genetic abnormalities causing the reduction or absence in globin chains synthesis and abnormal Hb or Hb variant in which the abnormal globin chains were produced (11, 12). Thailand has a high incidence of hemoglobinopathies, which include not only α-thalassemia, β-thalassemia, but also abnormal Hb or more precisely, "Hb variants" (11, 12).

3.1.1 Thalassemia

Thalassemia are a group of inherited autosomal recessive disorders because of the decreased or absent synthesis of a globin chain as a quantitative defect of globin chain production, lead to imbalanced globin chain synthesis, defective Hb production and damage to the red blood cells or their precursors(7, 13, 14). These defects lead to hereditary anemia with symptomatic varieties. Thalassemias are mainly classified, based on type of deficient globin chain into α-thalassemia and β-thalassemia.

3.1.1.1 Beta thalassemia

β-thalassemia syndromes are a group of hereditary disorders characterized by decreased or absent β globin chain synthesis. (15, 16). In the homozygous state, beta thalassemia (ie, thalassemia major) causes severe transfusion-dependent anemia. In the heterozygous state, the beta thalassemia trait (ie, thalassemia minor) causes mild-to-moderate microcytic anemia (17). In addition, hemoglobin (Hb) E, a common Hb variant found in Southeast Asia, is associated with a beta thalassemia phenotype, and this variant is included in the beta thalassemia category of diseases (14). Beta-thalassemia results in an excess of alpha globins that accumulate in the erythroblast (immature red blood cell). These aggregates are very insoluble and

precipitation interferes with erythropoiesis, cell maturation and cell membrane function, leading to ineffective erythropoiesis and anemia (14, 16, 18).

3.1.1.2 Alpha thalassemia

α-thalassemia is characterized by a reduced rate of α globin chain production, which is common hereditary anemia resulting from the deficient expression of the functional α1 and/or α2-globin genes. Although a small minority of cases involve point mutations of the α-globin genes, α-thalassemia is usually caused by deletions that remove one or both α-globin genes (16, 19-21). Individuals in whom one or two α-globin genes have been deleted or inactivated are mildly anemic, but otherwise healthy (α-thalassemia trait) (21). Loss of three α-globin genes results in an excess of beta globins, which leads to the formation of beta globin tetramers (β4) called Hb H (16).These tetramers are more stable and soluble, but under special circumstances can lead to hemolysis, generally shortening the life span of the red cell. Conditions of oxidant stress cause Hb H to precipitate, interfering with membrane function and leading to red cell breakage (19). Hb H-Constant Spring disease is a more severe form of this hemolytic disorder (22, 23). The most severe thalassemia is alpha thalassemia major, in which a fetus produces no alpha globins, which is generally incompatible with life (16).

3.1.2 Abnormal Hb or Hb variant

Hb variants are inherited disorders of the structural synthesis of globins cause alteration in the amino acids (2, 16). These changes may effect the structure of the hemoglobin, resulting in abnormal function such as oxygen transport or leads to hemolytic anemia (24). Some Hb variants are synthesized in reduced amount leading to thalassemia-like effect (8, 25). Most Hb variants themselves actually do not cause any abnormalities in function, since they occur at the surface of molecule (7). Some of high prevalent Hb variants have clinical implication, ether on their own or in association with thalassemia (25, 26). Many types of Hb variants have been found, depending on racial background (27, 28). The most common Hb variants worldwide are Hb S, Hb E, Hb C, Hb D. Approximately 8% of African-Americans descent carries mutation which produces Hb S (β6 (Glu>Val)), the incidence of the gene in some

African populations is as high as 40 %, the gene is also found with less frequency in non-Indo-European aboriginal peoples of India and in the middle East (29). Those with Hb S disease have two abnormal beta (β^S) chains and two normal alpha (α) chains. The presence of hemoglobin S causes the red blood cell to deform and assume a sickle shape when exposed to decreased amounts of oxygen such as might happen when someone exercises. Sickle red blood cells can block small blood vessels, causing pain and impaired circulation, decrease the oxygen-carrying capacity of the red blood cell, and decrease the cell's lifespan (29-31). A single β^S copy does not cause symptoms unless it is combined with another hemoglobin mutation (29-31).

In Thailand, since the discovery of Hb E in 1964, more than 30 different Hb variants have now been found (31, 68). Most are rare except for Hb E and Hb Constant Spring with frequency of 10- 53 % and 1-8 % respectively (31, 35). Most mutations are point mutations included point mutations in α chains and point mutations in β chains (2). But insertions, deletions, C-terminal elongations and unequal crossing-over with the production of fusion genes are also found (2). Some of them are shown in Table 3.1. The consequences of these genetic events depend on the type of amino acid substituted, the site of substitution or crossing-over, and the rate of production of the hemoglobin variant (69,70). In many cases the structural changes cause no disability (2, 31). In some cases they cause shortening of red cell survival by either altering the shape of the red cell or by causing precipitation of hemoglobin due to molecular instability (2, 24, 71). Because of their proximity to the hemepocket, produce permanent methemoglobinemia while others result in lack of heme/heme interaction and produce a high oxygen-affinity state which causes a genetically determined polycythemia (2, 24, 36, 71).

3.2 Genetic Classification of Hb variants in Thailand

3.2.1 Point mutations in α-globin gene

A point mutation changes only a single base by substitution of one nucleotide, loss of a nucleotide, or insertion of an additional nucleotide (2, 31). The point mutations include missense, nonsense, frameshift, and silent mutations. In Thai

individuals, Hb variants due to point mutations in α-globin genes were reported such as Hb Anantharaj, Hb siam, Hb Queens, Hb Mahidol or Hb Q-Thailand, Hb Constant Spring (Hb CS) and Hb Pakse′ (Hb PS) (52, 53, 72, 74-78).

Hb Anantharaj was characterized in 1974 as being the mutation in α11 (Lys>Glu) by S. Pootrakul (64) and the mutated amino acid was reexamined to be α11 (Lys>Gln) as Hb J-Wenchang-Wuming by J. Svati et al. in 1993 (53). Heterozygous Hb Anantharaj did not appear to produce any disturbance of hematological parameters (53).

Hb Siam or Hb Ottawa (α15 Gly>Arg) has been described in a Chinese male from Thailand by S. Pootrakul *et a*l. in 1974 (52). In previous reports, the hematological profile was normal in heterozygote (5, 46, 52) Hb typing by automated HPLC (VARIANT^{TM}; Bio-Rad Laboratories, Hercules, CA, USA) revealed unknown peak in S window (73).

Hb Queens also known as Hb Ogi (α34 Leu>Arg) has been reported in Oriental races including subject of Korean, Japanese, Chinese and Vietnamese (72, 74). The first report in Thailand was found in Thai male of Chinese ancestry by S. Yongsuwan et al. in 1987 (38). The hematological profile was normal in heterozygote and has a slightly increased oxygen affinity compared to Hb A (38, 66).

Hb Mahidol, also known as Hb Q-Thailand, Hb G-Taichung, Hb Kurashiki and Hb Asabara, was found mainly in China and South-East Asian countries (75). In Thai, it has been reported in 1970 by S. Pootrakul *et al*. (76). Hb Q-Thailand is resulting from a point mutation in codon 74 (Asp>His) of α1 globin gene occurs on a chromosome with the α2-globin gene deleted (4.2-kb deletion) (75). It has important implications in the identification because of the heterozygous form is associated with slight microcytosis due to linked α-thalassemia 2 determinant .Thus co-inheritance of Hb Q-Thailand with α-thalassemia 1 results in a thalassemia intermedia known as the Hb Q-H disease with similar clinical features of Hb H disease.

Hb CS and Hb Pakse′ are two abnormal Hbs resulting from point mutations at the stop codon of the α2-globin gene, TAA>CAA and TAA>TAT, respectively. These two mutant globin genes are the most prevalent nondeletional α-thalassemias among Southeast Asian populations. These mutations lead to the addition

of 31 amino acids to the normal α-globin sequence. The mRNAs of these two mutant genes are very unstable, and the rates of α-chain synthesis are reduced. Heterozygotes of these two variants are clinically and hematologically normal. Homozygotes show a clinical picture of thalassemia intermedia with mild anemia, jaundice, and hepatosplenomegaly. Interactions of them with α1-thalassemia lead to the Hb H disease, which is usually more severe than the Hb H disease caused by deletion of either α2 or α1 gene (61, 77, 78).

3.2.2 Point mutations in β-globin gene

A various mutation in β-globin gene reported in Thai individuals were Hb E, Hb C, Hb Siriraj, Hb J-Bangkok, Hb D-Punjab and Hb Hope (35, 63, 66, 79, 80-82, 84, 86).

Hb E (*β*26 Glu>Lys) is a variant Hb with a mutation causing substitution of glutamic acid for lysine at position 26 of the β globin chain (80). Hb E is the most common Hb variant in Thailand (80). Both heterozygotes and homozygotes are asymptomatic, minimally anemic, and have microcytic and hypochromic red blood cells (15, 34, 80, 82). Most of the problems with Hb E is found when it is associated with β-thalassemia, so that can cause mild to moderate and severe anemia (82).

Hb C (*β*6 Glu>Lys) is a Hb variant that causes mild hemolytic anemia in homozygotes but SC disease is a clinically significant such as having sporadic episodes of musculoskeletal (joint) pain (54, 83). Persons with hemoglobin C disease with continued hemolysis may produce pigmented gallstones, an unusual type of gallstone composed of the dark-colored contents of red blood cells (83, 84). The cause of pigmented gallstones is uncertain. Hemoglobin C comprises of two normal alpha chains and two variant beta chains in which lysine has replaced glutamic acid at position 6. This unstable hemoglobin precipitates in red blood cells to form crystals. These intracellular crystals lead to a decrease in red blood cell deformability and an increase in the viscosity of the blood (84, 85). The spleen effectively removes these crystal-containing cells. Much like the mechanism in sickle cell hemoglobin, the amino acid change in the hemoglobin C molecule impairs malaria growth and development (86). It reduces parasitemia and confers protection against mild malaria

attack. Therefore, persons who are heterozygous for Hb C have a survival advantage in endemic areas. The risk of malaria is lower still in persons who are homozygous for hemoglobin C (86). Hemoglobin C disease has a prevalence of 0.017% in African Americans and approximately 0.03 % in Northern Africa (87). The hematological changes are present of target cells in the peripheral smears, intraerythrocytic crystals in red blood cells of homozygotes and in the absence of other types of hemoglobin (54, 83, 84).

Hb Siriraj or Hb G-Siriraj (β7 Glu>Lys) was found in a few Thai and Chinese families(77). In Thailand, it has been reported by S. Tuchinda *et al*. in 1965 (63) In the heterozygous state has normal hematological parameters, with the amount of Hb variant of 33-40% (72). The variant has been found in combination with β-thalasemia and in homozygote. On HPLC Hb Siriraj is eluted in C window

Hb J-Bangkok (β56 Gly>Asp), also known as Hb J-Meinung, Hb J-Korat and Hb J-Manado, was found in Thai, Indonesian, Black and Chinese families (57, 88). The Hb variant quantity in heterozygote is approximately 40-45 % (73) and has normal hematological parameters (59). It also found in combination with Hb S (88).

Hb D-Punjab (β121 Glu>Gln), also known as Hb D Los Angeles, is primarily found in Northern India, especially in Punjab but also wide-spread (89). It is the fourth most frequently occurring Hb variants world wide (77). The amount of the variant is approximately 40% in heterozygote (72). It also found with Hb S, Hb C, Hb E, β-thalassemia, α-thalassemia and in the homozygous state (89). The hematological parameters are normal in heterozygotes and homozygotes (90).

Hb Hope (β136 Gly>Asp) was found in several Black families, Japanese, Thai, Loatain and Cuban families (36, 91). The variant amount of 40-50% has been found in heterozygote with normal hematological values (72, 91). Hb Hope has been found in combination with Hb S, Hb E, α-thalassemia 2 (3.7-kb deletion) and β-thalassemia (36, 42).Hb Hope is only hemoglobin variant eluted in P2 window on HPLC with the mean percentage of 45.9% (72).

3.2.3 Deletion

Gene deletion is a mutation in which a part of a chromosome or a sequence of DNA is missing. Deletions can be caused by errors in chromosomal crossover during meiosis. This causes several serious genetic diseases (2). In Thailand, Hb variant causes by gene deletion is Hb Khon Kaen (β123>125 deletion), which highly unstable thus cannot be distinguished by HPLC techniques (41).

3.2.4 Insertion and frameshift mutation

Insertion mutation is the addition of one or more nucleotide base pairs into a DNA sequence. Hb variant found in Thailand such as Hb Pak Nam Pho (α131/132 insertion) contains the additional 34 amino acids (49, 92). This Hb disorder, resulting in frame shift, gives rise to a highly unstable α-globin chain. This unusual α -globin variant clearly causes α thalassemia (92). Hb Tak is caused by an insertion and frameshift mutation of a dinucleotides AC after codon 146 that abolishes the normal stop codon at position 147. This insertion results in a frameshift causing elongation of the β-chain by 11 amino acids ((147) Thr-Lys-Leu-Ala-Phe-Leu-Leu-Ser-Asn-Phe-(157)Tyr-COOH). In heterozygote, the hematological values were normal and have 30-40 % of Hb Tak with normal or slightly high Hb level of 14-17 g/dl (47, 92, 93). The combination of Hb Tak/Hb E was not expressed as α-thalassemia but a mild polycythemia (37, 47, 26, 93). Hb Tak found in association with β-thalassemia is characteristically polycythemic or cyanotic condition such as congenital cyanotic heart disease which may be explained by the high oxygen affinity of the mutant hemoglobin (47, 93).

3.2.5 δ-β hybrid chain

An abnormal hemoglobin named Hb Lepore is resulted from a crossover between δ and β-globin gene loci (30, 33). Hb Lepore has a normal alpha chain combined with a non alpha chain that consists of the N-terminal residue of the delta chain fused with the C-terminal residue of the beta chain. Many different varieties of Hb Lepore have been described in which the transition from delta to beta amino acid sequences occurs at different points (56, 94, 95). Essentially the Lepore non-alpha

chain is a delta-beta fusion chain. Hb Lepore consists of 3 variants characterized by 3 different δ-to-β sequence transitions at the fusion junction (56, 94). Hb Lepore-Boston-Washington ($\delta^{aa1\text{-}87}\beta^{aa116\text{-}146}$) is the most common Hb Lepore type (56). Heterozygotes and homozygotes contain about 7-20 % of Hb variant with mild anemia, microcytosis, and hypochromia as in β^{+}-thalassemia (30, 33, 56) but Homozygotes state had severe anemia (95). Hb Lepore had elution peak in the A_2 window and the characteristic hump on the downward slope of the elution peak on HPLC and migrated in zone 6 on CZE, respectively (72, 94, 95).

Table 3.1 Hemoglobin variants in Thailand

Hb variants	%	Mutation	Hematology	refs
Hb Anantharaj (J-Wenchang-Wuming)	rare	α11 Lys>Gln	normal	Pootrakul *et al*, 1975(64) J. Svati *et al*, 1993(53)
Hb Siam (Hb Ottawa)	rare	α15 Gly>Arg	normal	Pootrakul *et al*,1974(52)
Hb Queens (Hb Ogi)	rare	α34 Leu>Arg	normal	Yongsuwan *et al*, 1987(38)
Hb Mahidol (Hb Q Thailand)	rare	α74 Asp>His, α1	Hb H ; α-thal1/Hb Mahidol	Pootrakul *et al*,1970(76)
Hb Siriraj	rare	β7 Glu>Lys	no anemia	Tuchinda *et al*,1965(63)
Hb Constant Spring	1-8 %	codon 142, TAA>CAA, α2	Hb H; α-thal1/Hb CS	Pongsamart *et al*,1975(96)
Hb Pakse	rare	codon 142, TAA>TAT, α2	Hb H; α-thal1/Hb PS	Fucharoen *et al*,2002(19)
Hb C	rare	β6 Glu>Lys	Target cells, Cystals in Rbc	Siriboon *et al*, 1993 (97)

Table 3.1 (cont.) Hemoglobin variants in Thailand

Hb variants	%	Mutation	Hematology	Refs
Hb E	10-53 %	β26 Glu>Lys	β-thalassemia/Hb E →thalassemia	Tuchinda *et al*,1964 (35)
Hb J Bangkok (J Meinung)	rare	β56 Gly>Asp	Normal	Pootrakul *et al*,1967 (98)
Hb D Punjab (D Los Angeles)	Rare	β121 Glu>Gln	Normal	Wasi *et al*,1968 (99)
Hb Hope	rare	β136 Gly>Asp	Normal	Pillers *et al*,1992 (100)
Hb Tak	rare	β147(+AC)	β thalassemia/HbTak, Polycythemia Hb Tak/Hb E, mild polycythemia	Flatz *et al*,1971 (93)
Hb Lepore Washington-Boston	rare	$\delta^{87}\beta^{116}$ fusion	β+-thal-like	Boontrakulpoontawee *et al*,1987(56)

3.3 Laboratory Diagnosis for Hb Variants

3.3.1 Hemoglobin typing

3.3.1.1 High Performance Liquid Chromatography (HPLC)

Automated HPLC analyzer, especially the Variant II, β-thalassemia short program (Bio-Rad, USA) has been widely used in Thailand for laboratory diagnosis of thalassemia and hemoglobin variants (73, 101-107). The samples are injected into the analysis stream. The separation principle based on the cation-exchange chromatography (73, 105). The cartridge consists of a phosphate ion gradient generated by mixing two buffers of different ionic strengths to elute the different hemoglobins (101, 106, 107). A dual wavelength filter photometer (415 and 690 nm) monitors the eluent from the cartridge and as it passes through the photometer cell, changes in optical density at 415 nm are measured. A secondary filter at 690 nm corrects the effects caused by mixing buffers of different ionic strengths. The data is processed and reported the chromatogram of time versus absorbance where each Hb is identified (due to its retention time) in a defined windows and their retention time to identify each hemoglobin type (Clinical Data Management software, Bio-Rad Laboratories) (Figure 3.2). For automated Variant II-HPLC, there are six “window” of A, F, A, D, S and C (Table 3.2). However the retention time can change with different lots of columns and reagents on the Variant system (73, 74, 101, 104, 106).

Table 3.2 Manufacturer-assigned windows for Bio-Rad, Variant II-HPLC system

Peak name	Retention time, min
P1 window	0.63-0.85
F window	0.98-1.20
P2 window	1.24-1.40
P3 window	1.40-1.90
A_0 window	1.90-3.10
A_2 window	3.30-3.90
D window	3.30-3.90
S window	3.90-4.30
C window	4.90-5.30

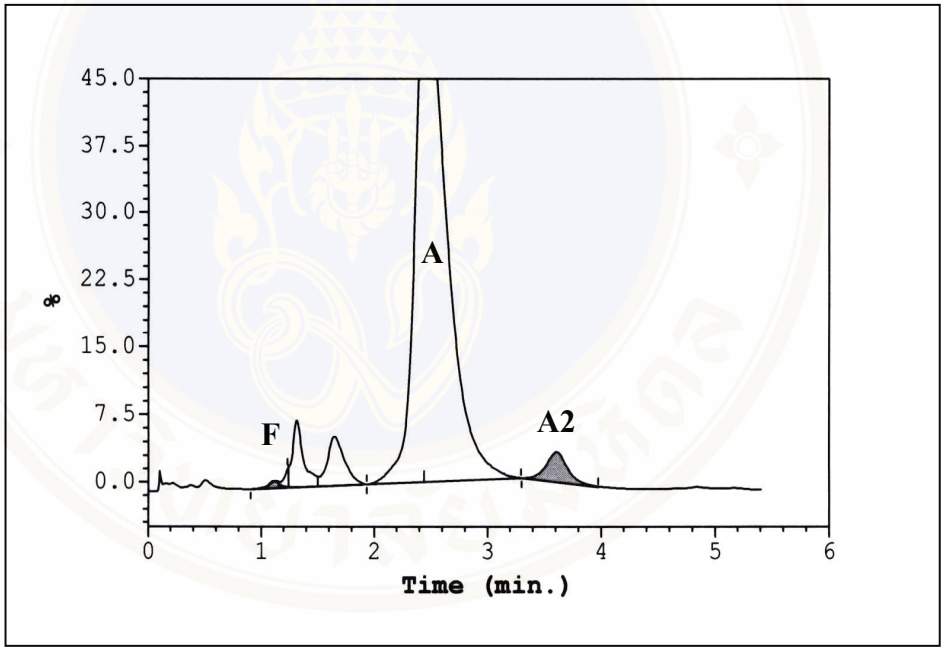

Figure 3.2 Chromatogram from Variant II-HPLC, β-thalassemia short program (Bio-Rad Laboratories, California, USA). Normal Hb typing shows Hb F, A and A_2 eluted in F window, A window and A_2 window, respectively.

3.3.1.2 Capillary electrophoresis (CE) or Capillary Zone Electrophoresis (CZE)

The recent technology of hemoglobin identification is automated capillary zone electrophoresis (CZE or CE) (99, 108). With this technique, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electro osmotic flow (EOF) (13, 109). A sample dilution is prepared with hemolysing solution and injected by aspiration at the anodic end of the capillary. A high voltage Hb separation is then performed and direct detection of the hemoglobin is made at 415 nm at the cathodic end of the capillary. The resulting electrophoregram is evaluated for Hb fraction and reported by dividing into 15 zones (Table 3.3 and Figure 3.3).

Table 3.3 Identification of zone in electrophroregram of Capillarys 2-CE system (Sebia Co., Ltd, France) (110)

zone	detection position (sec.)
Z 15	0-50
Z14	51-65
Z13	66-75
Z12	76-110
Z11	111-130
Z10	131-140
Z9	141-155
Z8	156-170
Z7	171-194
Z6	195-210
Z5	211-220
Z4	221-235
Z3	236-250
Z2	251-260
Z1	261-291

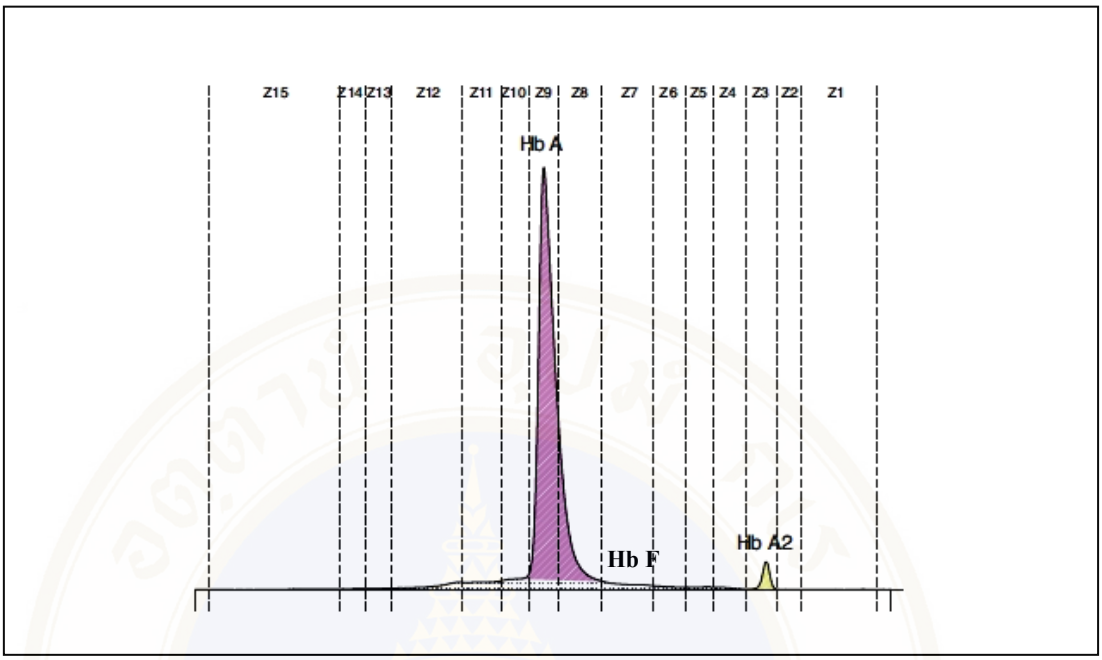

Figure 3.3 Electrophoregram from CE Capillarys 2-CE (Sebia Co., Ltd, France). Normal Hb typing shows Hb A, F and A_2 eluted in zone 9, 7 and 3, respectively.

3.4 DNA analysis for confirmation of Hb variants

However, by two Hb variants screening methods as HPLC and CE, many different Hb variants may be co-eluted in the same window on HPLC or the same zone on CE (108, 109). Thus, the definitive identification of Hb variants can be achieved only by DNA analysis of globin genes (2, 60).

DNA techniques have been used for definitive identification of Hb variants point mutation and small deletion detection (2, 12, 31, 111).

3.4.1 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR), invented by Kary B. Mullis, at the Cetus Corporation, who was awarded the 1993 Nobel Prize in chemistry (112). This technique allows a small amount of DNA to be amplified in vitro (112). The process is composed of cycles of the three following steps: perform heat denaturing to separate double-strand DNA target sequence into single strands, anneal each strand to the specific primers, and then extend the polymerase chain from the primer termini. Once there are enough of the DNA target sequences produced, further analysis can be performed. Gel electrophoresis is commonly done following the PCR to separate different DNA fragments. Many additional methods can be coupled with gel electrophoresis and PCR for detection of Hb variants such as direct DNA sequencing, the amplification refractory mutation system (ARMS-PCR), and single stranded conformation polymorphism (SSCP) (2, 31,112, 113).

In this research, the definitive identification of Hb variant was carried out by using multiplex amplification refractory mutation system polymerase chain reaction (MARMS-PCR) and then some of the positive PCR products were additionally confirmed by DNA sequencing at the research center of Ramathibodi hospital.

3.4.2 Multiplex amplification refractory mutation system polymerase chain reaction (MARMS-PCR)

The amplification refractory mutation system (ARMS-PCR), also known as allele specific PCR, is used to detect point mutations in the present or absence of

allele-specific primers (11, 12, 114). Gel electrophoresis is used to separate specific DNA bands. The Multiplex PCR was first described in 1988 by Chamberlain et al. The technique based on multiplex-PCR and ARMS which is named as MARMS (115). Basic principle of the multiplex PCR is a modification of polymerase chain reaction which enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers (11, 12, 116). More than one mutation can also be screened at the same time. Multiplex ARMS-PCR requires stringent optimization of primer annealing conditions and primer concentration (117). This technique has been applied to the diagnosis of β-thalassemia mutation in Taiwan and has been used for diagnosis of Hb variants (118).

3.4.3 Manual and automated DNA sequencing

An enzymatic DNA sequencing technique, known as dideoxynucleotide sequencing or the Sanger method (119). The use of 2',3'-dideoxynucleotide triphosphates (ddNTPs) molecules that differ from deoxynucleotides by having a hydrogen atom attached to the 3' carbon rather than an OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide. Double stranded DNA must be converted into single stranded DNA, by denaturing the double stranded DNA with NaOH, to be used as a template for in vitro synthesis by DNA polymerase (120). For manual sequencing, four reactions are set up, one for each of the four dideoxy chain terminators to be used. In addition, either the primer, used to start the reaction, or one of the normal deoxynucleotides is labeled with a radioactive atom or a fluorescent tag (120). The dideoxynucleotide is present at a concentration about 200-fold less than its competing nucleotide (120). There is therefore a competition between deoxynucleotides and dideoxynucleotides for incorporation into the growing chain leading to a statistical representation of lengths of DNA which correspond to the first 200-500 residues complementary to the template (120). Four separate reactions are run and these are loaded and their components separated within four separate lanes of a denaturing gel by electrophoresis. Labeled bands will appear at each location where the dideoxynucleotide brought that particular elongation reaction to a halt. Thus, the sequence can be read directly.

Similarly, an automated DNA sequencer is based on chain termination. The dideoxynucleosides are labeled with four different fluorescent dyes in a single tube and then the detection is performed with automated DNA sequencer (121).

CHAPTER IV
MATERIALS AND METHODS

4.1 Biological samples

4.1.1 Source of sample

Whole blood samples were collected in EDTA anticoagulant from routine laboratory service of Blood Disease Diagnostic Center, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. The total 59 peripheral blood samples with hemoglobin variants were collected from May 2005 to January 2009. The study protocol was approved by Committee on Human Rights Related to Researches Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University (MURA2009/1409)

4.2 Hematological analysis and Hb Identification

Hematological parameters were measured within a day after blood collection using a Bayer Advia120 (Bayer Diagnostic Division, Tarrytown, NY) automated blood cell counter. Hb identification was performed using an automated high performance liquid chromatography (HPLC) (Variant II, Bio-Rad Laboratories, California, USA) with β-thal short program. An automated Capillary Zone Electrophoresis (CE or CZE) (Capillarys 2, Sebia Co., Ltd, France) was used in comparative to the Variant II HPLC. The results from both methods were analyzed in combination as a guideline for fu rther identification by multiplex amplification refractory mutation system polymerase chain reaction (MARMS-PCR). The samples present an abnormal peak in Hb typing results either on Variant II HPLC or Capillarys 2-CE were further identified by MARMS-PCR and DNA sequencing. The normal Hb typing shows peaks of Hb F, Hb A, Hb A2 in F-window, A-window and A2-window, respectively on the Variant II HPLC, while on the Capillarys 2-CE, they migrate in

zone 7, zone 9 and zone 3, respectively (Figure 4.1). The samples with abnormal peak (s) were grouped according to the location related to Hb A on Variant II HPLC (Table 4.1). For group 1, Hb variant was eluted before Hb A on Variant II HPLC. The Hb variants in this group are possible to be Hb Hope, Hb J-Bangkok and Hb Anantharaj. Hb variants in group 2 were eluted after Hb A on Variant II HPLC, the Hb variants in this group are possible to be Hb Lepore, Hb D-Punjab, Hb Tak, Hb Siam, Hb Queens, Hb Q-Thailand, Hb C and Hb Siriraj. The retention time of abnormal peaks in group 3 were similar to group 2 but the samples were collected from the Middle East individuals. The Hb variants in this group are possible to be Hb S and Hb D-Punjab.

Table 4.1 Location of Hb variant peaks from Variant II HPLC and Capillarys 2-CE and groups of MARMS-PCR primers.

Location of Hb variant		Suspected Hb variants	MARMS-PCR Primers
(HPLC)	(CZE)		
P2, P3 window Between P2-P3	zone 10, zone 12	Hb Hope, Hb J-Bangkok, Hb Anantharaj	Group 1
A2-window, D-window, C-window, S-window	zone2, zone5, zone6, zone7	Hb Lepore, Hb D-Punjab, Hb C Hb Tak, Hb Siam, Hb Siriraj, Hb Queens, Hb Q-Thailand	Group 2
S-window, D-window	zone 5, zone6, between zone 5-6	Hb S, Hb D-Punjab [Middle East]	Group 3

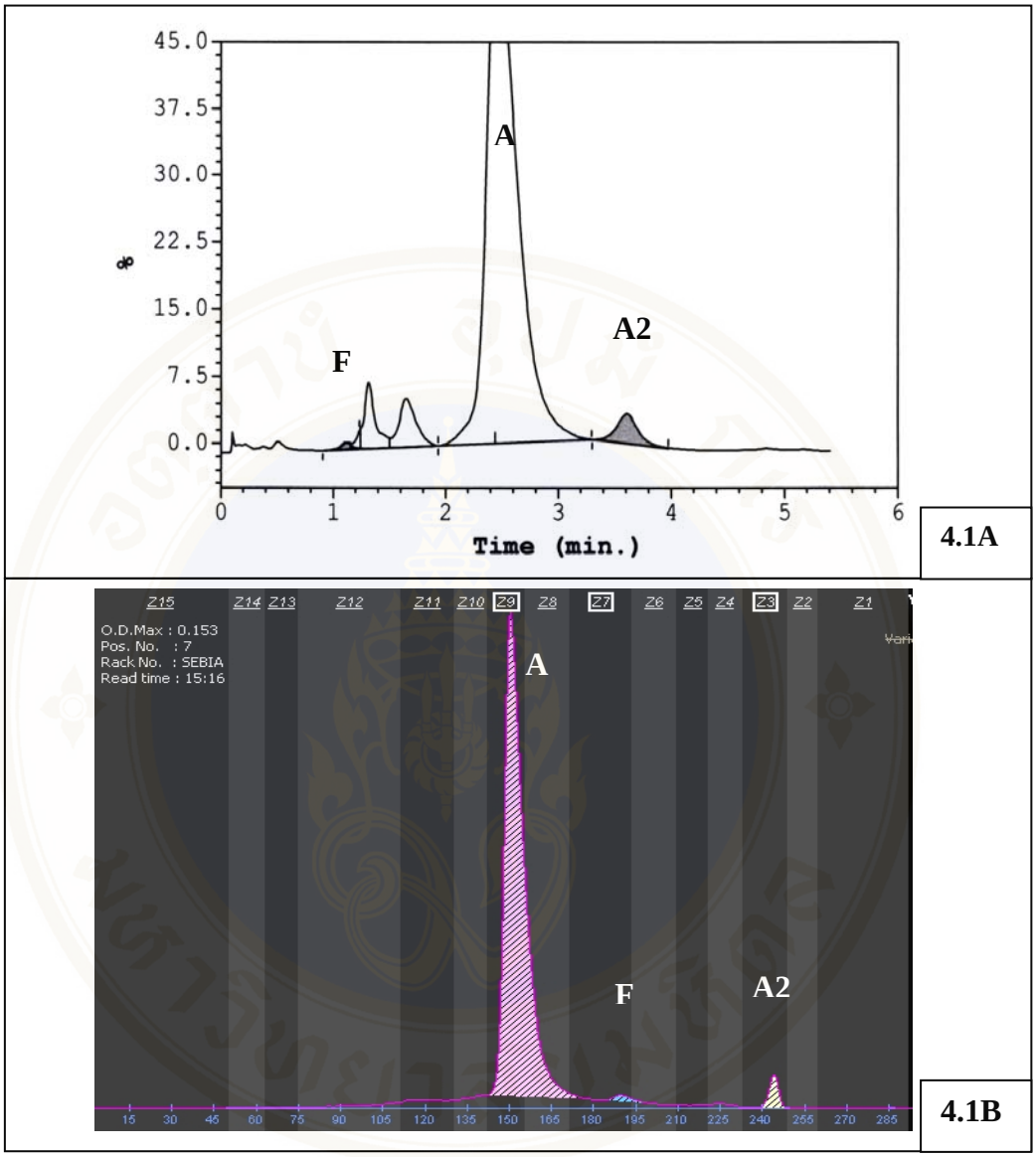

Figure 4.1 The chromatogram and electrophoregram from the Variant II HPLC (4.1A) and Capillarys 2-CE (4.1B) showed normal Hb typing. Hb F, Hb A and Hb A2 were eluted in the F window, A window and A2 window, respectively on the Variant II HPLC. While Hb A, Hb F and Hb A2 were migrated in zone 9, zone 7 and zone 3, respectively on the Capillarys 2-CE

4.3 DNA preparation

DNA was extracted from blood samples using Maxwell16® blood DNA purification kit and automated Maxwell16® instrument (Promega Corporation, USA) following the manufacturer protocol. The prepared DNA was kept in -30°C freezer for analysis.

4.4 Multiplex-PCR for α-thalassemia

All of the samples were examined for α-thlassemia genes including α-thal 1 deletions; i.e., SEA deletion ($--^{SEA}$), Thai deletion ($--^{Thai}$), FIL deletion ($--^{FIL}$), MED deletion ($--^{MED}$), 20.5 -kb deletion ($-\alpha^{20.5}$), α-thal 2 deletions; i.e., 3.7 -kb deletion ($-\alpha^{3.7}$), 4.2 -kb deletion ($-\alpha^{4.2}$) and non deletion α-thal 2; i.e., Hb CS and Hb PS.

4.5 Design of multiplex amplification refractory mutation system-polymerase chain reaction (MARMS-PCR) primers for hemoglobin variants

MARMS-PCR primers were designed for detection of various Hb variants using Bio Edit version 7.0.5 and Primer Express software (Applied Biosystems). The genomic sequences of β- and α-globin gene were obtained from the GenBank database under accession number NG_000007 and NG_000006, respectively. The primers were synthesized from the BioService Unit of the National Center for Genetic Engineering and Biotechnology (BIOTECH) and the National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. Three groups of primers were arranged according to the location of Hb variants' peaks present in Hb typing results. Forward and reverse primers of β-actin sequence were used as an internal control primers in each group as direct in Table 4.1. Group 1 primers were designed for detection of 3 Hb variants eluted before Hb A by Variant II HPLC. In this group, the suspected Hb variants found in Thais are Hb Hope (*β*136, Gly>Asp) and Hb J-

Bangkok (β56, Gly>Asp), which are the β-chain variants and Hb Anantharaj (α11, Lys>Gln) which is the α-chain variant. Figure 4.2 shows the locations of primers in group 1. Group 2 primers were designed for 8 suspected Hb variants eluted after Hb A by Variant II HPLC. They are possible to be 5 β-chain variants; Hb Lepore (δ-β hybrid chain), Hb D-Punjab (β121, Glu>Gln), Hb Tak (β147(+AC)), Hb C (β6,Glu>Lys) and Hb Siriraj (β7, Glu>Lys) and 3 α-chain variants; Hb Siam (α15, Gly>Arg), Hb Queens (α34, Leu>Arg) and Hb Q-Thailand (α74, Asp>His) The primers sequences and sizes of PCR products are indicated in Table 4.3. Figure 4.3A and 4.3 B shows the locations of primers in group 2. Group 3 primers are setting for Middle East individuals carried Hb variants with the location similar to Group 2. They are possible to be 2 β-chain variants; Hb S (β6, Glu>Val) and Hb D-Punjab (β121, Glu>Gln). Figure 4.4 shows the locations of the primers in group 3. The primers sequences and sizes of PCR products are indicated in Table 4.2-4.4.The grouping criterias depended on the locations of each Hb variant related to Hb A in the Variant II HPLC chromatogram, the previous reports of the each Hb variant found in Thai or Middle East population, and the databases from the Variant II HPLC and Capillarys 2-CE. MARMS-PCR are started from choosing primer sequence which length of 18-24 bp and a GC content of 35-60 % thus having an annealing temperature of 55-58^{o}C or higher. To calculate the melting point and test to possible primer-primer interaction, “Primer Express” was used.

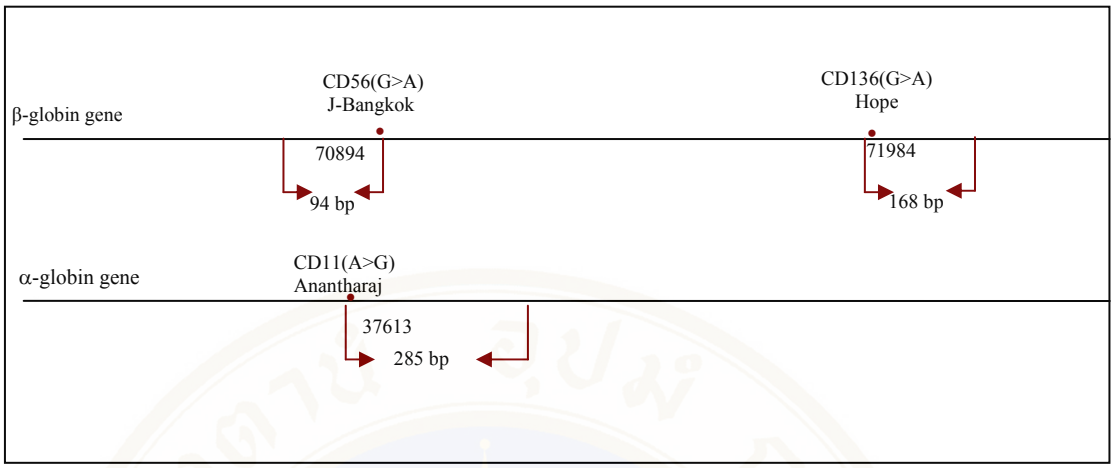

Figure 4.2 Locations of group 1 primers including of Hb J-Bangkok and Hb Hope as the β-chain variants and Hb Anantharaj as α-chain variant.

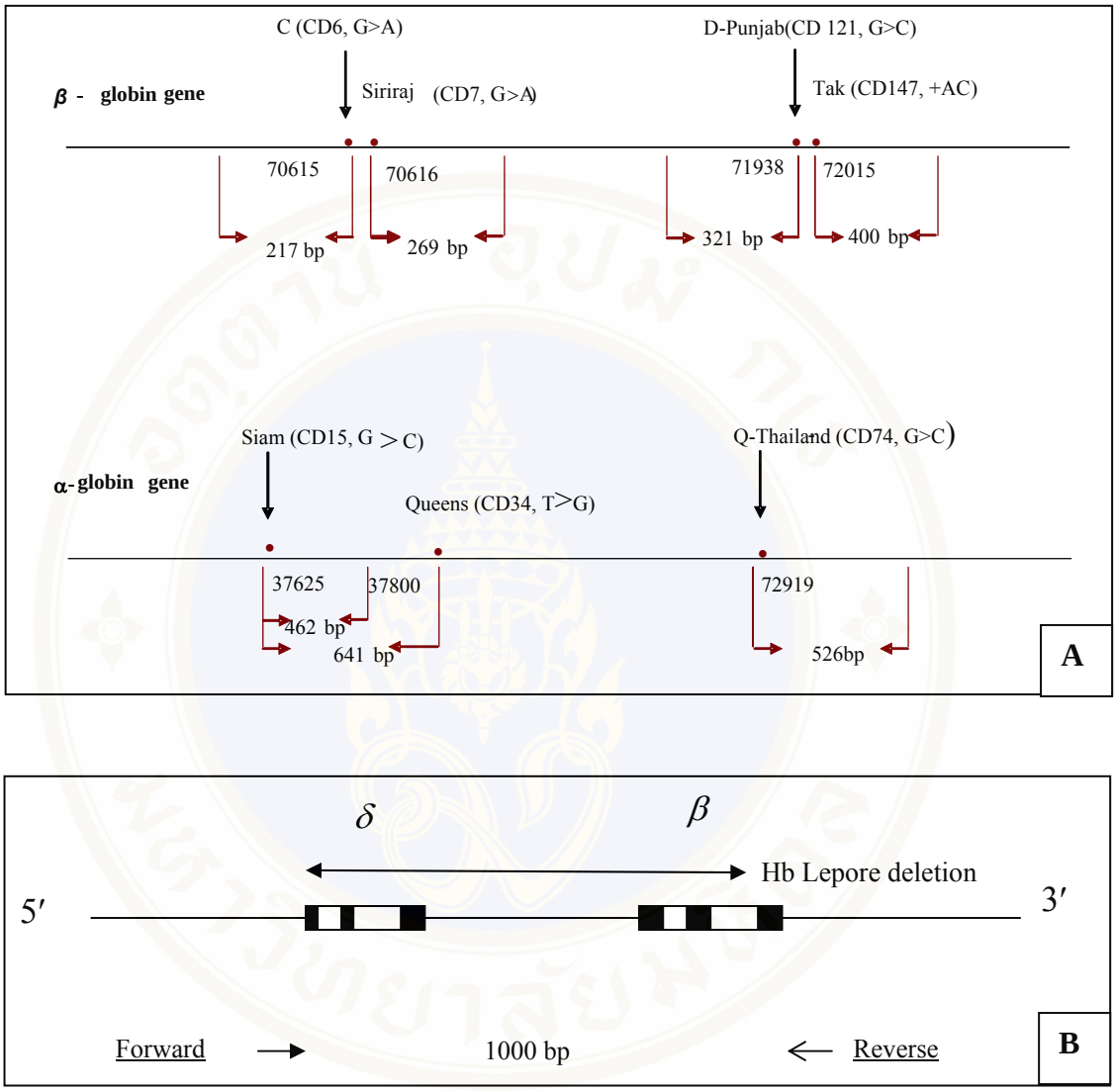

Figure 4.3 Locations of group 2 primers including of 5 β-chain variants; Hb C, Hb Siriraj, Hb D-Punjab, Hb Tak and 3 α-chain variant; Hb Siam, Hb Queens and Hb Q-Thailand (A) and Hb Lepore (B)

Figure 4.4 Locations of group 3 primers including of 2 β-chain variant; Hb S and Hb D-Punjab.

Table 4.2 Oligonucleotide sequences of group 1 primers and sizes of PCR products (Hb variants eluted before Hb A by Variant II HPLC)

Specific for Hb variants	Oligonucleotide Sequences (5'-3')	Accession no. (5'-3')	Sequence length (bp)	Tm (^{o}C)	%GC	PCR product (bp)
Internal control-F	CTAACACTGGCTCGTGGACAAG	NG_007992 (1671-1693)	23	58.6	52	751
Internal control-R	CATGCCTGAGAGGGAAATGAG	NG_007992 (2421-2401)	21	58.3	52	
J-Bangkok -F	TGGTGGTCTACCCTTGGACC	NG_000007 (70822-70841)	20	58.3	60	94
J-Bangkok -R	TGAGCCTTCACCTTAGGGTTGT	NG_000007 (70915-70894)	22	58.8	50	
Hope -F	CCTATCAGAAAGTGGTGGCTGAT	NG_000007 (71963-71985)	23	58.3	48	168
Hope -R	TTATTAGGCAGAATCCAGATGCTC	NG_000007 (72130-72107)	24	57.7	42	
Anantharaj -F	CCGACAAGACCAACGTCGA	NG_000006 (37596-37614)	19	59.2	58	285
Anantharaj -R	TCTTGCCGTGGCCCTTAAC	NG_000006 (37880-37862)	19	59.7	58	

Table 4.3 Oligonucleotide sequences of group 2 primers and sizes of PCR products (Hb variants eluted after Hb A by Variant II HPLC)

Specific for Hb variants	Oligonucleotide Sequences (5'-3')	Accession no. (5'-3')	Sequence length (bp)	Tm (^{o}C)	%GC	product (bp)
Hb Siriraj-F	ATGGTGCATCTGACTCCTGAGA	NG_000007 70595-70616	22	58.4	50	269
Hb Siriraj-R	CAAAGGACTCAAAGAACCTCTGG	NG_000007 70863-70841	23	58.1	48	
Hb Tak-F	CCTGGCCCACAAGTATCACAC	NG_000007 71997-72015	21	58.9	57	400
Hb Tak-R	ACTTTAACCTCCAAATCAAGCCTC	NG_000007 72394-72371	24	58	42	
Hb Lepore-F	GGCAAAGAAGAACTTTATATTGAGTCA	NG_000007 62734-62760	27	57.5	33	1000
Hb Lepore-R	CATTCGTCTGTTTCCCATTCTAAAC	NG_000007 71153-71130	25	59.0	40	
Hb C-F	GGCTGTCATCACTTAGACCTCACC	NG_000007 70420-70443	24	60	54	217
Hb C-R	GGCAGTAACGGCAGACTTCTCTT	NG_000007 70636 – 70614	23	59.9	52	

Table 4.3 (cont.) Oligonucleotide sequences of group 2 primers and sizes of PCR products (Hb variants eluted after Hb A by Variant II HPLC)

Specific for Hb variants	Oligonucleotide Sequences (5'-3')	Accession no. (5'-3')	Sequence length (bp)	Tm (^{o}C)	%GC	product (bp)
Hb D-Punjab-F	GTATCATGCCTCTTTGCACCATT	NG_000007 71636-71658	23	59	43	321
Hb D-Punjab-R	GCACTGGTGGGGTGAATTGT	NG_000007 71956-71937	20	58.3	55	
Hb Siam-F	GACTTTCTCCCTCGCTAGGGA	NG_000006 37177-37197	21	58.5	57	462
Hb Siam-R	GCGCCGACCTTACGCC	NG_000006 37638-37623	16	59.9	75	
Hb Queens-F	GACTTTCTCCCTCGCTAGGGA	NG_000006 37177-37197	21	58.5	57	641
Hb Queens-R	TGGTGGTGGGGAAGGACC	NG_000006 37817-37800	18	60.1	67	
Hb Q-Thailand -F	CGTGGCGCACGTGCAC	NG_000006 37906-37921	16	61.8	75	526
Hb Q-Thailand -R	ATGCCTGGCACGTTTGCT	NG_000006 38431-38414	18	58.8	56	

Table 4.4 Oligonucleotide sequences of group 3 primers and sizes of PCR products (Hb variants eluted after Hb A by Variant II HPLC , Middle East samples)

Specific for Hb variants	Oligonucleotide Sequences (5'-3')	Accession no. (5'-3')	Sequence length (bp)	Tm (^{o}C)	%GC	PCR product (bp)
Internal control-F	CTAACACTGGCTCGTGGACAAG	NG_007992 (1671-1693)	23	58.6	52	751
Internal control-R	CATGCCTGAGAGGGAAATGAG	NG_007992 (2421-2401)	21	58.3	52	
HbS-F	GGCTGTCATCACTTAGACCTCAC	NG_000007 (70420-70443)	24	60	54	215
HbS-R	CAGTAACGGCAGACTTCTCCAC	NG_000007 (70634-70613)	22	57.6	55	
D-Punjab-F	GTATCATGCCTCTTTGCACCATT	NG_000007 (71636-71658)	23	59	43	321
D-Punjab-R	GCACTGGTGGGGTGAATTGT	NG_000007 (71956-71937)	20	58.3	55	

4.6 Establishment of the MARMS-PCR assays for detection of Hb variants

Due to each primer group can have different requirements, so it is essential that all aspects of the procedures are optimized, particularly for multiple reactions where several primer sets are used. The following conditions require optimization in each group of primers to maximize product yields. The concentration of each primer is used at 0.2-1 μM and Taq DNA polymerase concentration are usually used at 1-1.5 units. The dNTP is used at the final concentration of 200 μM but some application can be accomplished using much lower concentration. The $MgCl_2$ concentration is used in the range of 1.5-2 mM. Cycle numbers for amplification are generally used at 30-40 cycles and the annealing temperature is ±5 ^{o}C from the melting temperature. These criteria need to be optimized for successful PCR.

The DNA samples were grouped according to the location of Hb variants in Hb typing results. The samples with Hb variants eluted before Hb A were identified using group 1 MARMS-PCR protocol. Those eluted after Hb A were identified using group 2 or group 3 protocols according to their races are Thai or Middle East individuals, respectively. The unidentified samples were submitted for DNA sequencing analysis.

4.6.1 Interpretation of the MARMS-PCR

The assay was considered satisfactory for interpretation when the 751-bp PCR product of internal control gene was present, the positive controls were positive, and the negative control showed no amplified product. A band was considered positive when it was in the expected size range.

4.6.2 Multiplex ARMS-PCR group 1 for detection of Hb variants eluted before Hb A by Variant HPLC

Group 1 primers were designed for detection of Hb Hope, Hb J-Bangkok and Hb Anantharaj as shown in Table 4.5 and 4.6.

Multiplex ARMS-PCR was performed using Veriti™ 96-Well Thermal cycler (Applied Biosystems, USA). The PCR reaction was carried out in a 30 µl reaction mixture of 2.5 µl DNA template, 1x PCR buffer, 1 units Go taq® Flexi DNA polymerase (Promega Corporation, USA), 0.67 mM $MgCl_2$, 0.2 mM dNTP. Internal control and Hb variant-specific primers were added to the reaction mixture in the concentration shown in Table 4.5. The optimal condition used is initial denaturation for 5 min at 94°C, follow by 30 cycles of 30s at 94°C, 45s at 62°C, 1 min at 72°C and final extension for 7 min at 72°C.

The amplified products were electrophoresed in 2-3 % agarose gel at 100 V for 35 min and stained with ethidium bromide and visualized with ChemiDoc™ XRS System (Bio-Rad Laboratories Ltd., USA). Then some of the positive PCR products were additionally confirmed by DNA sequencing.

Table 4.5 The optimal condition of MARMS-PCR group 1 for detection of Hb variants eluted before Hb A by Variant II HPLC

	Volume (μl)	Final concentration
5xbuffer	6	1x
25 mM $MgCl_2$	0.8	0.67 mM
1 mM dNTPs	6	0.2 mM
Taq	0.2	1 U
10 μM Primer: Internal control-F	1.4	0.47 μM
Internal control-R	1.4	0.47 μM
Hb Hope-F	1.6	0.53 μM
Hb Hope-R	1.6	0.53 μM
Hb J-Bangkok-F	0.9	0.3 μM
Hb J-Bangkok-R	0.9	0.3 μM
Hb Anantharaj-F	0.6	0.2 μM
Hb Anantharaj-R	0.6	0.2 μM
DNA sample	2.5	0.05 μg
ddH_2O	5.5	
Total volume	30	

Table 4.6 Protocol of MARMS-PCR group 1 for detection of Hb variants eluted before Hb A by Variant II HPLC

Protocol	temperature	time	cycle
Initial denaturation	94°C	5 min	
denaturation	94°C	30s	30 cycles
Annealing	62°C	45s	
Extension	72°C	1 min	
Final extension	72°C	7 min	

Separate on 2-3 % agarose gel at 100 V for 35 min.

4.6.3 Multiplex ARMS-PCR group 2 for detection of Hb variants eluted after Hb A by Variant II HPLC

Group 2 primers were designed for detection of Hb Lepore, Hb Q-Thailand, Hb Tak, Hb D-Punjab Hb C, Hb Siriraj, Hb Siam and Hb Queens as shown in Table 4.7 and 4.8.

Multiplex ARMS-PCR was performed using Veriti^{TM} 96-Well Thermal cycler (Applied Biosystems, USA). The PCR reaction was carried out in a 30 µl reaction mixture of 2.5 µl DNA template, 1x PCR buffer, 0.75 units Go taq® Flexi DNA polymerase (Promega Corporation, USA), 0.67 mM $MgCl_2$, 0.13 mM dNTP. Internal control and Hb variant-specific primers were added to the reaction mixture in the concentration shown in Figure 4.6. The optimal condition used is initial denaturation for 5 min at 94^{o}C, follow by 30 cycles of 30s at 94^{o}C, 45s at 62^{o}C, 1 min at 72^{o}C and final extension for 7 min at 72^{o}C.

The amplified products were electrophoresed in 2-3 % agarose gel at 100 V for 35 min and stained with ethidium bromide and visualized with ChemiDoc^{TM} XRS System (Bio-Rad Laboratories Ltd., USA). Then some of the positive PCR products were additionally confirmed by DNA sequencing.

Table 4.7 The optimal condition of MARMS-PCR group 2 for detection of Hb variants eluted after Hb A by Variant II HPLC

	Volume (µl)	Final concentration
5xbuffer	6	1x
25 mM $MgCl_2$	0.8	0.67 mM
1 mM dNTPs	4	0.13 mM
Taq	0.15	0.75 U
10 µM Primer: Hb Lepore-F	3	1 µM
Hb Lepore-R	3	1 µM
Hb Q-Thailand-F	2	0.67 µM
Hb Q-Thailand-R	2	0.67 µM
Hb Tak-F	1.3	0.43 µM
Hb Tak-R	1.3	0.43 µM
Hb D-Punjab-F	1.5	0.5 µM
Hb D-Punjab-R	1.5	0.5 µM
Hb C-F	0.7	0.23 µM
Hb C-R	0.7	0.23 µM
Hb Siriraj-F	0.6	0.23 µM
Hb Siriraj-R	0.6	0.23 µM
Hb Siam-F	0.6	0.23 µM
Hb Siam-R	0.6	0.23 µM
Hb Queens-F	0.6	0.23 µM
Hb Queens-R	0.6	0.23 µM
DNA sample	2.5	0.05 µg
ddH_2O	3.5	
Total volume	30	

Table 4.8 Protocol of MARMS-PCR group 2 for detection of Hb variants eluted after Hb A by Variant II HPLC

Protocol	temperature	time	cycle
Initial denaturation	94°C	5 min	
denaturation	94°C	30s	30 cycles
Annealing	62°C	45s	
Extension	72°C	1 min	
Final extension	72°C	7 min	

Separate on 2-3 % agarose gel at 100 V for 35 min.

4.6.4 Multiplex ARMS-PCR group 3 for detection of Hb variants eluted after Hb A by Variant II HPLC (Middle East individual)

Group 3 primers were designed for detection of Hb S and Hb D-Punjab as shown in Table 4.4. The PCR reaction was carried out in a 30 µl reaction mixture of 2.5 µl DNA template, 1x PCR buffer, 1 units Go taq® Flexi DNA polymerase (Promega Corporation, USA), 0.67 mM $MgCl_2$, 0.2 mM dNTP. Hb variant-specific primers were added to the reaction mixture in the concentration shown in Table 4.9 and 4.10.

Multiplex ARMS-PCR was performed using thermal cycler (Eppendorf, Germany) under the following conditions: Denaturation at 94°C for 5 min, followed by 30 amplification cycles consisting of denaturation at 94°C for 45 sec, primer annealing at 62°C for 45 sec and followed by extension at 72°C for 1 min, and a final extension at 72°C for 7 min. After amplification, the amplified products were electrophoresed in a 2-3 % agarose gel at 100 V for 35 min and stained with ethidium bromide and visualized with ChemiDoc™ XRS System (Bio-Rad Laboratories Ltd., USA). Then some of the positive PCR products were additionally confirmed by DNA sequencing.

Figure 4.8 is a summarized flowchart of analytical process for detection of Hb variants in this thesis.

Table 4.9 The optimal condition of MARMS-PCR group 3 for detection of Hb variants eluted after Hb A by Variant II HPLC (Middle East individual)

	Volume (μl)	Final concentration
5xbuffer	6	1x
25 mM $MgCl_2$	0.8	0.67 mM
1 nM dNTPs	6	0.2 mM
Taq	0.2	1 U
10 μM Primer: Internal control-F	1.4	0.47 μM
Internal control-R	1.4	0.47 μM
Hb S-F	1	0.33 μM
Hb S-R	1	0.33 μM
Hb D-Punjab-F	1.4	0.47 μM
Hb D-Punjab-R	1.4	0.47 μM
DNA sample	2.5	0.05 μg
ddH_2O	6.95	
Total volume	30	

Table 4.10 Protocol of MARMS-PCR group 3 for detection of Hb variants eluted after Hb A by Variant II HPLC (Middle East individual)

Protocol	temperature	time	cycle
Initial denaturation	94°C	5 min	
denaturation	94°C	30s	30 cycles
Annealing	62°C	45s	
Extension	72°C	1 min	
Final extension	72°C	7 min	

Separate on 2-3 % agarose gel at 100 V for 35 min.

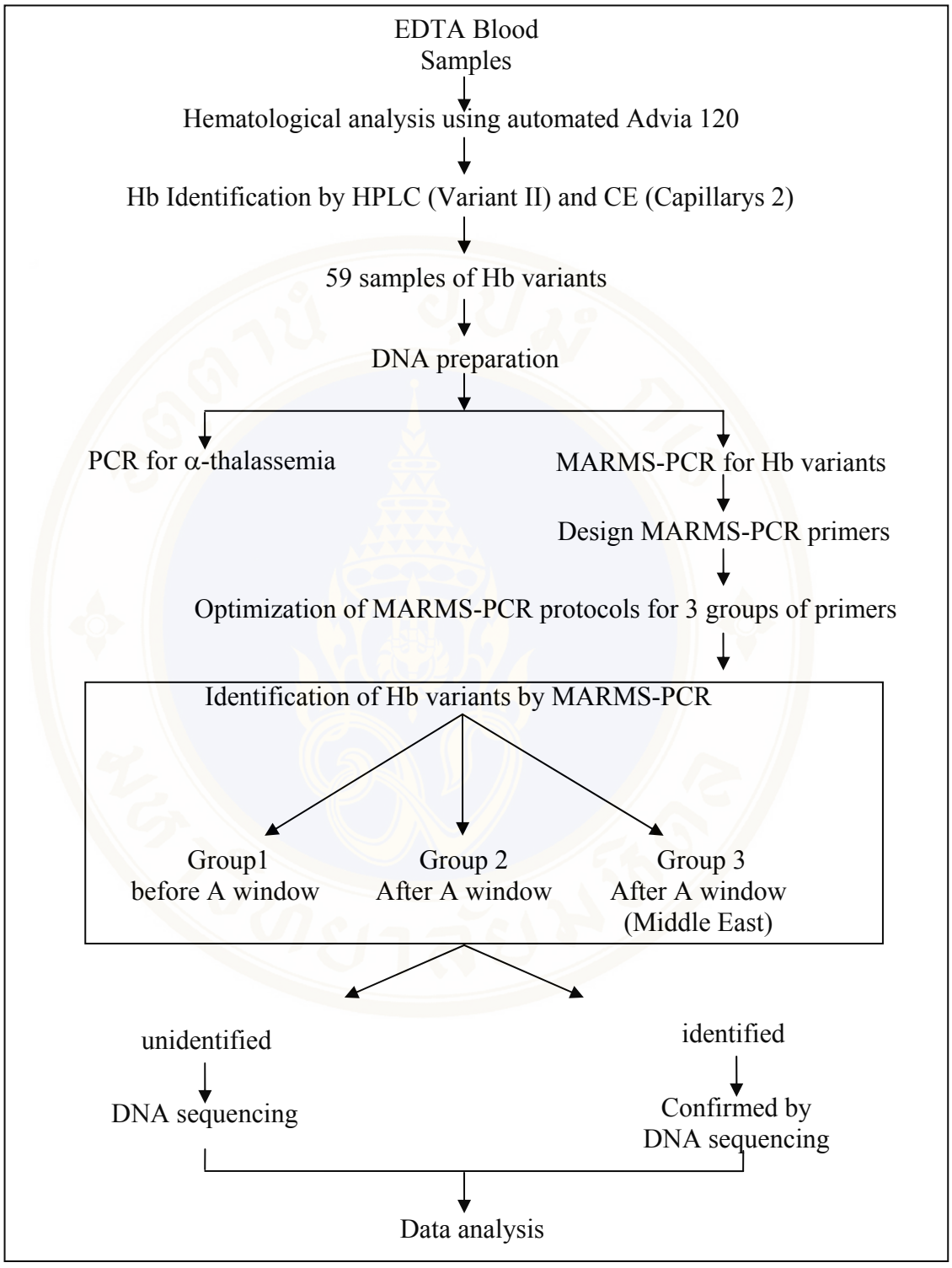

Figure 4.5 Analytical process for detection of Hb variants

CHAPTER V
RESULTS

The hematological parameters were measured in all whole blood samples using automated cell counter (Advia 120, Bayer Diagnostic Division, Tarrytown, NY) and Hb typing was performed using High performance liquid chromatography (HPLC) (Variant II, β-thalassemia short program, Bio-Rad Laboratories, California, USA) and Capillary Zone Electrophoresis (CZE or CE) (Capillarys 2, Sebia Co., Ltd, France). A total of 59 samples of Hb variants were collected and divided into three groups according to the results of Hb typing as indicated in the materials and methods. These Hb variants were identified by three groups of multiplex amplification refractory mutation system polymerase chain reaction (MARMS-PCR)**.** Each type of Hb variant and unidentified one was analyzed by DNA sequencing.

5.1 Multiplex ARMS-PCR

Multiplex amplification refractory mutation system polymerase chain reaction (MARMS-PCR) testing can detect Hb variants by allele specific primers for PCR reaction. The 3′ terminal nucleotide in the primer is either complementary to the normal sequence or the mutant sequence at a particular position. MARMS-PCR was tested on 59 Hb variants samples. We were successfully optimized PCR conditions for amplifications of twelve Hb variants, which were divided into 3 groups according to the variants location related to Hb A. Primer concentrations for each mutation were adjusted in the reaction in such a way that there was no possibility of obtaining false-positive results.

5.1.1 MARMS-PCR of Group 1 Hb variants

The primers of MAMRS-PCR group1 were designed for detection of Hb variants which eluted before Hb A from Variants II HPLC. These included Hb Hope (β136, Gly>Asp), Hb J-Bangkok (β56, Gly>Asp) which are the β-chain variants and Hb Anantharaj (α11, Lys>Gln) which is the α-chain variant. The specific primers for these variants and internal control were simultaneously amplified in a single tube, thereby producing the PCR products of 285, 168 and 94 bp for Hb Anantharaj, Hb Hope and Hb J-Bangkok, respectively and 751 bp for the internal control (Figure 5.1). Two Hb variants, Hb Hope and Hb J-Bangkok, were found in this study. No sample was positive for Hb Anantharaj.

In this study, we found Hb Hope in 12 samples, including the cases of heterozygous Hb Hope, heterozygous Hb Hope/heterozygous α- thalassemia 2 (3.7-kb deletion), heterozygous Hb Hope/homozygous α-thalassemia 2 (3.7-kb deletion) and heterozygous Hb Hope/heterozygous α-thalassemia 1 (--SEA deletion). From retrospectively analyzed the data, Hb Hope was eluted in P2 window on the Variant II HPLC and migrated in zone 10 and in between zone 10 and zone 11 on the Capillarys 2-CE (Figure 5.2).

In addition, we found heterozygous Hb J-Bangkok in 3 samples. Hb J-Bangkok was found eluted at the retention time before A0 window on the Variant II HPLC and migrated in zone 12 on the automated Capillarys 2-CE.

The specific mutations of Hb Hope and Hb J-Bangkok were confirmed by DNA sequencing method (Figure 5.3).

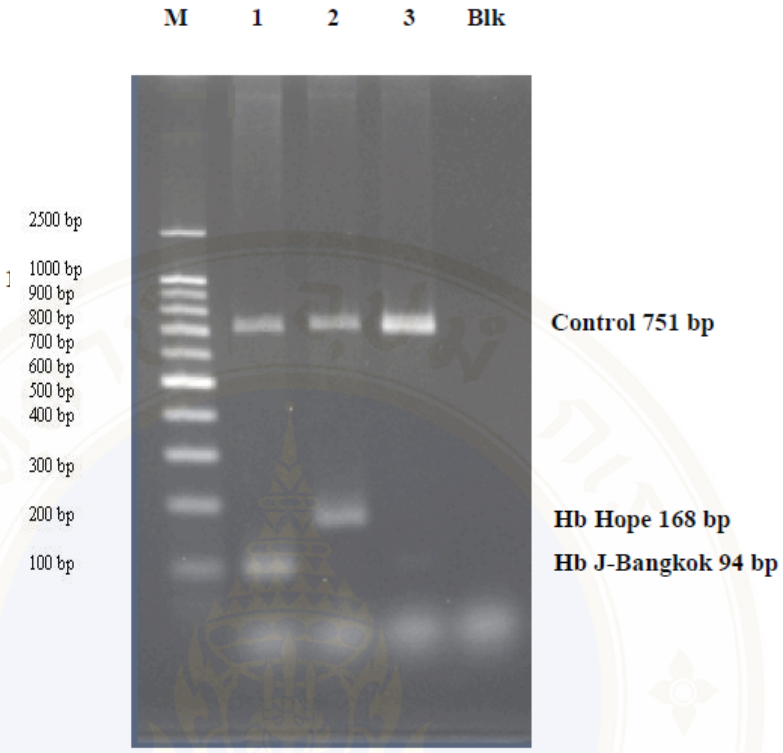

Figure 5.1 Agarose gel electrophoresis of amplified PCR products of MARMS-PCR group 1. Two different Hb variants were amplified from primer specific to mutations. The 94 bp fragment is Hb J-Bangkok and 168 bp fragment is Hb Hope. The 751 bp fragment is internal control. Lane 1 and 2 represent the PCR products from the samples of Hb J-Bangkok and Hb Hope, respectively. Lane 3 is a normal control case. M is a 100-2500 bp DNA ladder marker (Promega, USA).

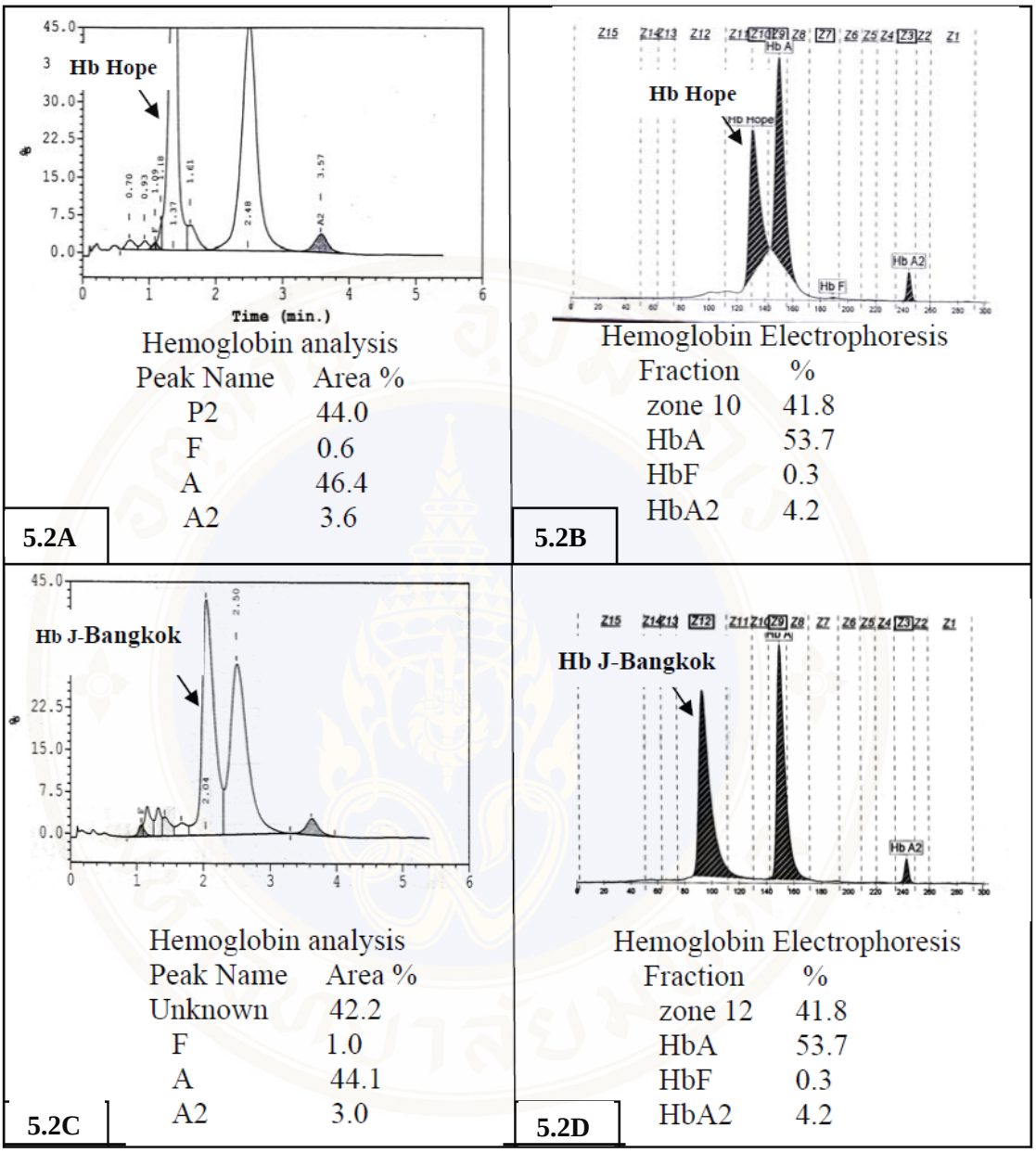

Figure 5.2 Hb identification of Hb Hope and Hb J-Bangkok. (5.2A) and (5.2B) are Hb Hope detected by the Variant II HPLC and the Capillarys 2-CE, respectively. Hb Hope is eluted in P2 window on Variant II HPLC and migrated in zone 10 and in between zone 10-11 on the Capillarys 2-CE. (5.2C) and (5.2D) are Hb J-Bangkok detected by the Variant II HPLC and the Capillarys 2-CE, respectively. Hb J-Bangkok is eluted before A0 window and migrated in zone 12 on the Capillarys 2 CE. Each Hb variant was pointed by an arrow

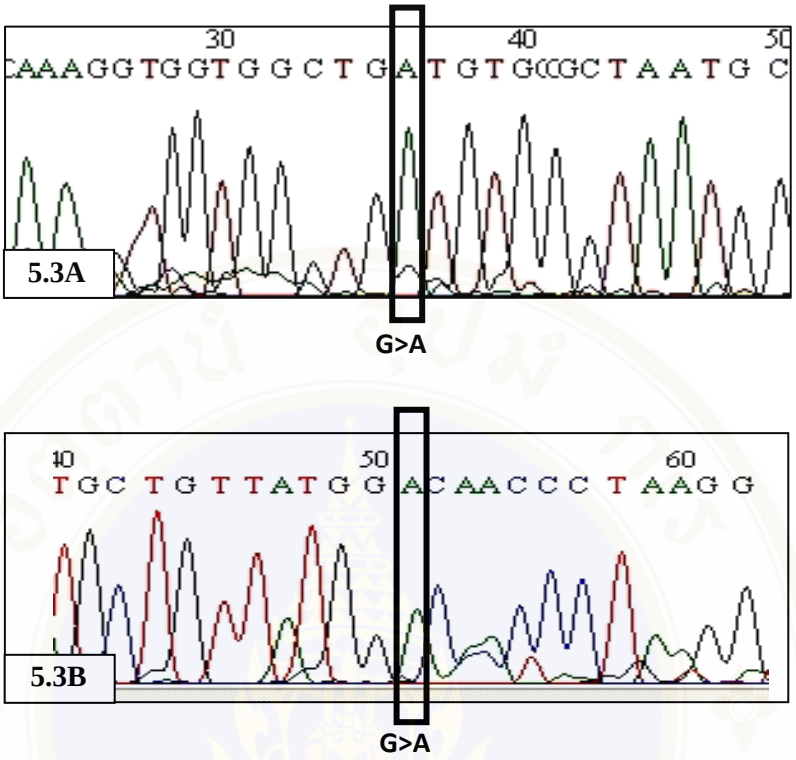

Figure 5.3 DNA sequencing of group 1 Hb variants. The cases positive for Hb Hope and Hb J-Bangkok by MARMS-PCR show G>A mutation at codon 136 for Hb Hope (5.3A) and G>A mutation at codon 56 for Hb J-Bangkok (5.3B).

5.1.2 MARMS-PCR of group 2 Hb variants

In group 2 MARMS-PCR, specific primers for 5 β-globin chain variants; i.e. Hb Lepore, Hb Tak, Hb D-Punjab, Hb C, Hb Siriraj and 3 α-globin chain variants; i.e. Hb Siam, Hb Queens, Hb Q-Thailand, were used for a single PCR reaction. The specific PCR products of Hb Lepore, Hb Queens, Hb Q-Thailand, Hb Siam, Hb Tak, Hb D-Punjab, Hb Siriraj and Hb C were 1000, 642, 526, 462, 400, 321, 269, 217 bp, respectively. The internal control of 758 bp produced from forward primer of Hb D-Punjab and reverse primer of Hb Tak (Figure 5.4)

From 26 samples in this study, we found 5 different Hb variants, 4 samples for Hb Lepore, 6 samples for Hb Q-Thailand, 7 samples for Hb Tak, 2 samples for Hb D-Punjab and 7 samples for Hb C. The others Hb variants in this group such as Hb Queens, Hb Siam and Hb Siriraj were not found in the present study. From retrospectively analyzed the data, Hb Lepore-Washington-Boston was eluted in A2 window on the Variant II HPLC and migrated in zone 6 on the Capillarys 2-CE, Hb Q-Thailand and Hb Tak were eluted in S window and migrated in zone 7, Hb D-Punjab was eluted in D window and migrated in zone 6, Hb C was eluted in C window and migrated in zone 2 (Figure 5.5 and 5.6).

The specific mutations of these Hb variants were confirmed by DNA sequencing method. as shown in Figure 5.7.

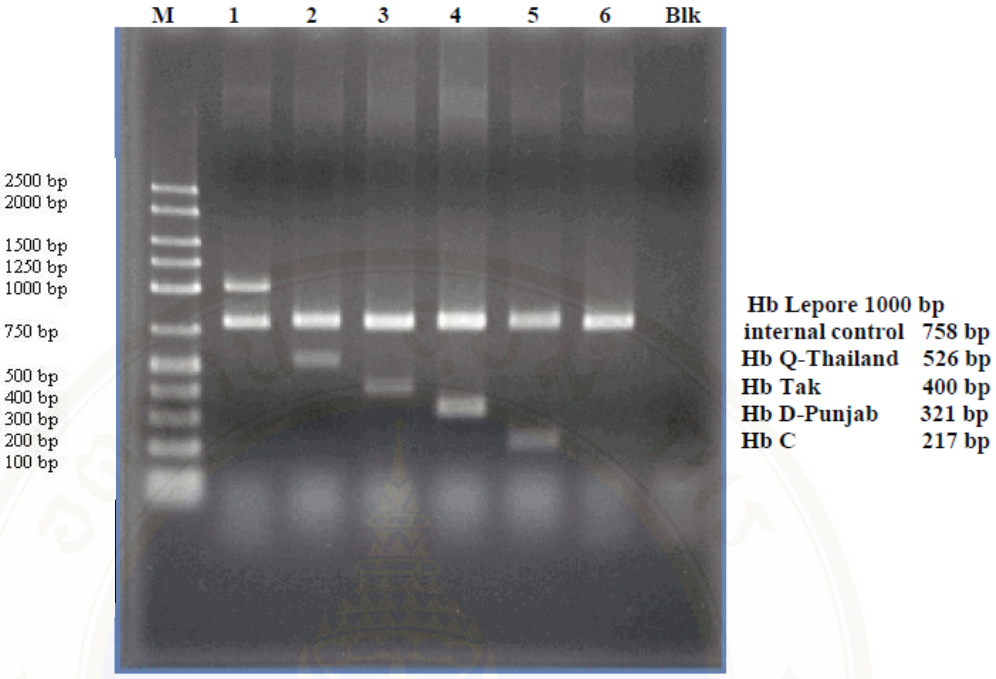

Figure 5.4 Agarose gel electrophoresis of amplified PCR products of MARMS-PCR group 2. Eight different Hb variants were amplified from primer specific to mutations. The PCR product of 1000 bp is Hb Lepore, 526 bp is Hb Q-Thailand, 400 bp is Hb Tak, 321 bp is Hb D-Punjab and 217 bp is Hb C. The 758 bp fragment is the internal control produced from forward primers of Hb D-Punjab and reverse primer of Hb Tak. Lane 1: Hb Lepore; lane 2: Hb Q-Thailand; lane 3: Hb Tak; lane 4: Hb D-Punjab; lane 5: Hb C and lane 6: normal control. M is a 100-2500 bp DNA ladder marker (Promega, USA).

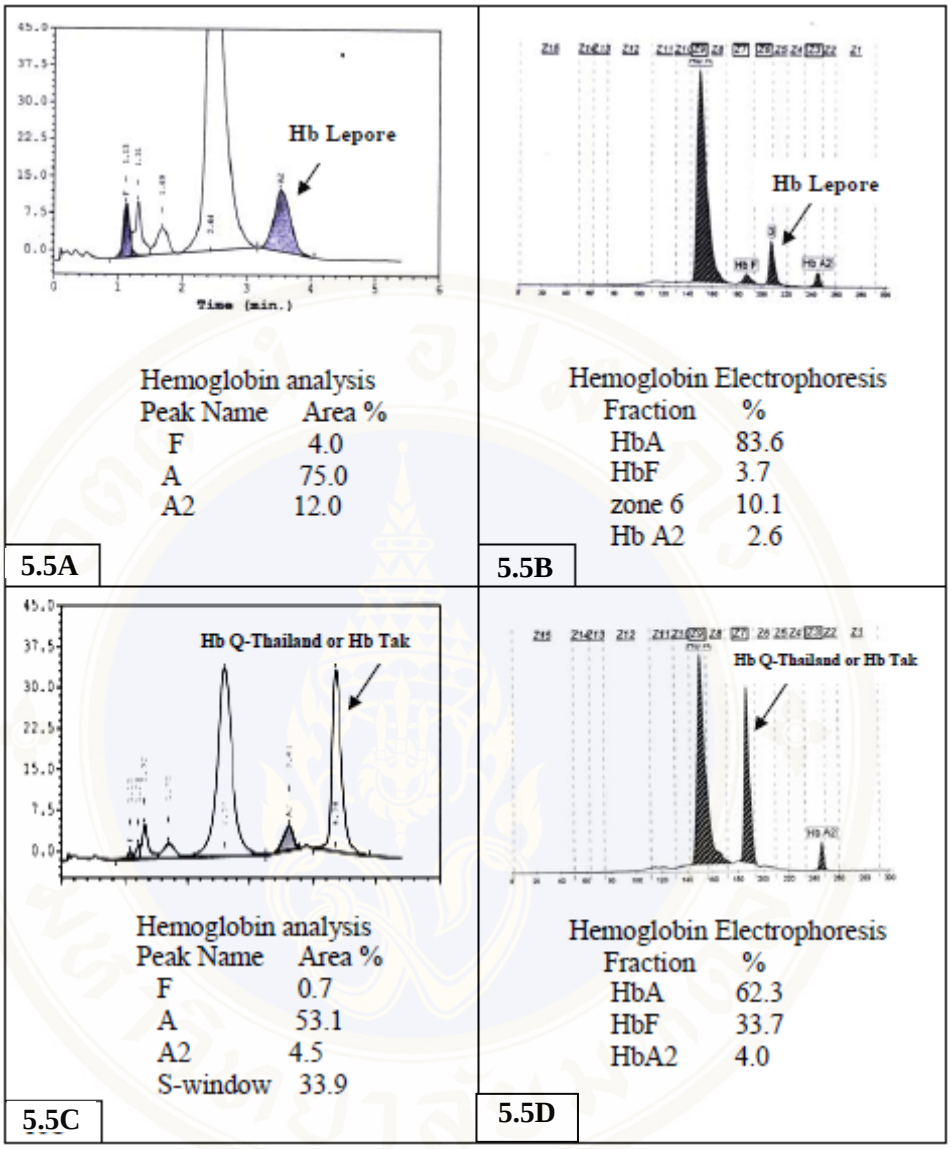

Figure 5.5 (5.5A) and (5.5B) are Hb Lepore detected by the Variant II HPLC and Capillarys 2-CE, respectively. Hb Lepore is eluted in A2 window on Variant II HPLC and migrated in zone 6 on the Capillarys 2-CE. (5.5C) and (5.5D) are Hb Q-Thailand and Hb Tak detected by the Variant II HPLC and the Capillarys 2-CE, respectively. Hb Q-Thailand and Hb Tak are eluted in S window on Variant II HPLC and co-migrated with Hb F in zone 7 on the Capillarys 2-CE.

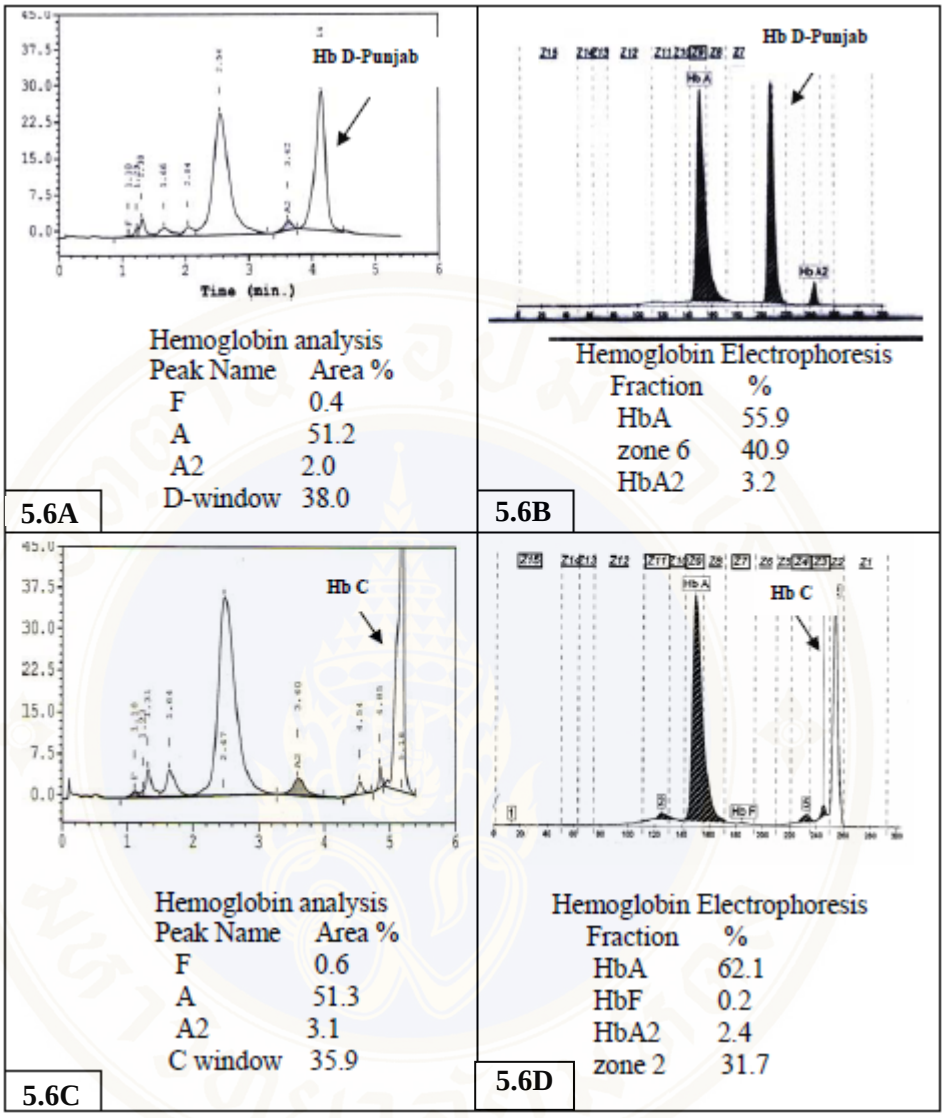

Figure 5.6 (5.6A) and (5.6B) are Hb D-Punjab detected by the Variant II HPLC and the Capillarys 2-CE, respectively. Hb D-Punjab is eluted in D window on Variant II HPLC and migrated in zone 6 on the Capillarys 2-CE. (5.6C) and (5.6D) are Hb C detected by the Variant II HPLC and the Capillarys 2-CE, respectively. Hb C is eluted in C window by the Variant II HPLC and migrated in zone 2 on the Capillarys 2-CE.

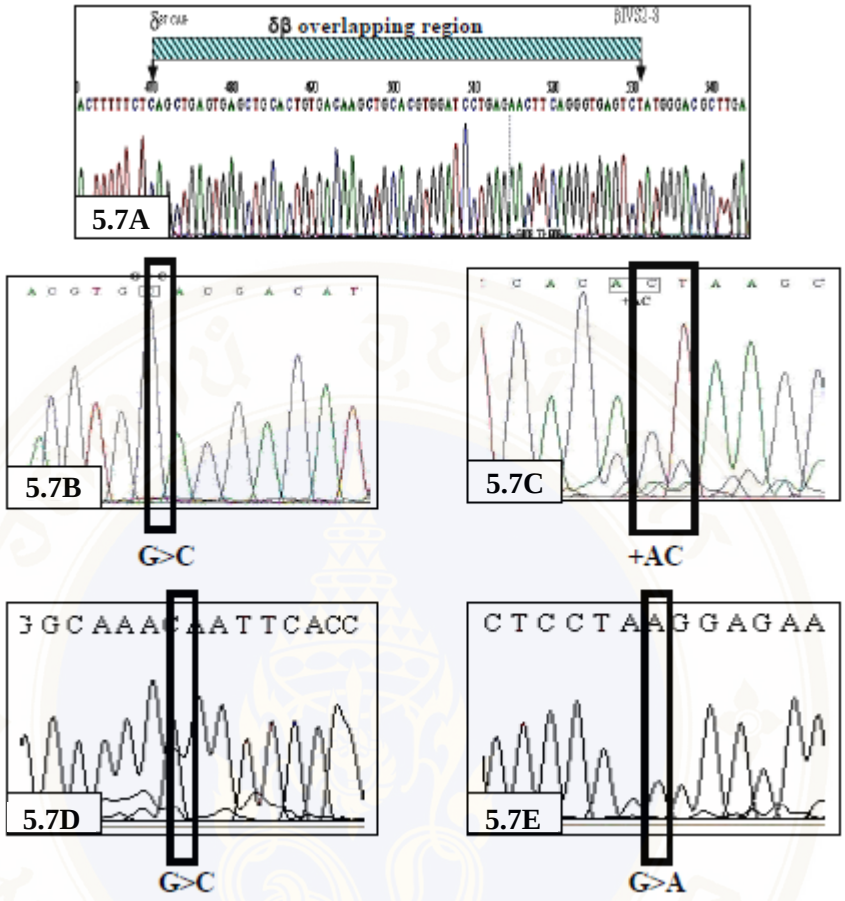

Figure 5.7 DNA sequencing of group 2 Hb variants. The cases positive for each variants by MARMS-PCR show specific mutations. (5.7A) *δβ* overlapping of Hb Lepore-Washington-Boston (*δ*87*β*IVS-II-8 hybrid) (shading box), (5.7B) the G>C mutation at α74 of α-globin gene in the case of Hb Q-Thailand, (5.7C) the addition of AC at β146 in the case of Hb Tak, (5.7D) the G>C mutation at *β*121 in the case of Hb D-Punjab and (5.7E) the G>A at *β*6 of β-globin chain in the case of Hb C.

5.1.3 MARMS-PCR of group 3 Hb variants

The primers in group 3 were designed for the most common β-globin chain variants of Middle East individual, Hb S (*β*6, A>T) and Hb D-Punjab (*β*121, G>C) eluted in S Window and D Window on Variant II HPLC. The specific PCR products of Hb S and Hb D-Punjab were 215 and 321 bp, respectively. The internal control fragment was 751 bp. (Figure5.8).

Hb S was eluted in the S-window on Variant II HPLC and migrated in zone 5 on Capillarys 2-CE (Figure 5.9). Hb D-Punjab was eluted in D window on Variant II HPLC and migrated in zone 6 on the Capillarys 2-CE (Table 4.1).

The specific mutations of Hb S and Hb D-Punjab were confirmed by DNA sequencing (Figure 5.7D and 5.10)

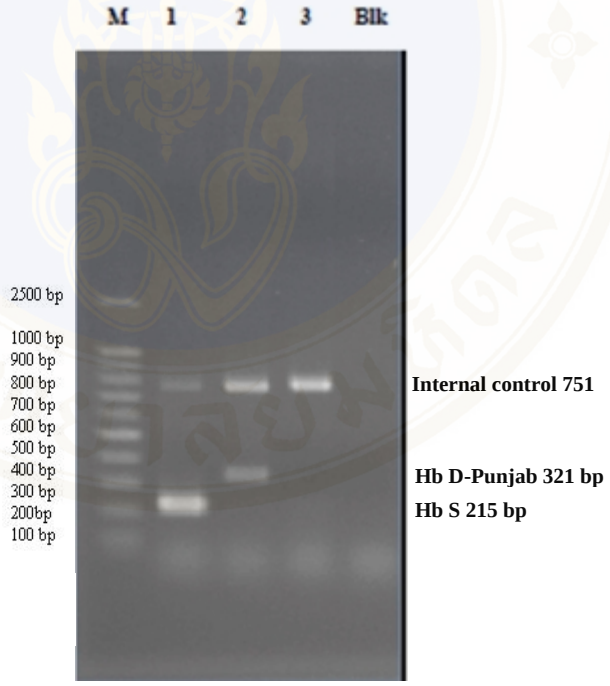

Figure 5.8 Agarose gel electrophoresis of amplified PCR products of MARMS-PCR group 3. Two different Hb variants were amplified from primer specific to mutations. The PCR product of 321 bp is Hb D-Punjab and 215 bp is Hb S. The 751 bp fragment is the internal control. Lane 1: Hb S; lane 2: Hb D-Punjab and lane 3: normal control. M is a 100-2500 bp DNA ladder marker (Promega, USA).

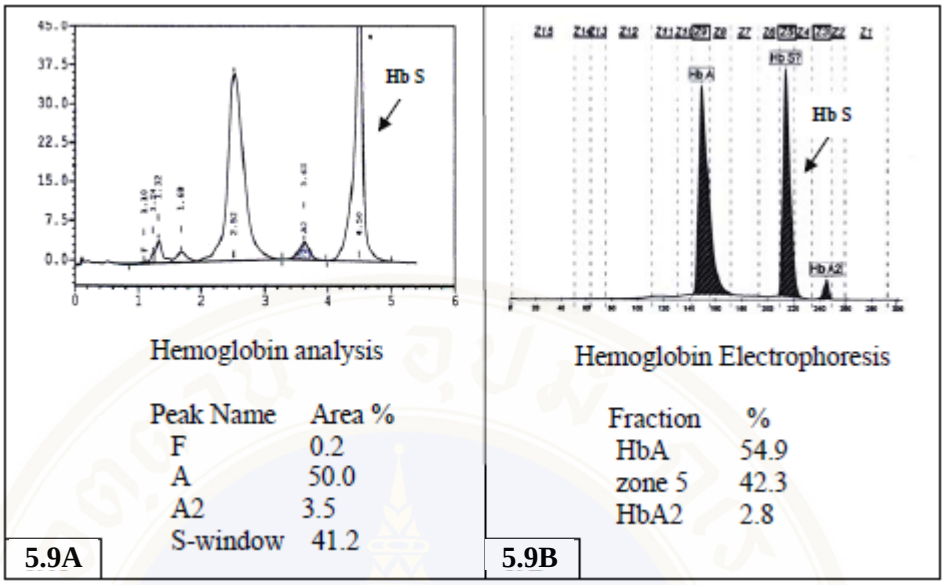

Figure 5.9 Hb S eluted in S-window on Variant II HPLC (5.9A) and migrated in zone 5 on the Capillarys 2-CE (5.9B).

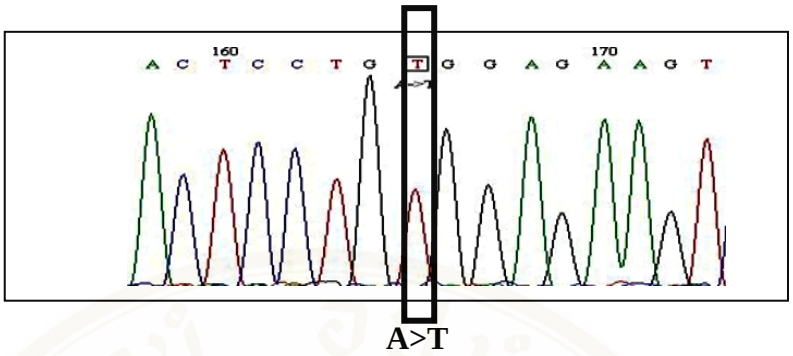

Figure 5.10 DNA sequencing of group 3 Hb variants. The cases positive for Hb S by MARMS-PCR show A>T mutation at β6 in β-globin gene

5.2 MARMS-PCR of α-thalassemia

The MARM-PCR of α-thalassemia includ five α-thal 1 deletions (SEA deletion ($--^{SEA}$), Thai deletion ($--^{Thai}$), FIL deletion ($--^{FIL}$), MED deletion ($--^{MED}$), 20.5 -kb deletion ($-\alpha^{20.5}$), two common α-thal 2 deletions (3.7 -kb deletion ($-\alpha^{3.7}$), 4.2 -kb deletion ($-\alpha^{4.2}$) and two non deletion α-thal 2 (Hb CS and Hb PS). In MARMS-PCR group 1 of Hb variant detection we found Hb Hope with various forms of α-globin genotype including one case of heterozygous Hb Hope/heterozygous α-thalassemia 2 (3.7-kb deletion), two cases of heterozygous Hb Hope/homozygous α-thalassemia 2 (3.7-kb deletion) and two cases of heterozygous Hb Hope/heterozygous α-thalassemia 1 (--SEA deletion). MARMS-PCR group 2 of Hb variant detection revealed one case of heterozygous Hb Lepore/ heterozygous α-thalassemia 1 (--SEA deletion), five cases of heterozygous Hb Q-Thailand/heterozygous α-thalassemia 2 (4.2-kb deletion), and one case of each combination of heterozygous Hb Q-Thailand coexistent with compound heterozygous α-thalassemia 2 (3.7-kb and 4.2-kb deletion), heterozygous Hb Tak/heterozygous Hb Constant Spring, heterozygous Hb C/ heterozygous α-thalassemia 2 (3.7-kb deletion), adult with Hb C/ heterozygous α-thalassemia 1 (--SEA deletion), new born with heterozygous Hb C/ heterozygous α-thalassemia 1 (--SEA deletion). In MARMS-PCR group 3 of Hb variant, we found Hb S with various forms of α-globin genotype including four cases of Hb S/heterozygous α-thalassemia 2 (3.7-kb deletion), five cases of heterozygous Hb S/homozygous α-thalassemia 2 (3.7-kb deletion) and homozygous Hb S coexistent with heterozygous α-thalassemia 2 (3.7-kb deletion). All of these results are summarized in Table 5.1-5.16.

5.3 Hematological Analysis and Hb Identification

Hematological parameters, Rbc, Hb, Hct, MCV, MCH, MCHC and RDW were measured by an automated cell counter, Bayer Advia 120 (Bayer Diagnostic Division, Tarrytown, NY). Among these parameters, MCV and MCH are most importance ones that can indicate the existence of thalassemia trait, i.e., when MCV<80 fL and MCH< 27pg. Hematological analysis of Hb Hope coinheritance with various α-globin genotype are summarized in Table 5.1. All of them were carriers and showed mild to moderate anemia. From retrospectively analyzed data, Hb Hope was eluted in P2 window on the Variant II HPLC ranging between 31.0-48.58 % and migrated in zone 10 and in between zone 10 and zone 11 on the Capillarys 2-CE ranging between 31.0-43.61 % with increased Hb A2 values (3.4-4.7 %) (Table 5.2). Hematological values of heterozygous Hb J-Bangkok were in the normal ranges (Table 5.3). Hb J-Bangkok eluted at the retention time before A0 window on the Variant II HPLC ranging between 41.4-43.6 % and migrated in zone 12 on the automated Capillarys 2-CE with the amount of 50.6 % (Table 5.4). In the cases of Hb Lepore-Washington-Boston heterozygote coexistent with heterozygous α-thalassemia (SEA deletion), they showed normal hematological values and eluted in A2 window on Variant II HPLC ranging between 11.1-13.3 % and migrated in zone 6 on Capillarys 2-CE ranging between 9.4-10.7 %. A case of Hb Lepore-Washington-Boston /Hb E showed hypochromic microcytic anemia (Table5.5). The variant eluted in A2 window on Variant II HPLC with the amount of 37.4 % and increased Hb F values (28.8 %). It migrated in zone 6 on Capillarys 2-CE with the variant levels of 4.3 %, increased Hb F values (31.3%), and 28.3 % of Hb E (Table 5.6). Hb Q-Thailand coexistent with heterozygous α-thalassemia 2 (4.2-kb deletion) showed normal hematological parameters. On Variant II HPLC, the variant level were 28.5-30.5 % in S window, however, it co-migrated with Hb F in zone 7 on Capillarys 2-CE in the level of 28.5-28.6 %. The variant amount increased to 42.4 % in a case of Hb Q-Thailand coinheritance with 4.2 kb and 3.7 kb α-globin deletions (Table 5.8) and hematological analysis showed mild anemia (Table5.7). The heterozygous Hb Tak with various form of α-globin genotypes had hemoglobin higher than 15.0 g/dL

(Table5.9). In heterozygous Hb Tak, the variant eluted in S window ranging between 33.8-35.7 % on Variant II HPLC with increased Hb A2 values (3.8-4.7 %) and increased to 84.5 % in homozygote with increased Hb F (8.6 %)and Hb A2 (6.0 %). In coinheritance with Hb E, the variant level were 55.0 %, and 59.5 % on Variant II HPLC and Capillarys 2-CE, respectively with 33.9-36.6 % Hb E and increased Hb A2 (4.8 %). For Hb Tak coinheritance with Hb CS, the variant amount decreased to 25.9 % with slightly high Hb A2 (Table 5.10). Hb D-Punjab cases showed mild to moderate anemia (Table 5.11). In heterozygous Hb D-Punjab, it eluted with amount of 36.4-41.1 % in D window on Variant II HPLC and eluted in zone 6 on Capillarys 2-CE ranging between 39.1-41.1 %. The variant amount increased to 92.0-94.8% in homozygous Hb D and 61.4-65.2 % in Hb D-Punjab/Hb E (Table 5.12). Hb C heterozygote with various α-globin genotype showed mild to moderate anemia except a new born patient who was also heterozygous for α-thalassemia 1 (SEA deletion) (Table 5.13). Hb C heterozygotes have the variant level of 31.7-40.6 % in C window and found 1.0-1.2 % of minor peak in S window on Variant II HPLC and eluted in zone 2 on Capillarys 2-CE with similar quantities (Table 5.14). Hb C coexistence with α-thalassemia showed slightly decreased in the variant amount 25.0-28.0 %. Hematological analysis of Hb S samples showed mild to moderate anemia except one case of Hb S homozygote coexistent with heterozygous α-thalassemia 2 (3.7-kb deletion) that showed normal values (Table 5.15). In Hb S heterozygote, the variant amount were between 35.2-42.3 % in S window on Variant II HPLC and in zone 5 on Capillarys 2-CE The variant amount increased to 62.4 and 75.2 % in homozygous Hb S and homozygous Hb S coexistent with heterozygous α-thalassemia 2 (3.7 deletion), respectively (Table 5.16).

Table 5.1 Mean ± SD of hematological parameters of heterozygous Hb Hope coinheritance with various α-globin genotypes

Hematological parameters	Heterozygous Hb Hope (β136, G>A) n=12			
	No α-thalassemia coexistence n=7	Coexistent with Heterozygous α-thalassemia 2 (3.7 Kb deletion) n=1*	Coexistent with homozygous α-thalassemia 2 (3.7 Kb deletion) n=2*	Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=2*
Rbc (10^6 cells/mL)	4.2±0.56	5.29	4.01,4.6	5.45,5.81
Hb (g/dL)	12.06±1.96	9.4	9.3,10.3	11.5,11.7
Hct (%)	36±5.29	33.1	29.3,37.0	35.9,37.6
MCV (fL)	85.04±4.67	62.5	73.0,80.4	64.7,65.8
MCH (pg)	28.76±2.1	17.8	22.4,23.3	20.2,21
MCHC (g/dL)	33.79±1.31	28.5	27.8,31.9	31.2,32
RDW (%)	13.7±0.69	34.7	15.1,16.3	14.1,18.1

* Raw data

Table 5.2 Mean ± SD of hemoglobin fraction in Hb Hope with various α-globin genotypes

Hemoglobin Fraction	Heterozygous Hb Hope (β136, G>A) n=12							
	No α-thalassemia coexistence n=7		Heterozygous α-thalassemia 2 (3.7 Kb deletion) n=1*		homozygous α-thalassemia 2 (3.7 Kb deletion) n=2*		heterozygous α-thalassemia 1 (SEA deletion) n=2*	
	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)
HbF	0.46±0.18	0.35±0.07	0.4	0	0.3	0	0.4,0.5	0
HbA	46.71±1.87	46.31±0.99	49.7	56.4	52.4,53.4	62.7,63.1	51.7,53.8	61.0,64.1
HbA2	3.29±0.160	4.42±0.60	2.3	3.4	3.1,3.2	4.5,4.7	3.2,3.5	4.5,4.6
Hb variant	P2 =46.71±1.87	Z11Z10,Z10 =40.78±2.83	P2 =46.7	Z10 =40.8	P2 =42.0,47.6	Z10 =31.9,32.5	P2 =37.5,39.7	Z10 =31.0,34.4

* Raw data

Table 5.3 Mean ± SD of hematological parameters in Hb J-Bangkok heterozygote

Hematological parameters	**Heterozygous Hb J-Bangkok (*β*56, G>A) n=3**
	Heterozygote
Rbc (10^6 cells/mL)	4.8±0.76
Hb (g/dL)	14.2±2.36
Hct (%)	41.6±6.8
MCV (fL)	86.5±0.53
MCH (pg)	29.53±0.21
MCHC (g/dL)	34.17±0.21
RDW (%)	14.3±2.1

* Raw data

Table 5.4 Mean ± SD of hemoglobin fraction in Hb J-Bangkok heterozygote

Hemoglobin Fraction	**Heterozygous Hb J-Bangkok (β56, G>A)**	
	Heterozygote n=3	
	HPLC (%)	**CZE (%)**
HbF	1.0-2.8	0
HbA	44.0-46.3	46.6
HbA2	3.0	2.8
Hb variant	Before A Window=41.4-43.6	Z12=50.6

* Raw data

Table 5.5 Mean ± SD of hematological parameters of heterozygous Hb Lepore-Washington-Boston with various α and β globin genotypes

Hematological parameters	**Heterozygous Hb Lepore-Washington-Boston (δ^{87}-$\beta^{IVS-II-8}$) n=4**		
	No α-thalassemia coexistence n=2*	**Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1***	**Coexistent with heterozygous Hb E n=1***
Rbc (10^6 cells/mL)	5.29,5.99	6.82	4.87
Hb (g/dL)	11.1,14.0	11.5	11.4
Hct (%)	35.0,41.9	40.2	23.5
MCV (fL)	66.2,70.0	74.7	66.8
MCH (pg)	21.0,23.4	21.4	23.5
MCHC (g/dL)	31.7,33.5	28.6	35.1
RDW (%)	15.2,16.9	14.4	21.8

* Raw data

Table 5.6 Mean ± SD of hemoglobin fraction parameters of heterozygous Hb Lepore-Washington-Boston with various α and β globin

Hemoglobin Fraction	Heterozygus Hb Lepore-Washington-Boston (δ^{87}-$\beta^{IVS-II-8}$) n=4					
	No α-thalassemia coexistence n=2*		Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1*		Coexistent with heterozygous Hb E n=1*	
	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)
HbF	2.6,4.0	2.3,3.7	3.5	3.1	28.8	31.3
HbA	75.0,76.1	83.6,84.1	77.9	84.1	27.6	33.7
HbA2	-	2.6,2.9	-	2.5	-	2.4
HbE	12.4,13.3	-	11.1	-	37.4	28.3
Hb variant	-	Z6 =10.1.10.7	-	Z6 =9.4	-	Z6 =4.3

* Raw data

Table 5.7 Mean ± SD of hematological parameters of heterozygous Hb Q-Thailand with various α-globin genotypes

Hematological parameters	Heterozygous Hb Q-Thailand (α 74, G>C) n=6	
	Coexistent with heterozygous α-thalassemia 2 (4.2 Kb deletion) n=5	Coexistent with compound heterozygous α-thalassemia 2 (3.7 Kb and 4.2 Kb deletion) n=1*
Rbc (10^6 cells /mL)	5.17±0.46	5.92
Hb (g/dL)	13.22±0.95	13.1
Hct (%)	39.98±4.7	37.5
MCV (fL)	77.22±3.12	63.4
MCH (pg)	25.62±0.63	22.1
MCHC (g/dL)	33.3±1.64	34.8
RDW (%)	13.48±0.55	16.0

* Raw data

Table 5.8 Mean ± SD of hemoglobin fraction parameters of heterozygous Hb Q-Thailand with various α-globin genotypes

Hemoglobin Fraction	**Heterozygous Hb Q-Thailand (α 74, G>C) n=6**			
	Coexistent with heterozygous α-thalassemia 2 (4.2 Kb deletion) n=5		**Coexistent with compound heterozygous α-thalassemia 2 (3.7 Kb and 4.2 Kb deletion) n=1***	
	HPLC (%)	**CZE (%)**	**HPLC (%)**	**CZE (%)**
HbF	0.2-0.3	28.5-28.6	0.4	ND
HbA	60.1-61.5	68.9-69.0	47.7	ND
HbA2	2.2-3.0	1.8	4.7	ND
Hb variant	S =29.6-30.5	-	S =42.4	ND

* Raw data

Table 5.9 Mean ± SD of hematological parameters of Hb Tak with various α-globin genotypes

Hematological parameters	Hb Tak (β147 (+AC): modified C-terminus: (147) Thr-Lys-Leu-Ala-Phe-Leu-Ser-Asn-Phe-(157)Tyr-COOH) n=7			
	No α-thalassemia coexistence n=4	Homozygote n=1*	Coexistent with heterozygous Hb Constant Spring n=1*	Coexistent with heterozygous Hb E n=1*
Rbc (10^6 cells/mL)	5.31±0.96	7.46	5.6	7.46
Hb (g/dL)	16.5±2.11	19.0	17.0	19.0
Hct (%)	45.33±4.65	55.0	45.4	55.0
MCV (fL)	85.83±3.42	73.6	80.2	73.6
MCH (pg)	31.3±1.69	25.5	30.0	25.5
MCHC (g/dL)	36.45±0.87	34.6	37.4	34.6
RDW (%)	14.45±0.87	13.0	15.5	20.7

* Raw data

Table 5.10 Mean ± SD of hemoglobin fraction parameters of Hb Tak with various α and β globin genotypes

Hemoglobin Fraction	**Hb Tak (β147 (+AC): modified C-terminus: (147) Thr-Lys-Leu-Ala-Phe-Leu-Ser-Asn-Phe-(157)Tyr-COOH) n=7**							
	No α-thalassemia coexistence n=4		**Homozygote n=1***		**Coexistent with heterozygous Hb Constant Spring n=1***		**Coexistent with heterozygous Hb E n=1***	
	HPLC (%)	**CZE (%)**	**HPLC (%)**	**CZE (%)**	**HPLC (%)**	**CZE (%)**	**HPLC (%)**	**CZE (%)**
HbF	0.3-0.9	33.7	8.6	ND	1.2	ND	1.9	59.5
HbA	52.9-54.7	62.7	1.8	ND	52.4	ND	3.2	1.8
HbA2	3.8-4.7	4.0	6	ND	3.6	ND	-	4.8
HbE	-	-	-	-	-	ND	36.6	33.9
Hb variant	S =33.8-35.7	-	S =84.5	ND	S =25.9	ND	S =55.0	-

* Raw data

Table 5.11 Mean ± SD of hematological parameters of Hb D-Punjab with various α and β globin genotypes

Hematological parameters	**Hb D-Punjab (*β*121, G>C) n=5**		
	No α-thalassemia coexistence n=3	**Homozygote n=1***	**Coexistent with heterozygous Hb E n=1***
Rbc (10^6 cells/mL)	5.08±0.25	6.32	6.32
Hb (g/dL)	13.1±1.68	15.8	15.8
Hct (%)	37.6±4.12	43.8	43.8
MCV (fL)	73.7±4.64	69.3	69.3
MCH (pg)	25.7±2.26	25.0	25.0
MCHC (g/dL)	34.8±1.23	36.1	36.1
RDW (%)	14.1±1.10	15.2	14.5

* Raw data

Table 5.12 Mean ± SD of hemoglobin fraction parameters of Hb D-Punjab with various α and β globin genotypes

Hemoglobin Fraction	Hb D-Punjab (β121, G>C) n=5					
	No α-thalassemia coexistence n=3		Homozygote n=1*		Coexistent with heterozygous Hb E n=1*	
	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)
HbF	0.2-1.4	0-1.3	0.3	0	1.4	0
HbA	50.1-53.2	54.6-58.2	4.3	1.0	4.3	0
HbA2	1.8-2.0	2.7-3.2	1.3	4.2	-	3.9
HbE	-	-	-	-	25.8	29.9
Hb variant	D =36.4-41.1	Z6 =39.1-41.1	D =92.0	Z5-6 =94.8	D =61.4	Z6 =65.2

* Raw data

Table 5.13 Mean ± SD of hematological parameters of heterozygous Hb C with various α-globin genotypes

Hematological parameters	**Heterozygous Hb C (β6, G>A)** n=7			
	No α-thalassemia coexistence n=4	Coexistent with heterozygous α-thalassemia 2 (3.7 Kb deletion) n=1*	Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1* (adult)	Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1* (< 1 year)
Rbc (10^6 cells/mL)	4.9±0.66	6.53	5.65	3.08
Hb (g/dL)	12.63±1.38	16.1	11.7	11.1
Hct (%)	36.3±3.78	45.8	35.3	34.2
MCV (fL)	74.68±6.99	70.1	62.5	111.1
MCH (pg)	26.03±3.13	24.4	20.7	35.9
MCHC (g/dL)	34.78±1.31	35.1	33.2	32.4
RDW (%)	16.2±1.39	18.5	18.5	15.5

* Raw data

Table 5.14 Mean ± SD of hemoglobin fraction parameters of heterozygous Hb C with various α-globin genotypes

Hemoglobin Fraction	**Heterozygous Hb C (β6, G>A) n=7**							
	No α-thalassemia Coexistence n=4		Coexistent with heterozygous α-thalassemia 2 (3.7 Kb deletion) n=1*		Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1* (adult)		Coexistent with heterozygous α-thalassemia 1 (SEA deletion) n=1* (< 1 year)	
	HPLC (%)	**CZE (%)**	**HPLC (%)**	**CZE (%)**	**HPLC (%)**	**CZE (%)**	**HPLC (%)**	**CZE (%)**
HbF	0.6-1.1	0.2-0.3	0.3	ND	0.5	0	71.8	74.4
HbA	49.9-62.1	58.7-62.1	57.6	ND	60.5	69.0	13.3	13.8
HbA2	3.1-4.0	2.4-3.1	3.5	ND	3.6	3.6	-	0.4
Hb variant	C =31.8-40.6	Z2 =31.7-37.9	C =31.2	ND	C =28.2	Z2 =25.0	C =15.5	Z2 =11.4
others	S=1.0-1.2	-	S=1.1	ND	S=0.8	-	-	-

* Raw data

Table 5.15 Mean ± SD of hematological parameters of Hb S with various α-globin genotypes

Hematological parameters	Hb S (β 136, G>A) n=15				
	No α-thalassemia Coexistence n=4	Coexistent with heterozygous α-thalassemia 2 (3.7 Kb deletion) n=4	Coexistent with homozygous α-thalassemia 2 (3.7 Kb deletion) n=5	Homozygote n=1*	Homozygous Hb S coexistent with heterozygous α-thalassemia 2 (3.7 deletion)
Rbc (10^6cells/mL)	4.64±0.28	5.62±1.07	4.94±0.76	4.5	3.77
Hb (g/dL)	12.08±2.23	11.13±3.89	10.56±0.86	10.3	11.1
Hc t(%)	35.1±4.67	35.28±9.39	32.64±2.73	31.2	32.5
MCV (fL)	75.58±5.72	62.98±11.92	66.78±5.41	69.3	86.1
MCH (pg)	25.93±3.33	19.7±4.92	21.56±1.81	22.9	29.5
MCHC (g/dL)	34.23±2.22	31.03±2.72	32.34±1.14	33.0	34.3
RDW (%)	15.2±3.23	19.8±4.29	15.86±2.03	18.9	16.2

* Raw data

Table 5.16 Mean ± SD of hemoglobin fraction parameters of Hb S with various α-globin genotypes

Hemoglobin Fraction	Hb S (β 136, G>A) n=15									
	No α-thalassemia Coexistence n=4		Coexistent with heterozygous α-thalassemia 2 (3.7 Kb deletion) n=4		Coexistent with homozygous α-thalassemia 2 (3.7 Kb deletion) n=5		Homozygote n=1*		Homozygous Hb S coexistent with heterozygous α-thalassemia2 (3.7 deletion) n=1*	
	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)	HPLC (%)	CZE (%)
HbF	0.2-0.4	0	0.1-0.7	0	0.3-1.3	0	18.8	ND	19.5	ND
HbA	48.5-54.3	54.9-56.7	55.1-59.8	62.0-71.2	57.8-61.7	58.2-66.5	5.1	ND	0	ND
HbA2	3.5-4.8	2.8-3.5	3.1-4.1	2.3-3.3	4.2	3.6-4.2	5.4	ND	4.2	ND
Hb variant	S =35.2-41.2	Z5 =39.8-42.3	S =23.1-35.1	Z5 =25.5-34.6	S =22.9-28.4	Z5 =28.9-29.9	S =62.4	ND	S =75.2	ND

* Raw data

CHAPTER VI
DISCUSSION

Hemoglobinopathies or inherited disorder of hemoglobin could involve either heme or globin parts of the molecule, which can be classified into two main groups, abnormal hemoglobin and thalassemia. Thalassemia syndromes are genetic disorders characterized by a reduced production rate of one or more of the globin chains of hemoglobin molecule (20). It causes imbalanced globin chain synthesis, defective hemoglobin production and damage to the red blood cells or their precursors. These defects lead to hereditary anemia with symptomatic varieties. Thalassemia are classified into two groups, based on type of deficient globin chain, i.e., α-thalassemia and β-thalassemia (2, 20). Abnormal hemoglobin is a hemoglobin variant resulting from the production of the defective globin subunit. The majority of hemoglobin variants result from the single amino acid substitution in either one of the globin chains (102). Although the majority of them do not cause clinical problems, the interaction with other thalassemic genes may potentiate hematological abnormalities (25, 26). Clinical and laboratory of these Hb variants could be useful for evaluations of new case and genetic counseling. Laboratory diagnosis of thalassemia and hemoglobin variants are generally inferred from automated HPLC and the recent technology of hemoglobin identification is automated capillary zone electrophoresis (CE). Thus, the definitive identification of hemoglobin variants present in either “window” or “zone” can be achieved only by DNA analysis or DNA sequencing.

Moreover, previous problems in laboratory for Hb variants diagnosis were observed in routine techniques such as automated Variant II HPLC, there are six “window” of A, F, S, C, D, and A_2/E in which many different hemoglobin variants may be eluted the same window. In CE method, the peaks of hemoglobin are reported within 15 zones and each zone may be occupied by several hemoglobin variants. Some of the carriers of Hb variants had represented hematologic phenotype similar to normal. The different types of Hb variants diagnosed by HPLC and CE demonstrated

in the same location of Hb fractions resulting in misdiagnosis. Old specimen caused the shift of Hb variants from their specific locations. The multiplex AMRS-PCR was used in this study to overcome those problems and definitely identify the Hb variants. In previous study, they developed multiplex allele-specific PCR assay for differential diagnosis of three common β-chain variants; Hb S, Hb D-Punjab and Hb Tak and for differential diagnosis of α-chain variants; Hb Queens and Hb Siam (62, 65).

To provide a simpler method for diagnosis of these Hb variants, we have developed and tested a multiplex ARMS-PCR. Our samples were tested by hematological analysis, Hb identification and PCR analysis for α-thalassemia. We developed three sets of multiplex AMRS-PCR for differential diagnosis of 12 Hb variants included either β or α-chain mutations.

In this present study, we have investigated 7 Hb variants in Thai population and 2 variants in the Middle East individuals.

The variants eluted before Hb A on Variant II HPLC were identified to be Hb Hope and Hb J-Bangkok. Hb Hope is a common hemoglobin variant found in Thailand (31, 66, 90, 99). The GGT>GAT conversion leads to a substitution of aspartate to glycine at codon 136 of the β-globin gene. The previous and our study found Hb Hope located in P2 window on Variant II HPLC as same as glycated hemoglobin which is increased higher more than in diabetes with a percentage of > 40 % (72, 122). Hb Hope migrated in zone 10 on the Capillarys 2-CE which similar to the previous reported (99). The separation of Hb Hope was correlated to the database of each methods (72, 90, 96, 110). In old specimens, we found the shift of the peak (between zone 10-11 on the Capillarys 2-ZE). The hematological data of Hb Hope heterozygote was normal. The association of Hb Hope and heterozygous α-thalassemia 2 (3.7 -deletion) present with microcytic anemia with Hb 9.4 g/dL, Hct 33.1 %, MCV 62.5 fL similar to previous report in black women (123). However, the previous report in 2 Thai patients found they were generally healthy (42). Differences in these finding may therefore be due to some other alteration in health status or small number of case in experiment. Hb J-Bangkok heterozygote was not associated with disease which similar to the previous report (31, 57). Hb J-Bangkok is eluted before A window on Variant II HPLC and migrated in zone 12 on the Capillarys 2-CE which similar to the previous study and the database of each methods (97, 99, 110).

Some Hb variants eluted after Hb A revealed co-elution with difference variant on the Variant II HPLC such as Hb Q-Thailand and Hb Tak. They were migrated in the zone 7 with Hb F on the Capillarys 2. The electrophoregram of both Hb Q-Thailand and Hb Tak may be similar to homozygous β-thalassemia and HPFH performed by Capillarys 2-CE method. Thus, in this case we suggests to use the DNA analysis for idetification. The amount of these two variants in heterozygotes were approximately 30-36 % when detected by both HPLC and CE methods, except in the cases of homozygotes and coinheritance of α-thalassemia or Hb E. We reported the increasing amount of the variant in homozygous Hb Tak (84.5 %), Hb Tak/HbE (55–57 %), and Hb Q-Thailand with 4.2 kb and 3.7 kb deletions (42.4 %) (47). Hb Lepore could be misdiagnosed on Variant II HPLC methods (94, 95, 101-103, 108) resulting from co-elution with Hb A2 but could be detect on the Capillarys 2- CE by elution in zone 6.By the Capillarys 2-CE Hb Lepore and Hb D-Punjab were co-eluted in the same area (zone 6). However, we could differentiate them by the amount of each variant, 4.3-10.4 % for Hb Lepore and approximately 40.0 % for Hb D-Punjab.

For hematological features, heterozygous state of Hb variants were not shown actually symptoms but in homozygous or association with thalassemia could be result in thalassemia intermedia with abnormalities of hematological parameters i.e., severe hypochromic microcytic anemia. From this study, we found Hb variants causing some clinical significances including Hb Lepore, Hb Tak, Hb D-Punjab, Hb C and Hb S (24, 30, 37, 47, 54, 56, 83, 90, 93, 124-126). Hb Lepore–Washington–Boston showed hypochromic microcytic red cells which similar to the previous study (30, 56, 95). Hb Lepore–Washington–Boston coexistent with heterozygous Hb E revealed hematological parameters and hemoglobin fraction parameters similar to those in β-thalassemia/Hb E. Thus, the identification in this case suggests the population screening or family studies. Hb Tak was shown symptomatic polycythemia, promotes the oxygenation leading to high oxygen affinity. Hb Tak in various combinations showed hemoglobin higher than 15.0 g/dL (31, 37, 47, 62, 90, 93). Hb D-Punjab showed clinical silence in the heterozygous state, but moderate anemia in Hb D-Punjab homozygote and Hb D-Punjab/Hb E compound heterozygote same as the previous documents (89, 90). Hb C showed mild anemia with MCV<80 fL and MCH< 27.0 pg.

In addition, the results from the Middle East samples also found Hb S and Hb D-Punjab. Hb S can cause clinical significance in homozygous or compound heterozygous states with other hemoglobinopathies and thalassemia. One case of Hb S homozygote coexistent with heterozygous α-thalassemia 2 (3.7-kb deletion) demonstrated normal hematological parameters contrast to other reports (14, 24, 29, 127). In major genotypes Hb S/S, Hb S/C, and less common genotypes, Hb S/D-Punjab and Hb S/Lepore, they produced a sickling phenotype; i.e., severity of disease varied (20, 24, 29, 127).

The identification of Hb variants can be summarized as shown in Figure 6.1**.** However, this multiplex ARMS-PCR still has limitation on the diagnosis of heterozygosity and homozygosity of each mutation. The result of DNA analysis is usually interpreted with Hb analysis data, and the presence or absence of Hb A to provide a definite diagnosis

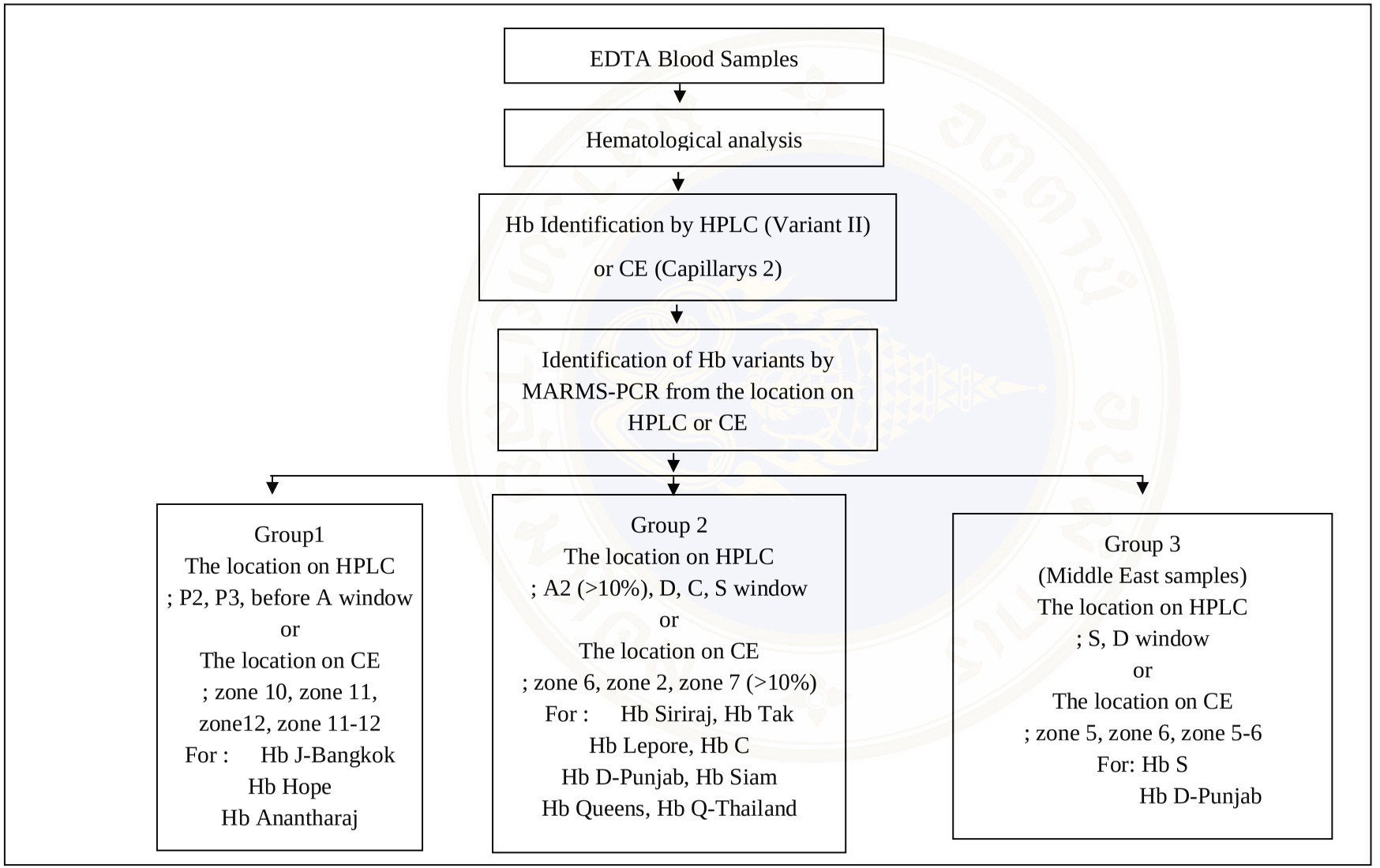

Figure 6.1 Summarized of protocol for identification the Hb variants

CHAPTER VII
CONCLUSION

In conclusion, DNA analysis is important for identification of Hb variants because the screening by HPLC and CZE found many different Hb variants co-eluted in the same window (on HPLC) or the same zone (on CZE). The multiplex AMRS-PCR is required for definitive identification in addition to routine Hb typing which is only a presumptive diagnosis. This thesis demonstrates the successful development of multiplex ARMS-PCR for common Hb variants in Thailand. The assay would greatly facilitate, less expensive, safer and additionally, this technique should prove useful in complementing routine Hb variant diagnosis in the region.

REFERENCES

1. Ho PJ, Thein SL. Gene regulation and deregulation: a β globin perspective. *Blood Reviews*. 2000; 14: 78-93.
2. Weatherall DJ, Clegg JB. Molecular Genetics of Human Hemoglobin. *Annual Review of Genetics*. 1976; 10(1): 157-78.
3. G S, FA G. An excellent up-to-date review on what is known about the mechanisms of the differential expression of the globin genes during development. *The Molecular Basis of Blood Disease New York*. 2001: 132-82.
4. Stamatoyannopoulos G. Control of globin gene expression during development and erythroid differentiation. *Experimental Hematology*. 2005; 33: 259-71.
5. Schechter. Hemoglobin research and the origins of molecular medicine. *Blood*. 2008; 112: 3927-8.
6. Cohen AR, Galanello R, Pennell DJ, Cunningham MJ, Vichinsky E. Thalassemia. *American Society of Hematology*. 2004.
7. Maxine Singer PB. Exploring Genetic Mechanisms. illustrated ed; 1997. p. 320-2.
8. Jameson JL. Principles of Molecular Medicine. Illustrated ed; 1998. p.188-90.
9. Weatherall DJ. Phenotype-genotype relationships in monogenic disease. The Thalassemias. *Nature review of genetics*. 2011; 2: 245-55.
10. Pinnock CA, Lin T, Smith T, Jones R. Fundamentals of Anaesthesia. In: Colin A. Pinnock RJ, editor. 2 ed; 1999. p. 261-3.
11. George P, Patrinos PK, Manoussos N, Papadakis. Molecular Diagnosis of Inherited Disorders: Lessons From Hemoglobinopathies. *Human mutation*. 2005; 26(5): 339-412.
12. Weatherall DJ. The molecular basis of blood diseases. *WB Saunder*. 2001:183-226.
13. Weatherall DJ. Control Mechanisms in Human Haemoglobin Synthesis. *Annual Review of Medicine*. 1968;19(1): 217-32.
14. Birgens H, Ljung R. The thalassaemia syndromes. *Scand J Clin Lab Invest*. 2007; 67: 11-26.

15. H. Thalassemia. Atlas of Genetic Diagnosis and Counseling: Humana Press; 2006. p. 950-4.

16. Herbert L, Muncie J, Campbell JS. Alpha and Beta Thalassemia. *Am Fam Physician*. 2009; 80(4): 339-44.

17. Galanello R, Origa R. Beta-thalassemia. *Orphanet Journal of Rare Diseases*. 2510; 5(1):11.

18. Cao A, Galanello R. Beta-thalassemia. *Genetics in Medicine*.2010; 12(2): 61-76

19. Fucharoen S, Winichagoon P. Thalassemia and abnormal hemoglobin. *International Journal of Hematology*. 2002; 76(0): 83-9.

20. Weatherall DJ, Clegg JB. The Thalassaemia Syndromes. Blackwell Science Ltd; 2008.

21. Ribeiro DM, Sonati MF. Regulation of human α-globin gene expression and α-thalassemia. *Genet Mol*. 2008; 7(4): 1045-53.

22. Chui DHK, Fucharoen S, Chan V. Hemoglobin H disease: not necessarily a benign disorder. *Blood.* 2003; 101(3): 791-800.

23. Ne W, Harano K, Harano T, Kyaw S, Aye Aye M, Khin Thander A, *et al*. Hb Constant Spring [alpha 142, Term-->Gln (TAA>CAA in alpha 2)] in the α-Thalassemia of Anemic Patients in Myanmar. *Hemoglobin.* 2008; 32(5): 454-61.

24. Huehns ER. Diseases due to abnormalities of hemoglobin of hemoglobin structure. *Annu Rev Med*. 1970; 21: 157-78.

25. Richard AS, Forget BG. The Thalassemias: Molecular Mechanisms of Human Genetic Disease. *Am J Hum Genet*. 1983; 35: 333-61.

26. Clark BE, Thein SL. Molecular diagnosis of haemoglobin disorders. *Chin Lab Haem*. 2004; 26:159-76.

27. Hartwell SK, Srisawang B, Kongtawelert P, Christian GD, Grudpan K. Review on screening and analysis techniques for hemoglobin variants and thalassemia. *Talanta*. 2005; 65(5): 1149-61.

28. Elles R, Old JM. Hemoglobinopathies. Molecular Diagnosis of Genetic Diseases. Humana Press; 1996. p. 169-83.

29. Monu JUV, Bohrer SP. The hemoglobinopathies: Sickle cell disease and thalassemia. *Radiology of Tropical Disease*. 1981: 430-86.

30. Chaibunruang A, Srivorakun H, Fucharoen S, Fucharoen G, Sae-ung N, Sanchaisuriya K. Interactions of hemoglobin Lepore ([delta][beta] hybrid hemoglobin) with various hemoglobinopathies: A molecular and hematological characteristics and differential diagnosis. *Blood Cells, Molecules, and Diseases*. 2010; 44(3): 140-5.

31. Fucharoen S, Fucharoen G. Common abnormal hemoglobins found in Thailand and laboratory in Thailand. *Journal of medical technology and physical therapy*. 2010; 22(2): 103-17.

32. Ngiwsara L, Srisomsap C, Winichagoon P, Fucharoen S and svasti J. Hb Kodaira II [beta146(HC3) His to Gln] detected in Thailand. *Hemoglobin*. 2003; 27: 37-9.

33. Viprakasit V, Pung-Amritt P, Suwanthon L, Clark K, Tanphaichtr VS. Complex interactions of deltabeta hybrid haemoglobin (Hb Lepore-Hollandia) Hb E (beta(26G-->A)) and alpha+ thalassaemia in a Thai family. *Eur J Haematol*. 2002; 68(2): 107-11.

34. Flatz G. Hemoglobin E: Distribution and Population Dynamics. *ltumangenetik*. 1967; 3: 189-234.

35. Tuchinda S, Beale D, Lehmann H. The suppression of Haemoglobin E synthesis. *Human Genetics*. 1967; 3(4): 312-8.

36. Svasti S, Yodsowon B, Sriphanich R, Winichagoon P, Boonkhan P, Wuwanban T, at el. Association of Hb Hope [beta136(H14)Gly to Asp] and Hb H Disease. *Hemoglobin*. 2001; 25(4): 429-35.

37. Teawtrakul N, Sirijirachai C, Chansung G, Fucharoen G. Compound Heterozygous Hb Tak/Hb E Causes Secondary Erythrocytosis in a Thai Family. *Hemoglobin*. 2010; 3 4(2): 165-8.

38. Yongsuwan S, Svasti J. and Fucharoen S. Decreased heat stability found in Hemoglobin Queens. *Hemoglobin*. 1987; 11(6): 567-70.

39. Svasti J, Srisomsap C, Winichagoon P, Fucharoen S. Detection and structural analysis of abnormal hemoglobins found in Thailand. *Southeast Asian J Trop Med Publ Hlth*. 1999; 30(2): 88-93.

40. Sawangareetrakul P, Svasti S, Yodsowon B, Winichagoon P, Srisomsap C, Svasti J, *et al*. Double heterozygosity for Hb Pyrgos [β 83(EF7) Gly to Asp]

and Hb E [β26(B8) Glu to Lys] found in association with alpha-thalassemia. *Hemoglobin*. 2002; 26: 191-6.

41. Fucharoen G, Fuchareon S, Fukumaki Y. Eight-base deletion in exon 3 of the beta-globin gene produced a novel variant (beta khon kaen) with an inclusion body beta-thalassemia trait. *Blood*. 1991; 78: 537-9.
42. Thanyachai Sura, Manisa Busabaratana, Supak Youngcharoen, Raewadee Wisedpanichkij, Vip Viprakasit, Trachoo O. Haemoglobin Hope in a northern Thai family: first identification of homozygous haemoglobin Hope associated with haemoglobin H disease. *European Journal of Haematology*. 2007; 79: 251-4.
43. Itchayanan D, Svasti J, Srisomsap C, Winichagoon P, Fucharoen, S. Hb G-Coushatta [beta-22(B4)Glu to Ala] in Thailand. *Hemoglobin*. 1999; 23: 69-72.
44. Ngiwsara L, Srisomsap C, Winichagoon P, Fucharoen S, Sae-Ngow B, Svasti J. Hb Kurosaki [alpha7(A5)Lys to Glu (AAG to GAG)]: an alpha2-globin gene mutation found in Thailand. *Hemoglobin*. 2005; 29: 155-9
45. Turbpaiboon C, Svasti S, Sawangareetakul P, Winichagoon P, Srisomsap C, Siritanaratkul N, *et al*. Hb Siam [alpha15(A13)Gly to Arg(alpha1) (GGT to CGT)] is a typical alpha chain hemoglobinopathy without an alpha-thalassemic effect. *Hemoglobin.* 2002, 26: 77-81.
46. Yodsowan B, Svasti J, Srisomsap C, Winichagoon P, Fucharoen S. Hb Siam [alpha15(A13)Gly to Arg] is a GGT to CGT mutation in the alpha1-globin gene. *Hemoglobin*. 2000; 24(1): 71-5.
47. Jindadamrongwech S, Tungbuppha N, Chuncharunee S, Butthep P. Hb Tak and Hb Q-Thailand in Thai patients are S-window hemoglobin variants revealed by high performance liquid chromatography. *Hemoglobin*. 2010; 34(2): 161-4.
48. Chaibunruang A, Fucharoen G, Jetsrisuparb A, Fucharoen S. Hemoglobin Lepore EF Bart's disease: a molecular, hematological, and diagnostic aspects. *Annals of Hematology*.2011; 1-4.

49. Vip V. Hemoglobin Pak Num Po (Hb PNP), a Novel Unorthodox α Hemoglobin Variant Causing Severe Form of α Thalassemia. *Siriraj Med J*. 2005; 57(11): 477-8.

50. Turbpaiboon C, Siritantikorn A, Thongnoppakhun W, Bunditworapoom D, Limwongse C, Yenchitsomanus P, *et al*. Hemoglobin Pakse: Presence on Red Blood Cell Membrane and Detection by Polymerase Chain Reaction-Single-Strand Conformational Polymorphism. *International Journal of Hematology*. 2004; 80(2): 136-9.

51. Singsanan S, Karnpean R, Fucharoen G, Sanchaisuriya K, Sae-ung N, Fucharoen S. Hemoglobin Q-Thailand related disorders: Origin, molecular, hematological and diagnostic aspects. *Blood Cells, Molecules, and Diseases*. 2010; 45(3): 210-4.

52. Pootrakul S, Srichiyanont S, Wasi P, Suanpan S. Hemoglobin Siam (alpha 2 15 arg beta 2): a new alpha-chain variant. *Humangenetik*. 1974; 23(3):199-204.

53. Svasti J, Surarit R, Srisomsap C, Pravatmuan P, Wasi P, Fucharoen S, Blouquit Y, Galacteros F, Rosa J. Identification of Hb Anantharaj [alpha11 (A9) Lys to Glu] as Hb J-Wenchang- Wuming [alpha11 (A9) Lys to Gln]. *Hemoglobin*. 1993; 17: 453-5.

54. Siriboon W, Srisomsap C, Winichagoon P, Fucharoen S, Svasti J. Identification of Hb C [beta6 (A3) Glu to Lys] in a Thai male. *Hemoglobin*. 1993; 17: 419-25.

55. Itchayanan D, Svasti J, Srisomsap C, Winichagoon P, Fucharoen S. Identification of Hb J-Buda [alpha-61 (E10)Lys to Asn] in a Thai female. *Hemoglobin*. 1999; 23, :183-6.

56. Boontrakoonpoontawee P, Svasti J, Fucharoen S., Winichagoon P. Identification of Hb Lepore-Washington-Boston in association with HbE [26(B8)GLU to LYS] in a Thai female. *Hemoglobin*. 1987; 11(4): 1.

57. Fucharoen S, Singsanan S, Sanchaisuriya K, Fucharoen G. Molecular and haematological characterization of compound Hb E/Hb Pyrgos and Hb E/Hb J-Bangkok in Thai patients. *Clinical & Laboratory Haematology*. 2005; 27(3): 184-9.

58. Saechan V, Nopparatana C, Nopparatana C, Fucharoen S. Molecular basis and hematological features of hemoglobin variants in Southern Thailand. *Int J Hematol.* 2010; 92: 445-50.

59. Winichagoon P, Higgs DR, Goodbourn SE, Clegg JB, Weatherall DJ, Wasi P. The molecular basis of alpha-thalassaemia in Thailand. *EMBO J*. 1984; 3(8): 1813-8.

60. Pranee W, Suthat F. Molecular mechanisms of thalassemia in Thailand. *J Sci Soc Thailand*. 1986; 12:9-21.

61. Pichanun D, Munkongdee T, Klamchuen S, Butthep P, Winichagoon P, Fucharoen S, *et al*. Molecular Screening of the Hbs Constant Spring (codon 142, TAA>CAA, α2) and Pakse (codon 142, TAA>TAT, α2) Mutations in Thailand. *Hemoglobin*.year 34(6): 582-6.

62. Sanchaisuriya K, Chunpanich S, Fucharoen G, Fucharoen S. Multiplex allele-specific PCR assay for differential diagnosis of Hb S, Hb D-Punjab and Hb Tak. *Clin Chim Acta.* 2004; 343(1-2): 129-34.

63. Tuchinda S, Beale D, Lehmann H. A new Haemoglobin in a Thai Family. A case of Haemoglobin Siriraj-Beta Thalassaemia. *Br Med J*. 1965, 9(1): 1583-5.

64. Pootrakul S, Kematorn B, Na-Nakorn S, Suanpan S. A new haemoglobin variant: haemoglobin Anantharaj alpha 11 (A9) lysine replaced by glutamic acid. *Biochim Biophys Acta*. 1975; 405(1): 161-6.

65. Fucharoen S, Singsanan S, Hama A, Fucharoen G, Sanchaisuriya K. Rapid molecular characterization of Hb Queens and Hb Siam: Two variants easily misidentified as sickle Hb. *Clinical Biochemistry*. 2007; 40(1-2): 137-40.

66. Svasti J, Srisomsap C, Itchayanan D, Limwuttiwong A, Siriboon W, Winichagoon P, *et al*. Recent studies on the abnormal hemoglogins found in Thailand. *J Chem Soc Pak*. 1999; 21: 281-8.

67. Sanguansermsri P, Shimbhu D, Wongvilairat R, Pimsorn C, Sanguansermsri T. Spontaneous mutation of the hemoglobin Leiden (beta 6 or 7-->GAG) in a Thai girl. *Haematologica*. 2003, 2003; 88(12): 35.

68. Boonsaa S, Sanchaisuriyab K, Fucharoenb G, Wiangnonc S, Jetsrisuparbc A, Fucharoena S. The Diverse Molecular Basis and Hematological Features of Hb H and AEBart's Diseases in Northeast Thailand. *Acta Haematologica*. 2004; 111: 149-54.

69. Weatherall DJ. Molecular Basis for Some Disorders of Haemoglobin Synthesis. *British Medical Journal*. 1974; 2: 451-4.

70. Huehns ER. Diseases due to abnormalities of hemoglobin structure. *Annu Rev Med*. 1970; 21: 157-78.

71. Wajcman H, Galactéros F. Abnormal hemoglobins with high oxygen affinity and erythrocytosis. *Hematology and Cell Therapy*. 1997; 38(4): 305-12.

72. Fucharoen S, Singsanan S, Hama A, Fucharoen G, Sanchaisuriya K. Rapid molecular characterization of Hb Queens and Hb Siam: two variants easily misidentified as sickle Hb. *Clin Biochem*. 2007; 40(1-2): 137-40.

73. Joutovsky A, Hadzi-Nesic J, Nardi MA. HPLC Retention Time as a Diagnostic Tool for Hemoglobin Variants and Hemoglobinopathies: A Study of 60000 Samples in a Clinical Diagnostic Laboratory. *Clin Chem*. 2004; 50(10):1736-47.

74. Hardison RC, Chui DHK, Giardine B, Riemer C, Patrinos GP, Anagnou N, *et al*. HbVar: A relational database of human hemoglobin variants and thalassemia mutations at the globin gene server. *Human Mutation*. 2002; 19(3): 225-33.

75. Zeng F, Fucharoen S, Huang S, Rodgers G. Hb Q-Thailand [alpha 74(EF3)Asp-->His]: gene organization, molecular structure, and DNA diagnosis. *Hemoglobin*. 1992; 16(6): 481-91.

76. Pootrakul S, Dixon GH. Hemoglobin Mahidol: a new hemoglobin alpha-chain mutant. *Canadian Journal of Biochemistry*. 1970; 48(9): 1066-78.

77. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. *Hemoglobin*. 1987; 11(1): 65-88.

78. Singsanan S, Fucharoen G, Savongsy O, Sanchaisuriya K, Fucharoen S. Molecular characterization and origins of Hb Constant Spring and Hb Paksé in Southeast Asian populations. *Annals of Hematology*. 2007; 86(9): 665-9.

79. Flatz G, Pik C, Sringam S. Haemoglobin E and β-thalassaemia: their distribution in Thailand. *Annals of Human Genetics*. 1965; 29(2): 151-70.

80. Vichinsky E. Hemoglobin E Syndromes. *American Society of Hematology*. 2007:79-83.

81. Borgna-Pignatti C, Galanello R. Thalassemias and related disorders: quantitative disorders of hemoglobin synthesis. *Wintrobe's Clinical Hematology*. 2004; 42:1319-65.

82. Bachir D, Galacteros F. Hemoglobin E. Orphanet Encyclopedia. 2004.

83. Spencer J, Sanders J, Ault B. Two cases of hematuria with hemoglobin C trait. *Pediatric Nephrology*. 2009; 24(12): 2455-7.

84. Bruno B, Dalens BJ. Hemoglobin C Disease. Syndromes: rapid recognition and perioperative implications 1976: 366-7.

85. Shibayama N, Sugiyama K, Park S-Y. Structures and oxygen affinities of crystalline human hemoglobin C (beta 6 Glu’ ’Lys) in the R and R2 quaternary structures. *Journal of Biological Chemistry*. 2011; 10: 1-14

86. Arie T, Fairhurst RM, Brittain NJ, Wellems TE, Dvorak JA. Hemoglobin C modulates the surface topography of Plasmodium falciparum-infected erythrocytes. *Journal of Structural Biology*. 2005;150(2):163-9.

87. Bruno Bissonnette BJD. Hemoglobin Disorders: Overview. Syndromes: rapid recognition and perioperative implications 1976; 1:366-7.

88. Gunay U, Pauli C, Shamsuddin M, Mason RG, Heinze WJ, Honig GR. Sickle Hemoglobin in Combination With Hb J-Bangkok (αA2β56 gly>asp2)). *Blood*. 1974, 44(5): 683-90.

89. Srinivas U, Pati HP, Saxena R. Hemoglobin D-Punjab syndromes in India: A single center experience on cation-exchange high performance liquid chromatography. *Hematology*. 2010; 15: 178-81.

90. Uaprasert N, Rojnuckarin P, Settapiboon R, Amornsiriwat S, Sutcharitchan P. Clinical and hematological characteristics of uncommon beta-globin variants in Thailand. *Int J Hemato*l. 2009; 89(5): 568-71.

91. Minnichi V, Hill RJ, Khuri PD, Anderson ME. Hemoglobin Hope: A Beta Chain Variant. *Blood*. 1965; 25: 830-8.

92. Viprakasit V. Hemoglobin Pak Num Po (Hb PNP), a Novel Unorthodox Hemoglobin Variant Causing Severe Form of Thalassemia. *Siriraj Med J*. 2005;57 : 477-8.

93. Flatz G, Kinderlerer JL, Kilmartin JV, Lehmann H. Haemoglobin Tak: a variant with additional residues at the end of the beta-chains. *Lancet*. 1971; 1(7702): 732-3.

94. Ropero P, Gonzalez FA, Sanchez J, Anguita E, Asenjo S, Del Arco A, *et al*. Identification of the Hb Lepore phenotype by HPLC. *Haematologica*. 1999; 84(12): 1081-4.

95. Viprakasit V, Pung-Amritt P, Suwanthon L, Clark K, Tanphaichitr VS. Complex interactions of δβ hybrid haemoglobin (Hb Lepore-Hollandia) Hb E (β26 G→A) and α+ thalassaemia in a Thai family. *European Journal of Haematology*. 2002; 68(2):107-11.

96. Hunt DM, Higgs DR, Winichagoon P, Clegg JB, Weatherall DJ. Haemoglobin Constant Spring has an unstable chain messenger RNA. *British Journal of Haematology*. 1982; 51(3).

97. Winichagoon P, Fucharoen S, Svasti J. Identification of Hb C [beta 6(A3) Glu-->Lys] in a Thai male. *Hemoglobi*n. 1993; 17(5): 419-25.

98 Pootrakul S, Wasi P, Na-Nakorn S. Haemoglobin J-Bangkok: A Clinical, Haematological and Genetical Study. *British Journal of Haematology*. 1967;13(3):303-9.

99 Wasi P, Pootrakul S, Na-Nakorn S, Beale D, Lehmann H: Haemoglobin D *β* Los Angeles (D Punjab, $\alpha_2\beta_2$ 121 Glu NH2) in a Thai Family. *Acta Haematol* 1968; 39:151-158

100. Pillers DA JM, Head C, Jones RT. Hb Hope [beta 136(H14) Gly→Asp] and Hb E [beta 26(B8)Glu→Lys]: compound heterozygosity in a Thai Mien family. *Hemoglobin*. 1992; 16(1-2).

101. Jean Riou, Christian Godart, Didier Hurtrel, Mireille Mathis, Catherine Bimet, Josiane Bardakdjian-Michau, *et al*. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. *Clinical Pathology*. 1997; 43: 34-9.

102. Colah RB, Surve R, Sawant P, D'Souza E, Italia K, Phanasgaonkar S, *et al*. HPLC studies in hemoglobinopathies. *Indian J Pediatr*. 2007; 74(7): 657-62.

103. Clarke GM, Higgins TN. Laboratory Investigation of Hemoglobinopathies and Thalassemias: Review and Update. *Clin Chem*. 2000; 46(8): 1284-90.

104. Galanello R, Barella S, Gasperini D, L. Perseu L, Paglietti E. Evaluation of an automatic HPLC analyser for thalassemia and haemoglobin variants screening. *Journal of Automatic Chemistry*. 1995; 17(2): 73-6.

105. Sachdev R, Dam AR, Tyagi G. Detection of Hb variants and hemoglobinopathies in Indian population using HPLC: report of 2600 cases. *Indian J Pathol Microbiol*.2010; 53(1):57-62.

106. Riou J, Godart C, Hurtrel D, Mathis M, Bimet C, Bardakdjian-Michau J, *et al*. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. *Clin Chem*.1997; 43(1): 34-9.

107. Fucharoen S, Winichagoon P, Wisedpanichkij R, Sae-Ngow B, Sriphanich R, Warangkana Oncoung, *et al*. Prenatal and postnatal diagnoses of thalassemias and hemoglobinopathies by HPLC. *Clinical Chemistry*. 1998; 44(4): 740-8.

108. Fucharoen G, Srivorakun H, Singsanan S, Fucharoen S. Presumptive diagnosis of common haemoglobinopathies in Southeast Asia using a capillary electrophoresis system. *International Journal of Laboratory Hematology*. 2011

109. Higgins T, Mack M, Khajuria A. Comparison of two methods for the quantification and identification of hemoglobin variants. *Clinical Biochemistry*. 2009; 42(7-8): 701-5.

110. SEBIA Instruction; Capillarys hemoglobin. 2008;1.

111. Mullis KB. The Unusual Origin of The Polymerase Chain Reaction. *Scientific American*. 1990: 56-65.

112. V K, Kakavas, Noulas A, Chalkias C, Hadjichristodoulou C, Georgiou I, *et al*. Identification of the Four Most Common β-Globin Gene Mutations in Greek β-Thalassemic Patients and Carriers by PCR-SSCP:Advantages and Limitations of the Method. *Journal of Clinical Laboratory Analysis.* 2006; 21(20): 1-7.

113. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of alpha -thalassemia. *Blood*. 2000; 95(1): 360-2.

114. Erilich HA. Polymerase Chain Reaction. *Journal of Clinical Immunology*. 1989; 9(6). 437-47.

115. S.Chamberlainl J, A.Gibbs R, E.Ranierl J, Nguyen PN, Caskey CT. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research*.1988; 16: 11141-56.

116. Markoulatos P, Siafakas N, Moncany M. Multiplex Polymerase Chain Reaction: A Practical Approach. *Journal of Clinical Laboratory Analysis*. 2002; 16: 47-51.

117. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*. 1975; 94(3): 441-6.

118. Chern JPS, Lin K-H, Lin M-YL-T, Jou S-T. β-Thalassemia Major Births After National Screening Program in Taiwan. *Pediatr Blood Cancer*. 2008; 50: 58-61.

119. White BA, Dicker AP, Volkenandt M, Bertino JR. Manual and Automated Direct Sequencing of Product Generated by the Polymerase Chain Reaction. PCR Protocols: Humana Press; 1993. p. 143-52.

120. Rees BWD. DNA sequencing. *The Nutrition Society*. 1996; 55: 605-12.

121 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*. 1977; 74(12): 5463-7.

122 Kotler L, Sobolev I, Ulanovsky L. DNA sequencing: modular primers for automated walking. *Biotechniques*. 1994;17(3): 554-9.

123. Rahbar S, Nozari G AY, Martin PA, Yeh CH, TD. L. Association of Hb Hope (beta 136(H14)Gly→Asp)and alpha-thalassemia-2 (3.7 Kb deletion) causing severe microcytic anemia. *Hemoglobin*. 1992; 16: 421-5.

124. Ali Ou Alla S, Allali F, Hajjaj-Hassouni N. Rheumatoid arthritis in patient with homozygous haemoglobin C disease. Rheumatology International. 2009:1-3.

125. Sharada AS. Thalassemia and related Hemoglobinopathies. *Indian Journal of Prediatics*. 2005; 72: 319-24.

126. Vella F, Hart PL. Sickle-cell anaemia in an Indian family in Malaya. *Med J Malaya*. 1959; 14: 144-50.

127. Harris JL. Sickle Cell Disease. illustrated ed; 2001. p. 32-3.

APPENDIX

1. Materials

1.1 Instruments and laboratory supplies for MARMS-PCR

- Bench-top microcentrifuge (Denville 260D)
- Horizontal gel electrophoresis (Mupid, Japan)
- ChemiDoc™ XRS System (Bio-Rad Laboratories Ltd., USA)
- Veriti™ 96-Well Thermal cycler (Applied Biosystems, USA)
- Automated DNA preparation Maxwell®16 (Promega Corporation, USA)
- Microwave (Sharp, Japan)
- Glassware (Pyrex, USA)
- Freezer (-20°C) (Mirage)
- Freezer (-30°C) (Sanyo, Japan)
- Refrigerator (4°C) (Sanyo, Japan)
- 1.5 ml and 0.5 ml Microcentrifuge tube (Treff, Switzerland)
- 1x8 Cap Strips, 0.2 mL (Corning Incorporated, USA)
- 1x8 Tube Strips, 0.2 mL (Corning Incorporated, USA)
- Automatic pipette, P2/10/20/100/200/1000µl (Gilson, France)
- Pipette tip 2-10 µl, white (Gilson)
- Pipette tip 20-200 µl, yellow (Coster)
- Pipette tip 1000 µl, blue (Treff)

1.2 Reagents for DNA isolation

- The Maxwell® 16 Blood DNA Purification Kit (Promega Corporation, USA)

1.3 Reagents for multiplex ARMS-PCR

- Go taq® Flexi DNA polymerase (Promega Corporation, USA)

- Oligonucleotide primers (Bioservice unit, National Science And Technology Development Agency (NSTDA), Thailand)
- 5X PCR buffer provide with Go taq® Flexi DNA polymerase (Promega Corporation, USA)
- 25 mM $MgCl_2$
- Deoxynucleotide triphosphate: 1mM each of dATP, dTTP, dCTP, dGTP
- Deionized water

1.4 Reagents for agarose gel electrophoresis

- 10X TBE buffer:
  60.5 g Tris base 30.85 g Boric acid, 7.445 g EDTA
- 1X TBE buffer:
  100 ml 10X TBE buffer, 900 ml distill water
  (Mix 10X TBE buffer with distill water, store at room temperature)
- Gel loading dye solution:
  0.1 g Bromophenol blue, 0.1g Xylene cyanol, 50 ml Glycerol, 50 ml 1X TBE
  Filter through Whatman paper#1, store at 4^oC
- Gel marker: contain linear double stranded DNA bands of 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 base pairs (Promega, USA)
- Aagarose gel (Sigma, USA)
- Ethidium bromide (Promega, USA)

BIOGRAPHY

NAME	Miss Noppawan Tangbubpha
DATE OF BIRTH	10 March 1975
PLACE OF BIRTH	Suphanburi, Thailand
INSTITUTIONS ATTENDED	Sukhothai Thammathirat University, 1997–2004: Bachelor of Public Health (Health Laboratory) Mahidol University, 2007–2011 : Master of Science (Clinical Pathology)
RESEARCH GRANT	Supported in part by the Thesis Grant, Faculty of Graduate Studies, Mahidol University, Bangkok Thailand
HOME ADDRESS	30/81 Regent Ratchada Condo, Soi Inthamara 49, Din Daeng, Bangkok, Thailand 10400 E-mail: npt_heracp@hotmail.com