การประยุกต์ใช้อินฟราเรดสเปกโทรสโกปีสำหรับการจำแนกธาลัสซีเมียและฮีโมโกลบินอี

นางสาวสมสมร สุขพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2559

APPLICATION OF INFRARED SPECTROSCOPY FOR CLASSIFICATION OF THALASSEMIA AND HEMOGLOBIN E

Somsamorn Sukpong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Sciences

Suranaree University of Technology

Academic Year 2016

APPLICATION OF INFRARED SPECTROSCOPY FOR CLASSIFICATION OF THALASSEMIA AND HEMOGLOBIN E

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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สมสมร สุขพงษ์ : การประยุกต์ใช้อินฟราเรดสเปกโทรสโกปีสำหรับการจำแนกธาลัสซีเมียและฮีโมโกลบินอี (APPLICATION OF INFRARED SPECTROSCOPY FOR CLASSIFICATION OF THALASSEMIA AND HEMOGLOBIN E) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ทัศนีย์ เสาวนะ, 83 หน้า.

ธาลัสซีเมียและฮีโมโกลบินผิดปกติเป็นโรคโลหิตจางที่ถ่ายทอดทางพันธุกรรม มีสาเหตุมาจากความผิดปกติของฮีโมโกลบิน การวินิจฉัยที่ครอบคลุมและถูกต้องมีหลากหลายวิธีซึ่งแต่ละวิธีมีความซับซ้อน ราคาแพง และต้องใช้บุคลากรที่มีความชำนาญในการตรวจวินิจฉัย จึงเป็นข้อจำกัดในหลาย ๆ ประเทศที่จะวินิจฉัยโรคนี้ ดังนั้นงานวิจัยนี้จึงประยุกต์ใช้อินฟราเรดสเปกโทรสโกปีสำหรับการจำแนกธาลัสซีเมียและความผิดปกติของฮีโมโกลบิน (โดยเฉพาะฮีโมโกลบินอีที่พบมากในประเทศไทย) เพราะเป็นวิธีที่ง่ายและมีข้อดีหลายประการคือ (ก) ไม่ต้องใช้น้ำยาทดสอบใด ๆ (ข) ใช้ปริมาณตัวอย่างน้อย (2 ไมโครลิตร) (ค) สามารถปรับใช้เป็นเครื่องมืออัตโนมัติได้ และ (ง) วิธีการไม่ซับซ้อน (ไม่ต้องการการฝึกฝนเพิ่มเติมมากนัก) ในงานวิจัยนี้ได้ทดสอบตัวอย่างทั้งหมด 124 ตัวอย่าง ประกอบด้วยกลุ่มของฮีโมโกลบินปกติ 24 ตัวอย่าง และ 20 ตัวอย่างของแต่ละกลุ่มของเบตาบวกธาลัสซีเมีย เบตาศูนย์ธาลัสซีเมีย ฮีโมโกลบินอี แอลฟาบวกธาลัสซีเมีย และแอลฟาศูนย์ธาลัสซีเมีย ผลจากการตรวจโดยอินฟราเรดสเปกโทรสโกปี (652 spectra) ได้ถูกนำมาวิเคราะห์จำแนกระหว่างกลุ่มของฮีโมโกลบินปกติและฮีโมโกลบินของเบตาบวกธาลัสซีเมีย เบตาศูนย์-ธาลัสซีเมีย ฮีโมโกลบินอี แอลฟาบวกธาลัสซีเมีย และแอลฟาศูนย์ธาลัสซีเมีย โดยใช้การวิเคราะห์ PCA และ UHCA ของโปรแกรม Unscrambler ผลของการวิเคราะห์มีการจำแนกความแตกต่างระหว่างกลุ่มฮีโมโกลบินปกติและฮีโมโกลบินผิดปกติที่เป็นธาลัสซีเมียได้ทุกชนิด โดยในธาลัส-ซีเมียกลุ่มต่าง ๆ กลุ่มของแอลฟาศูนย์ธาลัสซีเมียมีความแตกต่างจากกลุ่มปกติมากที่สุด รองลงมาคือกลุ่มของฮีโมโกลบินอี แอลฟาบวกธาลัสซีเมีย เบตาบวกธาลัสซีเมีย และเบตาศูนย์ธาลัสซีเมีย ตามลำดับ

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

SOMSAMORN SUKPONG : APPLICATION OF INFRARED SPECTROSCOPY FOR CLASSIFICATION OF THALASSEMIA AND HEMOGLOBIN E. THESIS ADVISOR : ASSOC. PROF. TASSANEE SAOVANA, Ph.D. 83 PP.

THALASSEMIA/ HEMOGLOBINOPATHY/ HEMOGLOBIN E/ INFRARED SPECTROSCOPY/BETA THALASSEMIA/ ALPHA THALASSEMIA/ FTIR

Thalassemia and hemoglobinopathy are the hereditary anemia. They are caused by hemoglobin (Hb) disorders. There are many comprehensive and accurate diagnostic methods but they were more complex, expensive and labor-intensive. In many countries, there are limited facilities to diagnose these diseases. Therefore, the application of infrared (IR) spectroscopy technique for classification of thalassemias and hemoglobinopathy (especially HbE which is common in Thailand) was studied because it is easy to use and has many advantages: (a) it is reagent free, (b) it uses a small amount of sample (2 μL), (c) it can be automated and (d) it is simple (not acquire more training). In this study, the total 124 samples were obtained, 24 samples were normal hemoglobin and each group with 20 samples of β^+ thalassemia, β^0 thalassemia, HbE, α^+ thalassemia and α^0 thalassemia hemoglobin. The results of this research by IR spectroscopy (652 spectra) were analyzed between normal hemoglobin and hemoglobin of β^+ thalassemia, β^0 thalassemia, HbE, α^+ thalassemia and α^0 thalassemia using PCA and UHCA analysis of Unscrambler software. The result found that this technique can be used to classify between normal hemoglobin and abnormal

hemoglobin of all thalassemias. Among these thalassemic groups, α^0 thalassemia was mostly differentiate from normal group, followed by HbE, α^+ thalassemia, β^+ thalassemia and β^0 thalassemia, respectively.

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Assoc. Prof. Dr. Tassanee Saovana for all the suggestions and giving me the opportunity for the research of my graduate study. I really appreciate her knowledge, support, encouragement, and patience. I would like to extend many thanks to Dr. Kanjana Thumanu for her expert contribution of the FTIR instrument and Dr. Yupin Jopang for counselling knowledge of thalassemias and Hb variants.

I would also like to thank Dr. Kanjana Thumanu and Asst. Prof. Naporn Uengarporn, M.D., for taking their times to serve on my thesis committee. I also wish to express my gratitude to the staff members of the Health Promoting Hospital, the Genome Laboratory Company and the Regional Blood Center for providing me the samples.

Many thanks to the Synchrotron Light Research Institute (Public Organization) for the opportunity they gave to take laboratory tests and support everything related to my study.

I am extremely grateful for the National Blood Center and Suranaree University of Technology thesis support grants for providing the financial support and giving me the opportunity to conduct and complete my thesis.

Finally, I want to extend a huge thanks to my family for their support, encouragement, and endless love.

Somsamorn Sukpong

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LISTS OF ABBREVIATIONS

aâ	=	amino acid
ALA	=	δ-aminolevulinic acid
DCIP	=	dichlorophenolindophenol precipitation test
DNA	=	deoxyribonucleic acid
DPG	=	2,3diphosphoglycerate
EDTA	=	ethylenediaminetetraacetic acid
Fe^{2+}	=	ferrous
Fe^{3+}	=	ferric
FTIR	=	fourier transform infrared spectroscopy
fL	=	femtoliter
Hb	=	hemoglobin
HPLF	=	hereditary persistence of fetal hemoglobin
HPLC	=	high performance liquid chromatography
IR	=	infrared
KBr	=	potassium bromide
L	=	liter
MCH	=	mean corpuscular hemoglobin
MCV	=	mean corpuscular volume
NaCl	=	sodium chloride
NSS	=	normal saline solution

LIST OF ABBREVIATIONS (Continued)

OF test	=	one tube osmotic fragility test
PBG	=	porphobilinogen
PCA	=	principal component analysis
PCR	=	polymerase chain reaction
PCS	=	principal components
pg	=	picogram
RBC	=	red blood cell
RES	=	reticuloendothelial systems
SD	=	standard deviation
SEM	=	standard error of mean
WHO	=	World Health Organization
UHCA	=	unsupervised hierarchical cluster analysis
µL	=	microliter
µm	=	micrometer
MCT	=	mercury cadmium telluride
α	=	alpha
β	=	beta
γ	=	gamma
δ	=	delta
ε	=	epsilon
ζ	=	zeta

CHAPTER I

INTRODUCTION

1.1 Rational of the study

Thalassemia and hemoglobinopathy are the hereditary anemia. They are caused by hemoglobin (Hb) disorders. It is called thalassemia when Hb decreases or does not synthesize, while it is called hemoglobinopathy when synthesized Hb is deformed. These unusual conditions of red blood cells, which have short life span lead to anemia. In 2008, the World Health Organization (WHO) reported the prevalence of carriers of hemoglobin gene variants and affected conceptions (Table 1.1). The prevalence of the population carrying in the significant variations, α^{+} thalassemia and any variant which were 5.2%, 20.7% and 24%, respectively. Thalassemia and hemoglobinopathy worldwide are at risk for having children with the hemoglobin disorders around 1.1% and 2.7 per 1000 conceptions are affected, whereas the prevalence of the affected births (under age 5 years mortality) were 3.4% (Modell and Darlison, 2008). In Thailand, about 1% of the population have the disease (Wibulpolprasert, 2011) and the prevalence in α thalassemia, β thalassemia and hemoglobin E (HbE) were 16-30%, 2-10% and 8-54%, respectively (Ratanasiri, 2013) (Figure 1.1). The only treatment of these diseases in most countries is regular blood transfusion and iron chelation that is recommended in the patients with iron overload. Therefore, the improvement of diagnosis, prevention and management have been done in many countries for preventing and controlling these diseases (Fucharoen and Winichagoon, 2010).

Table 1.1 Estimated prevalences of carriers of hemoglobin gene variants and affected conceptions (Modell and Darlison, 2008).

WHO region	Demography 2003				% of the population carrying			Affected conceptions (per 1000)			Affected births
	Population (millions)	Crude Birth rate	Annual births (1000s)	Under-5 mortality rate	Significant variant[a]	α^+ thalassemia[b]	Any variant[c]	Sickle-cell disorders[d]	Thalassemias[e]	Total	(% of under-5 mortality)
African	586	39.0	22895	168	18.2	41.2	44.4	10.68	0.07	10.7	6.4
American	853	19.5	16609	27	3.0	4.8	7.5	0.49	0.06	0.54	2.0
Eastern - Mediterranean	573	29.3	16798	108	4.4	19.0	21.7	0.84	0.70	1.54	1.4
European	879	11.9	10459	25	1.1	2.3	3.3	0.07	0.13	0.20	0.8
South East-Asian	1564	24.4	38139	83	6.6	44.6	45.5	0.68	0.66	1.34	1.6
Western - Pacific	1761	13.6	23914	38	3.2	10.3	13.2	0.00	0.76	0.76	2.0
World	6217	20.7	128814	81	5.2	20.7	24.0	2.28	0.46	2.73	3.4

[a] Significant variants include HbS, HbC, HbE, HbD etc. β thalassaemia, α^0 thalassaemia. [b] α^+ thalassaemia includes heterozygous and homozygous α^+ thalassaemia. [c] Allows for (1) coincidence of α and β variants, and (2) harmless combinations of β variants. [d] Sickle-cell disorders include SS, SC, S/β thalassaemia. [e] Thalassaemias include homozygous β thalassaemia, haemoglobin E/β thalassaemia, homozygous α^0 thalassaemia, α^0/α^+ thalassaemia (haemoglobin H disease).

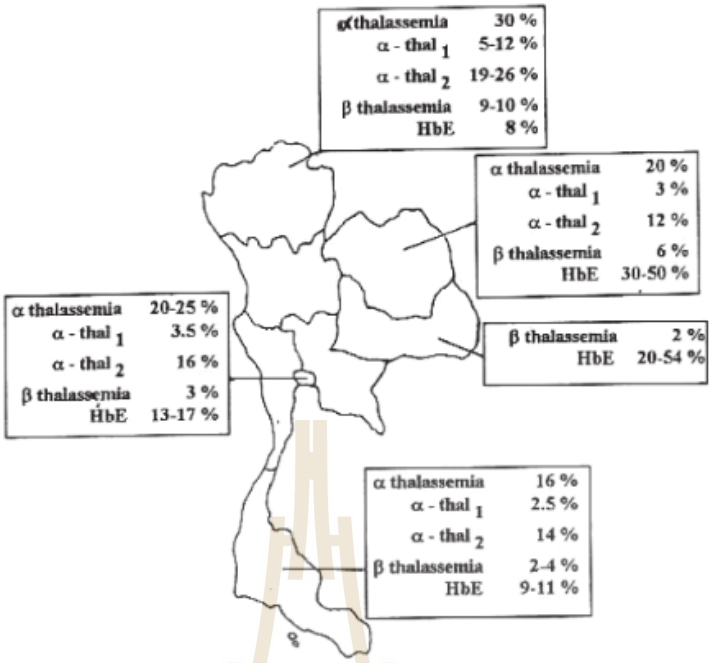


Figure 1.1 Prevalence of thalassemias in Thailand (Ratanasiri, 2013).

Normal Hb consists of 2 α globin chains and 2 non-α globin chains. Alpha globin chains are controlled by the gene cluster on chromosome 16 which synthesizes ζ and α chains and consists of 141 amino acids. The other chains are controlled by the gene cluster on chromosome 11 which establishes ε, γ, β and δ chains and consists of 146 amino acids. Synthesis of Hb chains depend on the patterns and the quantity of the periods. During the gestation period, there are Hb Gower I, Hb Gower II and Hb Portland, respectively. After birth, they turn to be HbF, HbA and HbA_2. Later, HbF decreases and HbA increase until adulthood. It is found that HbA is the highest form of Hb in adult with approximately 95-97.5%, followed by HbA_2 (2.5-3.5%) and HbF (less than 1%). Thus, each Hb form composes of 2 α globin, and 2 non- α globin such as Hb Gower I ($\zeta_2\varepsilon_2$), Hb Gower II ($\alpha_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$), HbF ($\alpha_2\gamma_2$), HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$) (Bain et al., 2010; Bunn et al., 1986).

Thalassemias are the result of autosomal recessive genes inherited from both father and mother. They are result in an imbalance of Hb by decreasing some Hb chains and increasing the other Hb chains which cause the hypochromic red blood cell (RBC). These hypochromic RBCs are destroyed rapidly, so the thalassemic patients show spleen and liver enlargement and anemia (Bank, 1978).

Hemoglobinopathy is an abnormal structure of Hb. It is caused by mutation of the gene which change the type or amount of amino acid in the Hb polypeptides. For example, HbE is unusual amino acid in β chain at position 26 that changes from glutamic acid to lysine. HbS is abnormal in the same β chain that deforms amino acid at position 6 and changes glutamic acid to valine. Hb Constant Spring has added 31 amino acids in α chain (Scott et al., 2013). Types of thalassemia are named according to the genotypic diagnosis, for example, alpha thalassemia is an abnormality of α globin chain and beta thalassemia is the deformation of β globin chain.

Diagnosis of thalassemia starts with the screening methods which include one tube osmotic fragility (OF) test, dichlorophenolindophenol (DCIP) precipitation test and red blood cell indicies [mean corpuscular volume (MCV) < 80 femtoliter (fL) and mean corpuscular hemoglobin (MCH) < 27 picogram (pg)]. Later, high performance liquid chromatography (HPLC) or electrophoresis will be used to separate types of Hb, and then molecular testing techniques are finally used to affirm the diagnosis (Bain, 2011; Fucharoen et al., 2004a). These techniques are more complex, costly, and require special instruments. From the research of Kan-Zhi Liu et al., they found the novel approach of infrared spectroscopy (IR) which was used to distinguish the normal Hb, β thalassemia and HbH. The principle of this technique is using the chemical groups to vibrate spectra insight into the binding of ligands. The main IR absorption bands arise

from N-H, C=O, C-H and P=O bonds found in proteins, lipids and nucleic acid. It is easy to use and has many advantages: (a) it is reagent free, (b) it uses a small amount of sample, (c) it can be automated and (d) it is simple (not acquire more training) (Liu et al., 2003; Wan et al., 2013a).

1.2 Research objective

To use the IR technique in classification the abnormal Hb of thalassemia and hemoglobin E patients.

1.3 Research hypothesis

The IR technique can be used to classify between normal Hb and abnormal Hb of thalassemia and hemoglobin E patients.

1.4 Expected result

The IR technique can be used to classify the abnormal Hb of thalassemia and hemoglobin E patients.

CHAPTER II

LITERATURE REVIEW

2.1 Hemoglobin

Hemoglobin (Hb) is the O_2 and CO_2 carrier which is in the red blood cells (RBCs). It is approximately 97% of RBC dry weight (Weed et al., 1963). Life span of RBC is 120 days (Shemin and Rittenberg, 1946), they are destroyed by macrophages and subsequent phagocytosis in the reticuloendothelial systems (spleen, liver and bone marrow) (Seaman et al., 1977).

Hb consists of heme and globin. Heme is a prosthetic group which seizes the O_2. Each heme has ferrous (Fe^{2+}) which is the main component at the middle and loosely captures the O_2. Heme synthesis begins by condensation of glycine and succinyl-CoA followed by decarboxylation to form δ-aminolevulinic acid (ALA). Two molecules of δ-aminolevulinic acid (ALA) form porphobilinogen (PBG) by ALA dehydratase or porphobilinogen synthase. Four PBG molecules condense into a cyclic tetrapyrrole to form uroporphyrinogen I or III. The type III isomer is converted from coproporphyrinogen III and protoporphyrinogen to protoporphyrin, then protoporphyrin and iron form heme (Figure 2.1) (Dailey and Meissner, 2013). Globin is a protein that consists of 4 polypeptide chains, then combines with heme to form hemoglobin which is the globular tetramer (Figure 2.2).

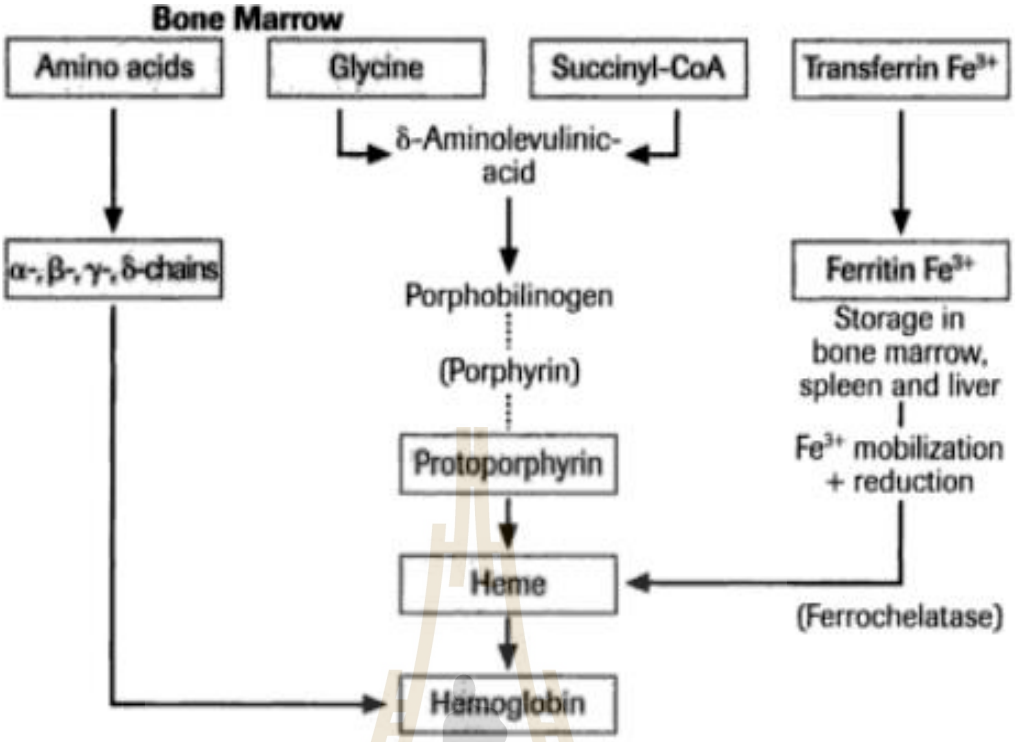


Figure 2.1 Hemoglobin synthesis (Dailey HA and Meissner PN, 2013).

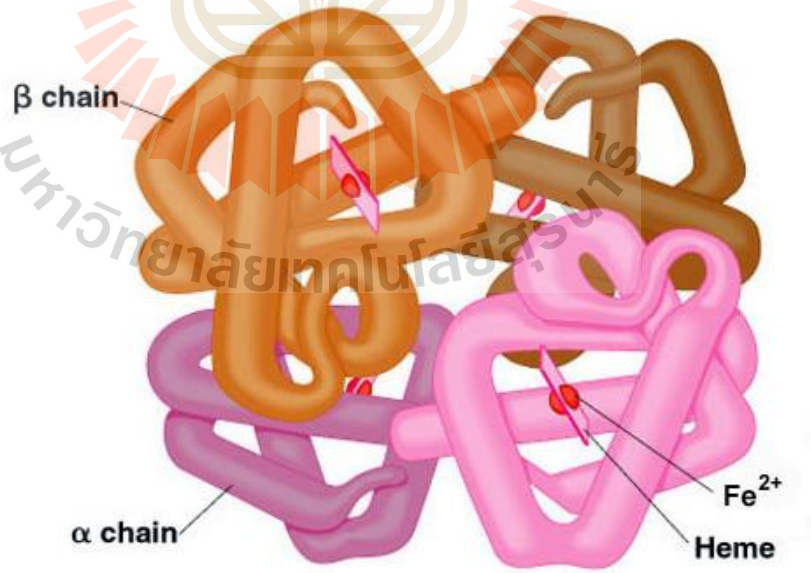


Figure 2.2 Structure of hemoglobin (http://themedicalbiochemistrypage.org/hemoglobin-myoglobin.php).

The tertiary structure of each globin has helical regions with 8-9 fragments depends on the type of polypeptides named A to H. Positions of amino acid in all conserved polypeptides are F8 (histidine) and CD1 (phenylalanine) which captures heme by covalent bond at helical region. These regions are more sensitive to mutate easily than other positions (Figure 2.3) (Thom et al., 2013b; Winslow, 2006).

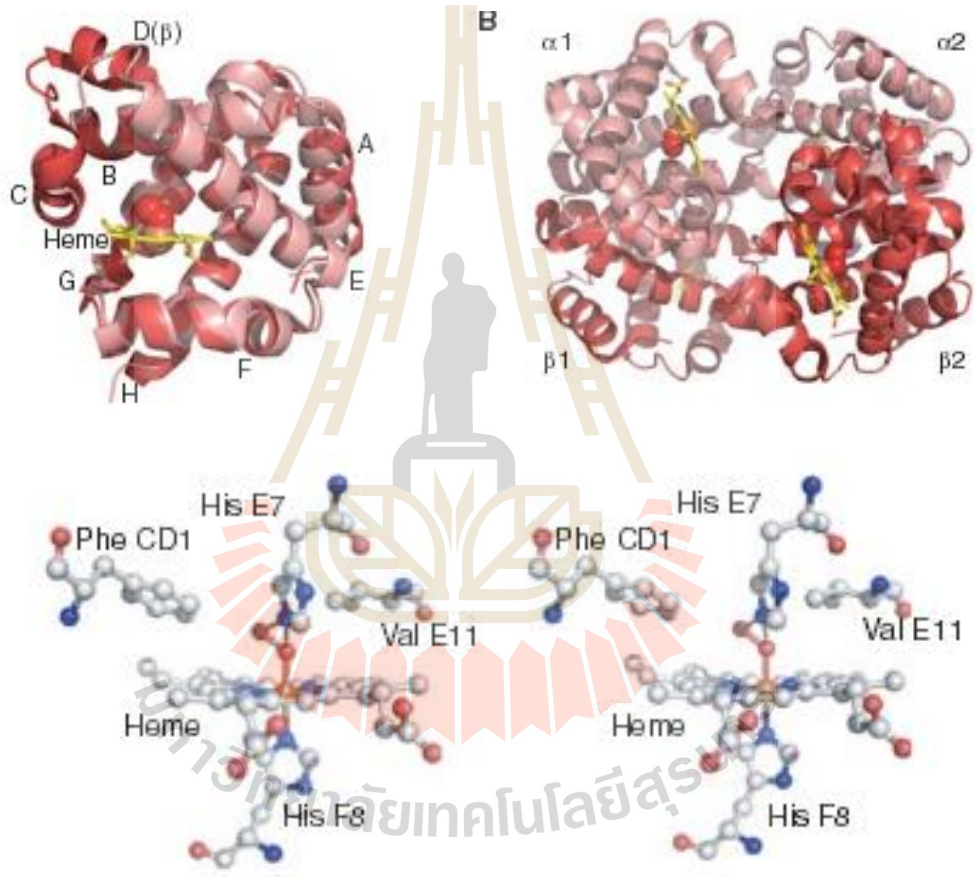


Figure 2.3 The tertiary structures of Hb (Thom et al., 2013).

The polypeptides found in Hb have many types and synthesize from genes which control the polypeptide synthesis, which depend on different stages of development. Previous studies found that genes which control polypeptide synthesis could distinguish into 2 groups: 1) α like genes (α globin like cluster) that are located

on chromosome 16 (nearly telomere) with 141 amino acids (aâ). These α like genes such as α and ζ gene synthesize α and ζ globin chain, respectively, and 2) β like genes (β globin like cluster) that have more genes than the first cluster, located on chromosome 11 with 146 aâ. These β like genes such as ε, γ, δ and β gene synthesize ε, γ, δ and β globin chain, respectively. There are also two groups of pseudogenes which can synthesize the polypeptides at a previous time, but have since mutated and do not synthesize the polypeptides anymore (Figure 2.4) (Weissbluth, 1974).

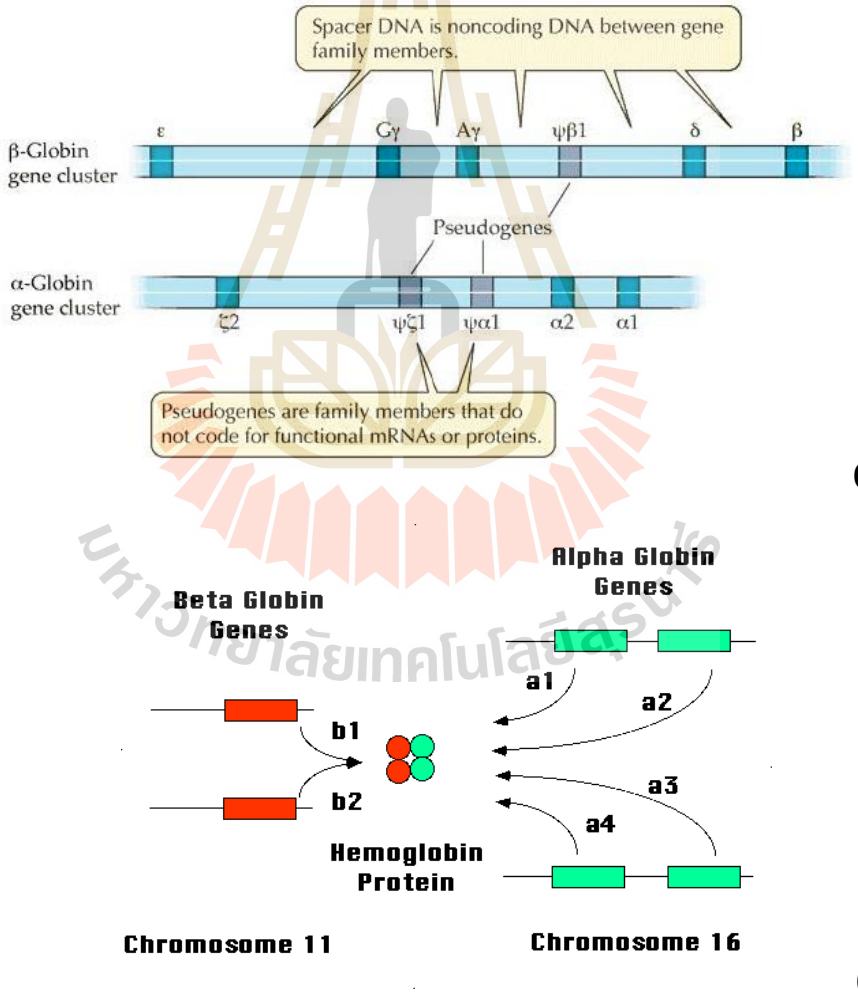


Figure 2.4 Location of β globin gene cluster and α globin gene cluster (a) to form hemoglobin protein (b) (http://sickle.bwh.harvard.edu/thal_inheritance.html).

These cluster genes (α like gene and β like gene) may have the same ancestral genes which produce the same number of amino acids. But δ gene has 10 amino acids and γ gene has 39 amino acids different from 146 amino acids of β gene (Figure 2.5) (Huisman, 1972).

```
        10         20         30         40         50         60
MVHLTPEEKS AVTALWGKVN VDEVGGEALG RLLVVYPWTQ RFFESFGDLS TPDAVMGNPK

        70         80         90        100        110        120
VKAHGKKVLG AFSDGLAHLD NLKGTFATLS ELHCDKLHVD PENFRLLGNV LVCVLAHHFG

       130        140
KEFTPPVQAA YQKVVAGVAN ALAHKYH
```

(a) β gene

```
        10         20         30         40         50         60
MVHLTPEEKT AVNALWGKVN VDAVGGEALG RLLVVYPWTQ RFFESFGDLS SPDAVMGNPK

        70         80         90        100        110        120
VKAHGKKVLG AFSDGLAHLD NLKGTFSQLS ELHCDKLHVD PENFRLLGNV LVCVLARNFG

       130        140
KEFTPQMQAA YQKVVAGVAN ALAHKYH
```

(b) δ gene

```
        10         20         30         40         50         60
MGHFTEEDKA TITSLWGKVN VEDAGGETLG RLLVVYPWTQ RFFDSFGNLS SASAIMGNPK

        70         80         90        100        110        120
VKAHGKKVLT SLGDAIKHLD DLKGTFAQLS ELHCDKLHVD PENFKLLGNV LVTVLAIHFG

       130        140
KEFTPEVQAS WQKMVTGVAS ALSSRYH
```

(c) γ gene

```
        10         20         30         40         50         60
MVLSPADKTN VKAAWGKVGA HAGEYGAEAL ERMFLSFPTT KTYFPHFDLS HGSAQVKGHG

        70         80         90        100        110        120
KKVADALTNA VAHVDDMPNA LSALSDLHAH KLRVDPVNFK LLSHCLLVTL AAHLPAEFTP

       130        140
AVHASLDKFL ASVSTVLTSK YR
```

(d) α gene

Figure 2.5 Amino acid sequences of β, δ, γ and α gene products (http://www.ncbi.nlm.gov/omim/?term=hemoglobin).

The products of the genes are globin polypeptides which are tetramers that consist of two α and two β chains. Occurrence of globin chains depends on the organ that synthesize the red blood cells during the development periods (Figure 2.6) (Rosenberg and Rosenberg, 2012).

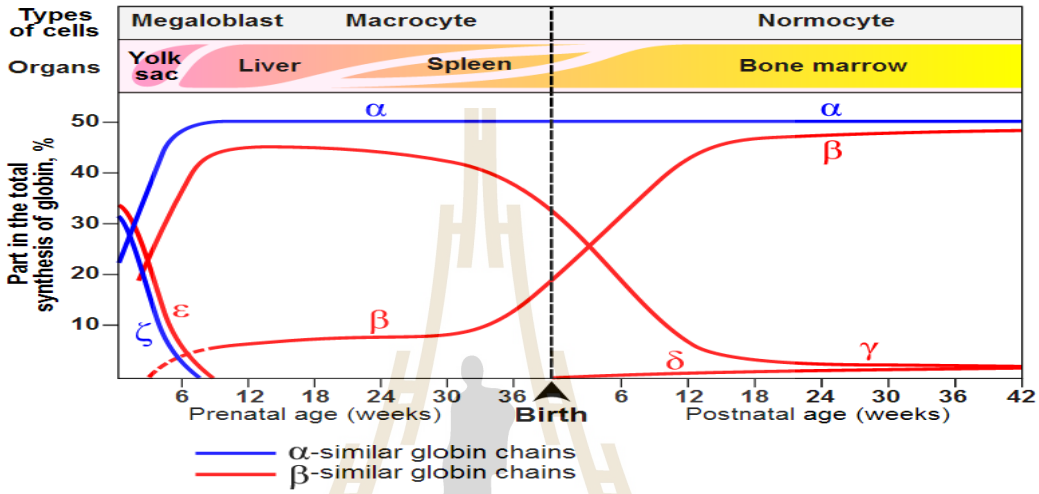


Figure 2.6 Gene expression of Hb before and after birth (Wood, 1976).

The development periods are

Period 1 Embryonic period is the period of red blood cell synthesis in the yolk sac. Types of Hbs in this period have 3 types as Hb Gower I ($\zeta_2\varepsilon_2$), Hb Gower II ($\alpha_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$).

Period 2 Fetal period (red blood cell synthesis in the liver, spleen, and bone marrow) is 5 weeks of gestation change from ζ chain to α chain found Hbs in 3 types as HbF ($\alpha_2\gamma_2$) 60-90%, HbA ($\alpha_2\beta_2$) 10-40% and HbA_2 ($\alpha_2\delta_2$) < 1%.

Period 3 After birth 3 months of age to adulthood (red blood cell synthesis in the bone marrow). Early synthesis of β chain in early birth until the adult period is HbA ($\alpha_2\beta_2$) found 95-97.5%, HbA_2 ($\alpha_2\delta_2$) 2.5-3.5% and HbF ($\alpha_2\gamma_2$) < 1% (Bunn et al., 1986; Nienhuis, 1987; Old, 2013; Wood et al., 1976).

Table 2.1 The types and amount of amino acids in β globin chain, α globin chain and δ globin chain of normal Hbs (HbA_2A).

Amino acid	Amount		
	β globin chain	**α globin chain**	**δ globin chain**
Alanine	15	21	15
Arginine	3	3	4
Asparagine	6	4	8
Aspartate	7	8	7
Cysteine	2	1	2
Glutamate	8	4	7
Glutamine	3	1	5
Glycine	13	7	13
Histidine	9	10	7
Isoleucine	-	-	-
Leucine	18	18	18
Lysine	11	11	11
Methionine	1	2	2
Phenylalanine	8	7	8
Proline	7	7	6
Serine	5	11	6
Threonine	7	9	5
Tryptophan	2	1	2
Tyrosine	3	3	3
Valine	18	13	17
Total	**146**	**141**	**146**

Table 2.1 displayed the types and amount of amino acids in the β, α and δ globin chain which were the component of normal Hbs (HbA_2A). There are difference in quantity of HbA_2 (2 δ globin chains combined with 2 α globin chains: less than or equal 3.5%) and HbA (2 β globin chains combined with 2 α globin chains: approximately 97%).

2.2 Definition and types of hemoglobinophathy and thalassemia

Deformity of Hb can be distinguished into two groups as 1) deformity of globin structures into many variant forms of globins called hemoglobinopathy and 2) decrease or failure to synthesize the globin chain (but structure is normal) called thalassemia. The pattern of these diseases is a recessive hereditary autosome, so the patient receives the deformed genes from both parents.

Hemoglobinopathy

Hemoglobinopathy is caused by mutated genes changing the amino acids in the globin structure while the number of the globin chains is normal. Changing of globin structures affect the O_2 capture of heme in RBCs. Most structural variants of Hb are caused by point mutation. Therefore, more than 1,000 variant forms can be found; half of these mutations are the normal phenotypes (Giardine et al., 2007). Gene mutations have many types, such as deletion of one or more bases, frameshift, or nonsense. Deletion is caused by missing cross over. Frameshift mutation makes the longer or shorter Hb and the nonsense mutation leads to finish the Hb synthesis. For example, in the β globin chains, HbS (sickle cell anemia) and HbC are the mutation from glutamic acid to valine and lysine at position 6, respectively. HbE is a mutation from glutamic acid to lysine at position 26. In α globin chain, Hb Mahidol is a mutation from aspartic acid to histidine at position 74. Other abnormal Hbs may occur by the addition of some amino acids resulting in longer chains, such as Hb Talk has 156 amino acids (β chain) and Hb Constant Spring has 172 amino acids (α chain). The hemoglobinopathy may mix with thalassemia such as β-thalassemia/HbE which is mostly found in Thailand (Fucharoen and Weatherall, 2012).

The hemoglobinopathy that was first found is sickle cell anemia, where the RBCs are sickle shape (Figure 2.7) in low O_2 condition resulting to be chronic anemia while normal RBCs are biconcave shape. These abnormal RBCs lead to the obstruction of the capillaries which are destroyed rapidly and may cause death. Genes that control sickle cell anemia compose of two alleles which are HbSHbS. Normal genes are HbAHbA and HbAHbS. The heterozygous gene (HbAHbS) shows asymptomatic but can be inherited. Molecules of HbA and HbS have different amino acid which is valine that replaces glutamic acid at position 6 (β chain) in HbS (Kaul, 2008).

Sickle cell trait (HbAHbS) shows asymptomatic except in low O_2 condition where partial RBCs may change to be sickly form and results in anemia. The patient with homozygous gene (HbSHbS) will encounter death of the fetal period. Furthermore, the HbS gene occurs in partial populations located in Africa where malaria is most prevalent. African populations have HbAHbS genotype approximately 30% (Nnaji et al., 2013). These people will not be infected with malaria because HbAHbS RBCs have an inappropriate condition for malaria growth.

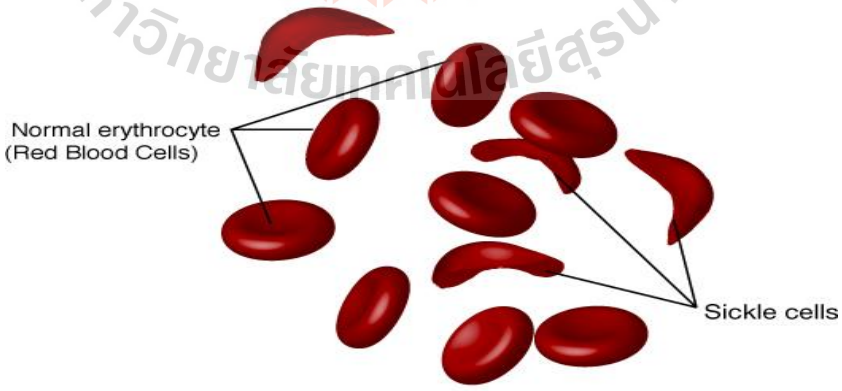
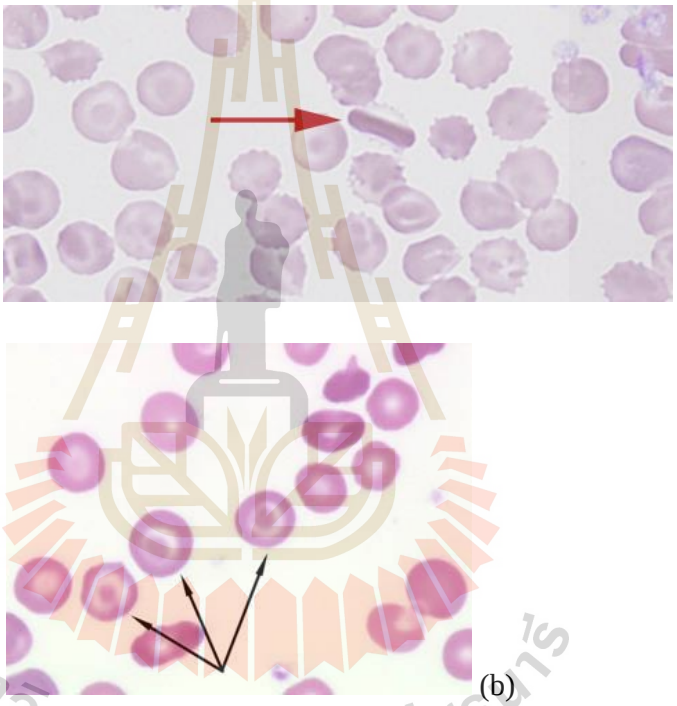


Figure 2.7 Sickle red blood cells (http://www.genome.gov/glossary/).

HbC is also prevalent in the African population but less frequency than sickle cell anemia (Williams and Weatherall, 2012). HbC RBCs have intracellular blunt ended crystalloids (Figure 2.8a) which cause less survival time and may cause vaso-occlusive. However, hemoglobinopathy with HbC is not as violent as sickle cell anemia. The HbC trait is asymptomatic like sickle cell trait. Homozygous HbC often shows many target cells (codocytes) in the blood smear (Figure 2.8b).



(a)

(b)

Figure 2.8 (a) Intracellular blunts ended crystalloids of HbC.

(b) Target cells (codocytes) (Ford, 2013).

The HbC gene from the father (or mother) may inherit with HbS gene from another, then occurs HbSC disease that has clinical severity of visual damage due to retinal vascular lesions which is worse than sickle cell anemia. The intracellular bodies

are the hybrids of the blunt ended crystalloids of HbC and the sharp-pointed tactoids of HbS (Nagel et al., 2003).

HbE is a very common β chain mutation and it is mostly found in Southeast Asian population (Fucharoen et al., 2004). The heterozygous gene is asymptomatic but causing microcytosis without anemia while the homozygous gene shows severe microcytosis and the little hypochromic RBCs.

Thalassemia

Thalassemias have the Hb disorder with reduction or absence of the Hb caused by the gene mutations. In case of decreased α globin chain, this is called α-thalassemia. On the other hand, decreasing of β globin chain is called β-thalassemia. Pathology of RBCs is caused by imbalance of Hb chains. For example, β-thalassemia patient has over production of the α globin chains, thus free globin chains can precipitate, resulting in hemolytic anemia with shortened RBCs life span. The quantity of RBCs is insufficient, so the patient must increasingly synthesize the RBCs from the bone marrow, then the bone marrow will expand and become fragile.

The Hb gene on chromosome 16 contains the α chain genes and the other on chromosome11 contains β chain genes. The linkage of genes on each chromosome turns to be inherited in a group which assort independently due to crossing over during gametogenesis. A linkage mutation affects the rate of β chain production but does not affect the rate of α production (Kato and Gladwin, 2009). Thalassemia has 2 types which are β thalassemia and α thalassemia.

Beta thalassemia is not common. The first description was written by Dr. Thomas Cooley in 1925. Cooley's anemia has been used synonymously with clinically severe forms of β thalassemia. Cooley's anemia was a fatal microcytic

anemia in Mediterranean children. The name thalassemia comes from ‘thalassa’ which is the classical Greek name for the Mediterranean Sea. Today, thalassemias in general turn to affect races of people from the tropical belt, especially Southeast Asia (Galanello and Origa, 2010).

There are two groups of β thalassemia based on the amount of β globin chain production. Different β thalassemia genes cause the disease with heterogeneous spectrum ranging from asymptomatic expression to classical, deadly Cooley’s anemia which are

1) β^0 thalassemia is the disease caused by the abnormal gene which do not produce the β chains. These genes produce very little of δ and γ chains after six months of age, leading to unstable α tetramers. These RBCs are destroyed while they are still in the bone marrow. Thus, the patient with this disease can survive if HbF is produced increasingly.

2) β^+ thalassemia is the disease caused by the abnormal genes that produce some of β chains. Homozygous β^+ thalassemia produces subnormal amounts of HbA, thus the α tetramers in RBCs are destroyed in the bone marrow, so the level of HbA is decreased.

Beta Thalassemia can be divided into three types from the genetic classification and the clinical presentation.

1. Heterozygous β thalassemia or thalassemia minor/ thalassemia trait is a complex of normal β gene and β thalassemia gene. β thalassemia gene synthesizes low β chain which reduces the HbA production and give the mild anemia, then cause high HbA_2 level (> 3.5%) whereas HbF level is normal (< 2.0%) (Ryan et al., 2010). Furthermore, peripheral blood smear shows target cells, ovalocytes and basophilic

stippling which are shown in Figure 2.9. For β-δ thalassemia trait, it has a deletion of β and δ globin gene which is novel found. RBCs morphology is similar to β thalassemia trait as microcytosis. Conversely, the patient with β-δ thalassemia has normal HbA_2 level and high HbF level (5-20%) (Hoyer et al., 2002).

2. Thalassemia intermedia is a puzzle complex that molecules are homozygous or compound heterozygous (β^0/β^+ or β^+/β^+) which reduce the β chain synthesis and lead to an imbalance of Hb chains due to an excess number of α globin chains. Since β globin chains do not produce the severe condition but still have more anemia than β thalassemia trait, thalassemia intermedia cannot be defined by a precise Hb level. The patients may require transfusion to maintain Hb levels to prevent bone abnormalities or splenic enlargement.

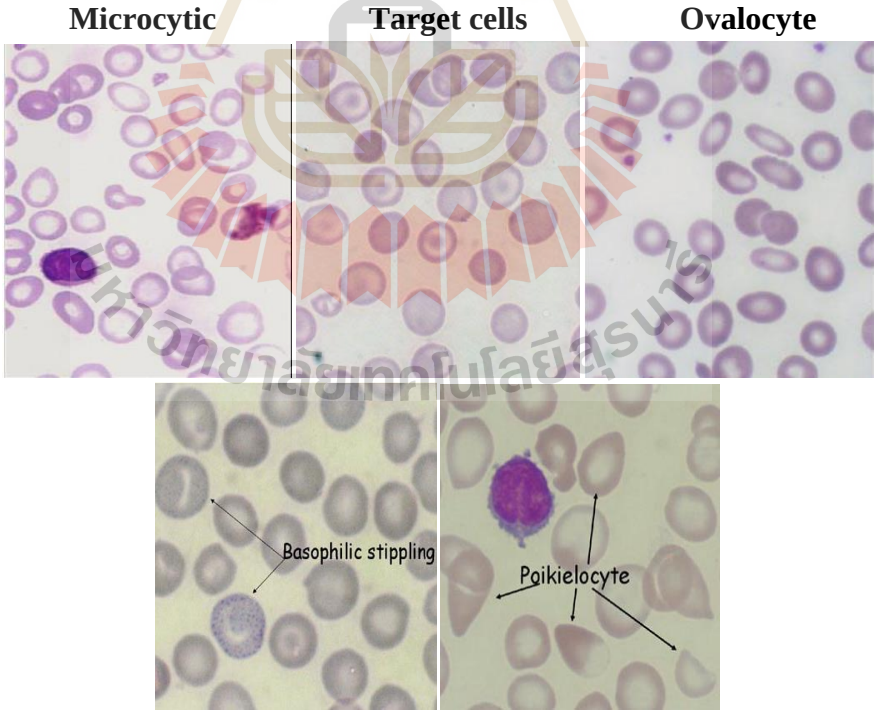


Figure 2.9 Characteristic of abnormal peripheral blood smear (http://home.kku.ac.th/acamed/kanchana/bsi.html).

3. Homozygous β thalassemia or thalassemia major or Cooley's anemia caused by β globin genes deletion, then it cannot produce β globin chains. HbA is not synthesized or synthesized very low by β^0/β^+ genes, then δ globin chains are produced a lot causing HbA_2 increase. The RBCs from homozygous thalassemia are strikingly with marked hypochromia, many microcytes, bizarre poikilocytes, and target cells (Figure 2.9) (Aster et al., 2012).

The disproportionate Hb synthesis is an excess of α globin chain leading to unstable RBCs. These abnormal erythrocytes precipitate in blood and are trapped into the reticuloendothelial systems (RES). This condition causes rapid destruction within the bone marrow which causes severe anemia. The clinical onset happens 6 months after birth caused by the normal postnatal Hb switching from γ globin chain to β globin chain.

In addition, the presence of HbE combines with β thalassemia is mostly found in Thailand varying from 3-50% (Weatherall and Clegg, 2001). Other hemoglobinopathies usually manifest when couple with β thalassemia gene and cause severe diseases, such as sickle β thalassemia which is merged between HbS gene and β thalassemia gene.

Alpha thalassemia occurs by the deletion of one or more of the four α globin genes on chromosome 16. Four α globin genes can be denoted as αα/αα. Each haplotype (αα) is inherited in couple from the parent unlike β thalassemia which can be single. Alpha thalassemia is present in fetus since the α chain is integral to all hemoglobins. Therefore, it is difficult to detect in heterozygous and an asymptomatic homozygous. The (α-) gene is called the α thalassemia 2 gene or α^+ thalassemia gene while the (--) gene or α^0 thalassemia gene is termed the α thalassemia 1. Thus,

α thalassemias have been divided into 4 types followed by the deletion of α globin genes (Figure 2.10) (Singer, 2009).

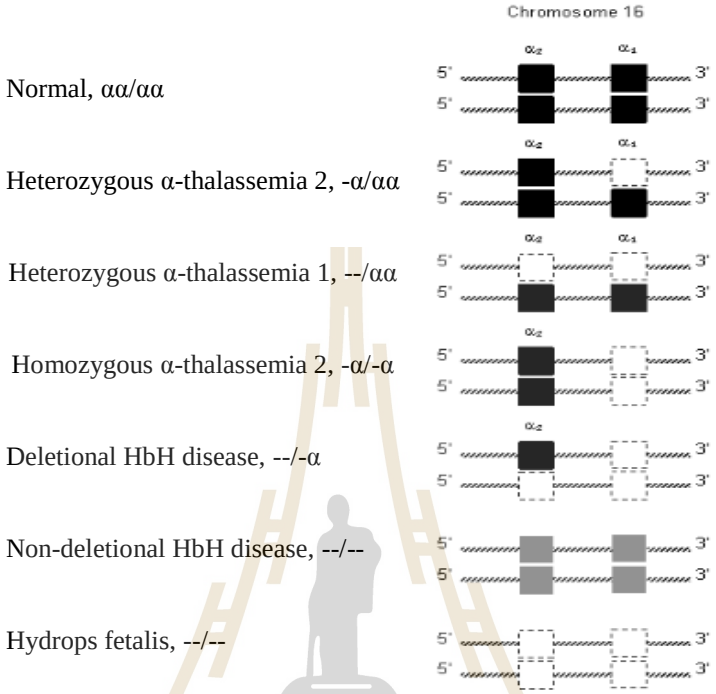


Figure 2.10 Diagrammatic representation of α thalassemia gene deletions. Black: normal gene, open dashed squares: gene deletion, solid gray squares: variable gene expression (Singer, 2009).

1. Alpha thalassemia 2 trait (-α/αα) is the deletion of one α globin gene. Clinical and hematological expressions are normal. Determination of the number of α globin genes by DNA analysis is the important diagnosis.

2. Alpha thalassemia 1 trait or homozygous α thalassemia 2 (-α/-α or --/αα) is deleting two genes from four α globin genes. The clinical sign is not usually anemia but may has microcytosis. Diagnosis is only to eliminate other causes of microcytosis and/or check RBCs indicies and finally confirm with DNA analysis.

3. Hemoglobin H disease (--/-α) is the deletion of three α globin genes. The proportion of α and β globin chains is an imbalance which causes an excess of free β globin chains. This occurrence is called hemoglobin H. Hemoglobin H is unstable and precipitate within RBCs. This precipitated RBCs demonstrate clumps, called Heinz bodies which are positive when staining with brilliant cresyl blue. The RE cells remove the damaged erythrocytes cause chronic microcytic and hemolytic anemia. Many patients with hemoglobin H disease have a hemoglobin variant as Hb Constant Spring (--/$\alpha^{CS}\alpha$) with splenomegaly and require more blood transfusion.

4. Hb Bart's hydrops fetalis (--/--) is the most severe because of the deletion of all α globin genes, then free γ globin chains are produced during the intrauterine period. The high O_2 affinity of Hb Bart's makes ineffective O_2 transport, causing the fetus to die in the uterine or in the immediate postnatal period. The severe anemia causes heart failure and subsequent massive total body edema which is called hydrops fetalis (Olivieri and Weatherall, 2007).

Besides, in case of β thalassemia, γ globin gene can act as β globin gene that is absent on chromosome 11. The surviving persons produce HbF instead of HbA which is called hereditary persistence of fetal hemoglobin (HPFH). This involves the genetic alterations that the patient produces HbF longer than the normal person. The non deletional forms of HPFH derive from point mutation in the promoter region of the γ genes. It overrides the signals of normally shut off γ globin gene transcription. Sometimes, patients with β thalassemia major have mild anemia because of substitute HbF production (Hagh et al., 2011).

2.3 Pathophysiology

Hemoglobinopathies

Hemoglobinopathies are the diseases where the protein chains have been substituted leading to changes of the Hb integrity. For example, substitution of glutamic acid by valine or lysine at position 6 of β chain produces HbS or HbC, respectively. Substitution of glutamic acid by lysine at position 26 of β chain produces HbE. The HbE-β thalassemia is found mostly in Thailand. These substitutions change the structures of Hb causing premature RBCs destruction. Abnormal globin structures manifest different RBCs function as follows.

1. Mutations at any portions of aâ sequences, 1) the regions of contact between α and β chains, 2) the C terminal regions, and 3) the regions that form the pocket which bind 2,3 diphosphoglycerate (DPG) will increase the O_2 affinity (Figure 2.11). The Hb picks up the O_2 from the alveoli but stingily gives up to the tissue which causes hypoxia to the kidney. Then, erythropoietin increases and produces more RBCs (Tosqui, 2010).

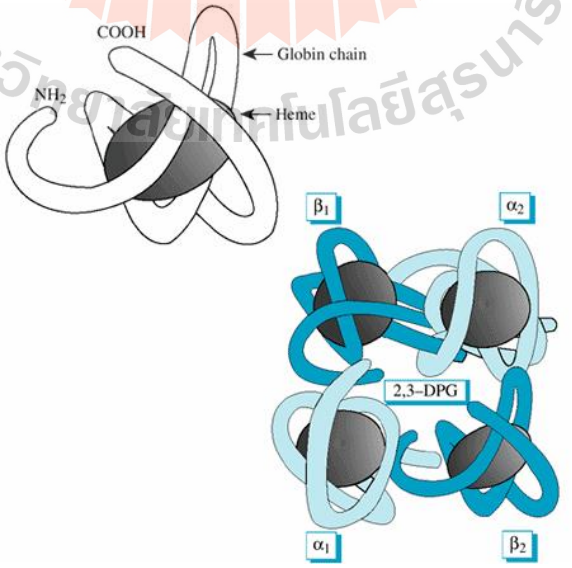


Figure 2.11 Position of 2,3-DPG molecule in hemoglobin chain (Hillman et al., 2005).

2. On the other hand, when the lung is dysfunctional, Hb will decrease the O_2 uptake which causes the low O_2 affinity. The reduced O_2 proportion of Hb is oxygenated at a given pO_2 to be the deoxygenated Hb. These RBCs are blue which cause cyanosis, bluish discoloration of skin and mucous membranes (Mounts et al., 2010).

3. Methemoglobinemia is a class of low O_2 affinity where Hb contains iron in the form of ferric (Fe^{3+}) oxidation state more than the normal ferrous (Fe^{2+}) state. The affected patients have cyanosis. Since methemoglobin is a brown pigment, the patients manifest brown blood (Thom et al., 2013a).

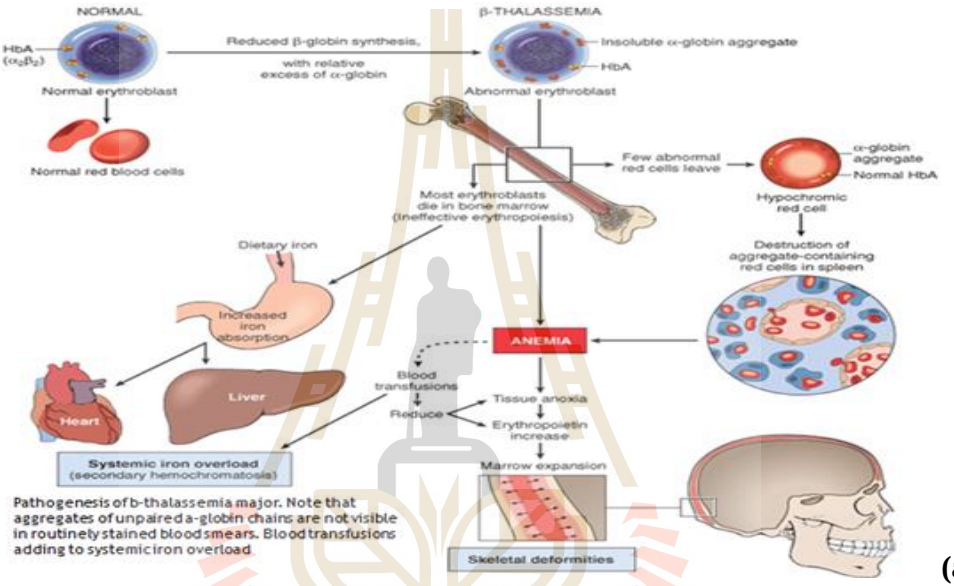
4. Unstable Hb is caused by abnormalities in the globin chain sequences. It forms the erythrocytic inclusions called Heinz bodies , then RBCs are destroyed in the spleen leading to anemia. (Asakura et al., 1975).

5. Sickling and crystallized RBCs are the phenomena in HbS and HbC, respectively. These forms are the most important abnormal Hbs. HbS causes sickle cell anemia that is chronic hemolysis and a vaso-occlusion. Effects of chronic hemolysis are anemia, jaundice, cholelithiasis, aplastic crisis and hemolytic crisis. The other clinical findings of vaso-occlusion are dactylitis, autosplenectomy, priapism, renal papillary necrosis, infarctive crisis, sequestration crisis and leg ulcers (Rees et al., 2010). HbC is less hemolysis and vaso-occlusion than HbS. Severity is great when HbC combines with HbS (Dalibalta et al., 2010).

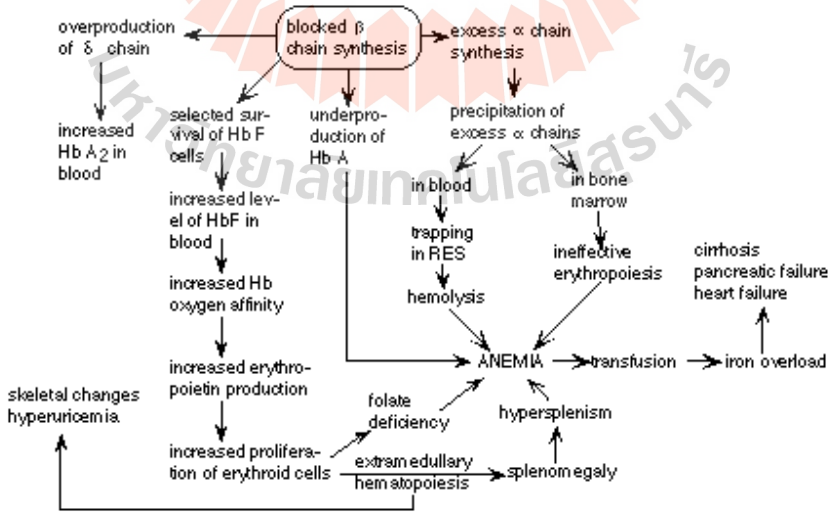
Thalassemias

β and α thalassemias are the public health problem and 20.7% of these diseases have been eliminated worldwide. Among this, it occurs in Southeast Asia approximately 44.6% (Modell and Darlison, 2008). These diseases need blood

transfusion because the incomplete RBCs appear hypochromic and will be destroyed before due time. Therefore, the patient has enlarged spleen and liver. Then, to produce more RBCs, bone marrow expands and erythropoietin increases which creates the skeleton deformities. The heart will enlarge because it works hard to pump sufficient blood (Figure 2.12a).



(a)



(b)

Figure 2.12 (a) Effects of Thalassemia disease to each organ. (b) Diagram of pathophysiology of β thalassemia major (Ed Uthman, 2009).

From figure 2.12b, the pathophysiology of β thalasssemia major is the best example, which are

1. When β chain synthesis decreases, HbA also decreases and causes the microcytic anemia.

2. The body will produce δ chains to compensate β chains, so HbA_2 increases.

3. In some cases, there are attempts to compensate by maintaining some production of HbF. Retention of HbF production is not as common as increased HbA_2. This condition is called HPFH.

4. In severe forms, there are many erythrocytic destruction caused by insufficient β globin chains and incomplete RBCs. Thus, over production of RBCs makes the marrow to expand. Extramedullary hematopoiesis and hemolysis cause splenomegaly.

5. The deficiency of folate causes megaloblastic anemia.

6. A treatment of thalassemia in major patients, blood transfusions are used to maintain Hb levels since children until ten years old. Overload of iron happens, when there are too frequent transfusions because the body cannot excrete the iron fast enough. The pancreas, liver, myocardium, adrenals and gonads are the most sensitive to iron toxicity. The results are diabetes mellitus, hepatic cirrhosis, congestive heart failure and adrenal failure. Therefore, the patients need to excrete the iron by using chelating agents (Higgs et al., 2012).

Alpha Thalassemia is an extreme disease that shows asymptom with mild laboratory abnormalities or the most severe symptom which causes death. The severity occurs from lack of RBCs because they are destroyed immediately. There are no

α globin like chains to combine with β globin like chains, so RBCs are incomplete since the fetus stage (Vichinsky, 2013).

2.4 Diagnosis of thalassemia and hemoglobinopathy

There are many methods to be screening tests, hemoglobin typing tests and DNA analysis of thalassemia and hemoglobinopathy. Screening tests employ 3 methods which are 1) red blood cell indices compose of mean corpuscular volume (MCV) less than 2 fold of standard deviation (SD) which depends on age and sex shown in Table 2.1 (Camitta and Jean Slye, 2012) and mean corpuscular hemoglobin (MCH) less than 27 picograms (Karimi and Rasekhi, 2002; Karnpean et al., 2011), 2) one tube osmotic fragility test (OF test) which measures the hemolysis in 0.36% NaCl. The thalassemia patients show partial hemolysis while normal people show complete hemolysis. This OF test gives approximately 3% of false positive (Chow et al., 2005; Winichagoon et al., 2002), and 3) dichlorophenol indophenols precipitation test (DCIP) which stains and precipitates the unstable hemoglobin. It has been used to screen hemoglobinopathies such as HbE and HbH (Fucharoen and Winichagoon, 2012).

Table 2.2 Mean levels of Hb and MCV at different ages. (Nathan and Oski, 1993)

Age	**Hb (g/dl) Mean (-2 SD)**	**MCV (fL) Mean (-2 SD)**
Birth (cord blood)	16.5 (13.5)	108 (98)
1 to 30 days	18.5 (14.5)	108 (95)
1 week	17.5 (13.5)	107 (88)
2 weeks	16.5 (12.5)	105 (86)
1 month	14.0 (10.0)	104 (85)
2 months	11.5 (9.0)	96 (77)
3 to 6 months	11.5 (9.5)	91 (74)
0.5 to 2 years	12.0 (10.5)	78 (70)
2 to 6 years	12.5 (11.5)	81 (75)
6 to 12 years	13.5 (11.5)	86 (77)
12 to 18 years		
Female	14.0 (12.0)	90 (78)
Male	14.5 (13.0)	88 (78)
Adults		
Female	14.0 (12.0)	90 (80)
Male	15.5 (13.5)	90 (80)

MCV is the technique for measuring the average volume of a red blood cell by dividing the hematocrit with the red blood cell count and multiplying by 10. The MCV categorizes red blood cells by size. Normal size of RBC is called normocytic RBC, microcytic RBC is a smaller cells and the larger cell is called macrocytic RBC. Normal range is 80-100 femtolitre.

$$\text{MCV} = \frac{\text{Hematocrit} \times 10}{\text{RBC} \times 10^{12}\ /\text{L}}$$

MCH is the average weight of hemoglobin per red blood cell which is calculated by using the amount of total hemoglobin per red blood cell multiplied by 10. The lower amount of hemoglobin is called hypochromic while the higher is called hyperchromic. Normal range is 27-31 picogram/cell (Bunn, 2012).

$$\text{MCH} = \frac{\text{Hb (g/dL)} \times 10}{\text{RBC} \times 10^{12}\ \text{/L}}$$

Hemoglobin typing tests have 2 principles which are pressure liquid chromatography (high and low performance liquid chromatography: HPLC and LPLC) and capillary electrophoresis. These methods are used to analyse the hemoglobin types and their results are quantitative. The most common test is HPLC. It can separate the hemoglobin by motility rate between stationary phase and mobile phase. Stationary phase has anion on the surface which will exchange hemoglobin. Mobile phase is the elution buffer which has higher ionic strength than hemoglobin. Thus, mobile phase will replace the hemoglobin. This hemoglobin will be eluted and passed through the column at the different times. The type of each hemoglobin is eluted, depending on the retention time. Quantity of hemoglobin is examined by the absorbance from the photometer and calculated from the area of each hemoglobin graph (Orsini et al., 2010). The results are the hemoglobin types in HbA, HbA_2/E and HbF with quantitative data. The interpretation of these data will be used to demonstrate the amount of HbA_2/E to diagnose thalassemia and HbE as shown in Table 2.2 (Department of Medical Science, 2010a).

Table 2.3 Summarizes the results of Hb typing (Department of Medical Science, 2010).

Hb typing	Interpretation	Screening test				Thalassemia types
		OF	DCIP	MCV (fL)	MCH (pg)	
A_2A, $HbA_2 < 4\%$	normal or non clinical significant thalassemia	-	-	≥ 80	≥ 27	α^+ thalassemia carrier
A_2A, $HbA_2 < 4\%$	normal Hb typing, not rule out α thalassemia	+	-	< 80	< 27	α^+ thalassemia carrier, α0 thalassemia carrier or homozygous α^+ thalassemia
A_2A, HbA_2 4-8%	β thalassemia trait with or without α thalassemia	+	-	< 80	< 27	β thalassemia trait with or without α thalassemia
EA, HbE ≥ 25%	HbE trait	-/+	+	< 80 or normal	< 27 or normal	HbE trait
EA, HbE < 25%	HbE trait with or without α thalassemia	+	+	< 80	< 27	HbE trait with or without α thalassemia
EE, HbE ≥ 80% HbF ≤ 5%	homozygous HbE with or without α thalassemia	+	+	< 80	< 27	homozygous HbE with or without α thalassemia

Table 2.3 Summarizes the results of Hb typing (Department of Medical Science, 2010) (Continued).

Hb typing	Interpretation	Screening test				Thalassemia types
		OF	DCIP	MCV (fL)	MCH (pg)	
EE/EF, HbE > 75% HbF > 5% (not sure EE or EF ↑)	suspected homozygous HbE or β thalassemia/HbE with or without α thalassemia (family study)	+	+	< 80	< 27	Suspected homozygous HbE (clinical thalassemia) suspected β thalassemia/HbE (no clinical thalassemia)
CS A_2A	suspected Hb Constant Spring	-/+	-	< 80 or normal	< 27 or normal	Hb Constant Spring
CS A_2A Bart's	suspected homozygous Hb Constant Spring	+	-	< 80	< 27	homozygous Hb Constant Spring
A_2A H or A_2A Bart's H	HbH disease (α^0 thalassemia/ α^+ thalassemia)	+	-/+	< 80	< 27	HbH disease (α^0 thalassemia/ α^+ thalassemia)
CS A_2A H or CS A_2A	HbH-CS disease (α^0 thalassemia/Hb Constant Spring)	+	-/+	< 80	< 27	HbH-CS disease (α^0 thalassemia/ Hb Constant Spring) Bart's H
A_2F (age > 1 year)	Homozygous β^0 thalassemia with or without α thalassemia	+	-	< 80	< 27	Homozygous β^0 thalassemia with or without α thalassemia

Table 2.3 Summarizes the results of Hb typing (Department of Medical Science, 2010) (Continued).

Hb typing	Interpretation	Screening test				Thalassemia types
		OF	DCIP	MCV (fL)	MCH (pg)	
EF, HbE 40-80%, HbF 20-60% or	suspected β^0 thalassemia/HbE or HPFH/HbE with or without α thalassemia	+	+	< 80	< 27	suspected β^0 thalassemia/HbE with or without α thalassemia (clinical thalassemia) suspected HPFH/HbE with without α thalassemia (no clinical thalassemia)
A_2FA, HbF 10-30%	suspected β^0/β^+ or β^+/β^+ or HPFH trait or $(\delta\beta)^0$ trait with or without α thalassemia	+	-	< 80	< 27	suspected β^0/β^+ or β^+/β^+ thalassemia with or without α thalassemia (clinical thalassemia) suspected HPFH trait or $(\delta\beta)^0$ trait with or without α thalassemia
EFA	β^+ thalassemia/HbE with or without α thalassemia	+	+	< 80	< 27	β^+ thalassemia/HbE with or without α thalassemia
EA Bart's	EA Bart's disease	+	+	< 80	< 27	HbH disease with HbE trait (α^0/α^+ thalassemia- β^E/β^A)

Table 2.3 Summarizes the results of Hb typing (Department of Medical Science, 2010) (Continued).

Hb typing	Interpretation	Screening test				Thalassemia types
		OF	DCIP	MCV (fL)	MCH (pg)	
EE Bart's or EF Bart's	EF Bart's disease	+	+	< 80	< 27	HbH disease with β^0 thalassemia/HbE or HbH disease with homozygous HbE (α^0/α^+ thalassemia-β^0/β^E or β^+/β^E)
EFA Bart's	EF Bart's disease	+	+	< 80	< 27	HbH disease with β^+ thalassemia/HbE (α^0/α^+ thalassemia- β^+/β^E)
CS EA Bart's	CS EA Bart's disease	+	+	< 80	< 27	HbH-CS with HbE trait (α^0/α^{CS}- β^E/β^A)
CS EE Bart's or CS EF Bart's	CS EF Bart's disease	+	+	< 80	< 27	HbH-CS with β^0/ HbE or HbH-CS disease with homozygous HbE(α^0/α^{CS}-β^0/β^E or β^E/β^E)
CS EFA Bart's	CS EFA Bart's disease	+	+	< 80	< 27	HbH-CS with β^+/ HbE(α^0/α^{CS}- β^+/β^E)
Rare Abnormal Hb	suspected abnormal Hb	depend on abnormal Hb				suspected abnormal Hb

From the above results, the user needs to have more knowledge and experience to diagnose thalassemia and HbE. However, HPLC does not confirm the types of thalassemia especially α thalassemia. The confirm tests are DNA analysis and DNA sequencing. The polymerase chain reaction (PCR) of DNA analysis is the most common test. It can diagnose abnormal genes that caused α^0, α^+, β^0, β^+ thalassemia and hemoglobinopathy (Abou-Diwan et al., 2009).

DNA is extracted from peripheral blood leukocytes by the salting out method (Miller et al., 1988). Genomic DNA is being amplified in the DNA Thermal Cycler with gap primers (specific deletions or mutation regions in α thalassemia) and allele specific primers (specific mutations in β thalassemia) to produce complementary DNA. Principle of the DNA Thermal Cycler is repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short single strand DNA) containing sequences complementary to the target region are used with the DNA polymerase to repeat the amplification. After that, these products are being used to confirm by agarose gel electrophoresis method (Nuntakarn et al., 2009; Yamsri et al., 2011). In Thailand, SEA that is 17.5 kilo base (kb) deletion gene and THAI (more than 38 kb deletion gene) are found in α^0 thalassemia, whereas α^+ thalassemia is -3.7 kb and -4.2 kb deletion gene. Most of β thalassemia, cause by point mutation (Fukumaki et al., 1991). Point mutations occur by base substitution, small deletion or insertion and lead to the frameshift mutation. The various types of mutations of β thalassemia in Thai population are shown in Table 2.3 (Department of Medical Science, 2010b).

Table 2.4 Types of mutations of β thalassemia in Thai population (Department of Medical Science, 2010b).

Mutations	**Type**	**Mechanism**
-28 (TAAA-TAGA) -31 (GCAT-GCGT) -86 (CACCC-CACCG) -87 (CACCC-CACAG)	β^+	Transcriptional mutations
+1 (A-C)	β^+	Cap site mutation
Init.Codon (ATG-AGG)	β^0	Initiation codon mutation
Codon 8/9 (+G) Codon 14/15(+G) Codon 15(-T) Codon 27/28 (+C) Codon 41 (-C) Codon 41/42 (-TTCT) Codon 71/72 (+T) Codon 71/72 (+A) Codon 95 (+A) Codon 123 (-ACCCCACC)	β^0	Frameshift mutations
IVS1-1 (G-T) IVS1-1 (G-A) IVS1-5 (G-C) IVS2#654 (C-T) Codon 19 (AAC-AGC) Codon 26 (GAG-AAG) Codon 126 (GTG-GGG)	β^0 β^0 β^+ (severe) β^+ (severe) β^+ (Hb Malay) β^E (HbE) β^0	RNA processing mutations
Codon 15 (TGG-TAG) Codon 17 (AAG-TAG) Codon 26 (GAG-TAG) Codon 35 (TAC-TAA) Codon 43 (GAG-TAG)	β^0	Nonfunctional mRNA mutations (nonsense mutations)

Table 2.4 Types of mutations of β thalassemia in Thai population (Department of Medical Science, 2010b) (Continued).

Mutations	**Type**	**Mechanism**
PolyA (AATAAA-AATAGA)	β^+	Polyadrenylation mutation
105 bp deletion		
619 bp deletion	β^+	Transcriptional mutations
3485 bp deletion		
12.5 kb deletion	β^0	Gene deletions
45 kb deletion	β^+	Cap site mutation
Asian india inversion	β^0	Initiation codon mutation

There are many thalassemia and Hb variants diagnosis tests. They use many reagents, time consuming, require expensive machines and require an expert person for analysis (Hartwell et al., 2005) (Figure 2.13).

Furthermore, Kan-Zhi Liu et al. (2003) reported the determination of hemoglobin structures and the hemoglobin disorders by infrared spectroscopy (IR). It showed different spectra between normal Hb and β-thalassemia Hb clearly. Jun-Hui Wan et al. (2013) established an IR spectroscopic method to be an additional tool for screening of thalassemias by comparing with MCV and MCH (Liu et al., 2003; Wan et al., 2013b).

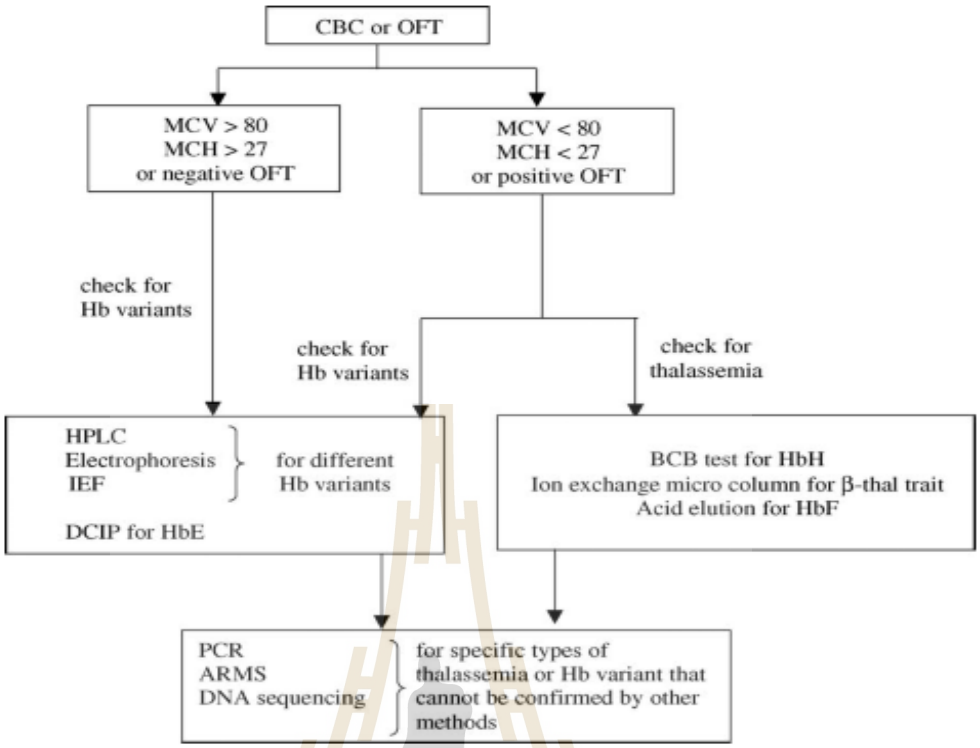


Figure 2.13 Summary of the normal processes of thalassemia and Hb variants diagnosis (Hartwell et al., 2005).

Infrared spectroscopy was discovered in 1800s by William Herschel who studied the refrangibility of the invisible rays of the sun (Ring, 2000). Later, the development of this technique was used for the detection of various substances. It has been used widely in industries, agricultures, medicines, food and pharmacies. In industries, IR was studied for the quantitative substances modeling, analysis the complicated samples, analysis the qualitative materials, determination of the composition and monitoring the qualitative products (de Oliveira et al., 2009; Hughes et al., 1995; Khanmohammadi et al., 2012; Liu et al., 2011; Pasquini and Bueno, 2007). In agriculture, it is mostly used to detect the extraction of herbs and fruits. These detections will be the useful applications in the future (Abdullah et al., 2012; Adiana

and Mazura, 2011; Liu et al., 2006; Liu et al., 2012). The IR can indicate the chemical substances in pharmacology and drug actions (Boiret et al., 2011; Roggo et al., 2005). In microbiology, it can identify the bacteria by the differentiation of the chemical structures (Lamprell et al., 2006; Mietke et al., 2010). Another advantage of IR is that it is non-invasive, it can be applied directly to the creature without any effects, so it is used for cerebral scans to evaluate the blood oxygenation change (Yokose et al., 2010), cerebral perfusion and blood concentration (Klaessens et al., 2005). It can also be used to prognose cancer by observing the protein-DNA structure changes (Gao et al., 2005). In hematology, many scientists studied about hemoglobin (Vályi-Nagy et al., 1997), hemoprotein (Dörr et al., 2008), molecular and chemical characterization of blood cells (Kan-Zhi Liu et al., 2005). Screening of β Thalassemia was evaluated by Kan-Zhi Liu et al. (2003). This IR method is very useful in Thailand because there is a high incidence of Thalassemia in this country. It is easy to use and has many advantages. These advantages are: (a) it is reagent free, (b) it uses a small amount of sample), (c) it can be automated and (d) it is simple (not acquire more training). Even it is useful to screen the β Thalassemia but nowadays, it has not be used in α Thalassemia and HbE which both are found mostly in Thailand (Wasi et al., 1980).

Previous IR spectroscopic studies of Hb had focused on many spectral features, for example the FTIR examined the cyanide ligated human methemoglobin (Hb-CN) in the CN stretching region (Al-Mustafa, 2002), the 2,3 bisphosphoglycerate (BPG) in the deoxy-HbA (NH-OOC hydrogen bond is formed between β82Lys and the carboxylate group of BPG) (Nadolny et al., 1993) and the carbonmonoxy hemoglobin in the cysteine SH stretching and the CO bond (Chen and Spiro, 2002). The technique has been useful for differentiating between CO bound to different subunits in human

Hb mutants, where marked changes in heme environment produce distinctly different spectral bands for CO bound to α- and β-subunits (Potter et al., 1983). The assignment of different -SH stretching bands in the IR spectrum of human Hb to individual cysteine residues was achieved in an elegant way by comparison with the spectrum of horse Hb, which is devoid of cysteine β-112, and that of bovine Hb, which contains only β-93 cysteine (Moh et al., 1987). The -SH stretch vibrations of cysteine residues in HbA are sensitive to the structural changes that result from the binding of ligands, such as O2, CO, and NO, at the heme iron (Dong and Caughey, 1994; Sampath et al., 1994).

More relevant to investigate are studies of individual Hb variants. For example, the IR difference spectrum of HbA and Hb Kempsey (a mutant in which Asp β-99 is replaced by Asn) revealed a negative band at 1697 cm-1 originating with the C=O stretch of carboxylic acid, ascribed to the side chain of Asp β-99 based on its known mutation (Gregoriou et al., 1995). Wallace et al. (1976) revealed that the *ν*CO of carbon monoxide bound to the α- and β-chains in Hb Zurich (Hb Z; β-63 His-Arg) shifted to 1950 and 1958 cm^{-1}, respectively, compared with that of normal HbACO (1951 cm^{-1}). Substitution of the distal histidine (β-63) of HbA by arginine in HbZ enhances its susceptibility to auto-oxidation in the presence of "oxidant drugs". This observation arranged a correlation of structure with the pathologic manifestations of HbZ and supported the contention that IR spectroscopy is able to provide an understanding of the origins of Hb diseases attributable to abnormal Hb structure.

2.5 Principle of infrared spectroscopy

The infrared (IR) spectrum is a part of the electromagnetic spectrum which locates in a long wavelength and lower frequencies than visible light (Figure 2.14). It

can be divided into three regions, the near-, mid- and far- infrared. The higher-energy near-IR is the frequency between 12800-4000 cm^{-1}, the mid-IR has a frequency between 4,000-200 cm^{-1} which is the most useful region and far-IR lying between 200-10 cm^{-1} (Colthup et al., 1990). The infrared vibrates the bonds of the molecules in stretching and bending patterns. Stretching pattern is the vibration of two atoms of a molecule which result is changing the distance. Bending vibration is a curve of bond bends from the axis, then changes the bond length and angle. Whenever, the molecule absorbs infrared radiation with stretching or bending pattern, each bond increases the amplitude of vibration to the excited state and releases heat when it turns to the ground state (Figure 2.15).

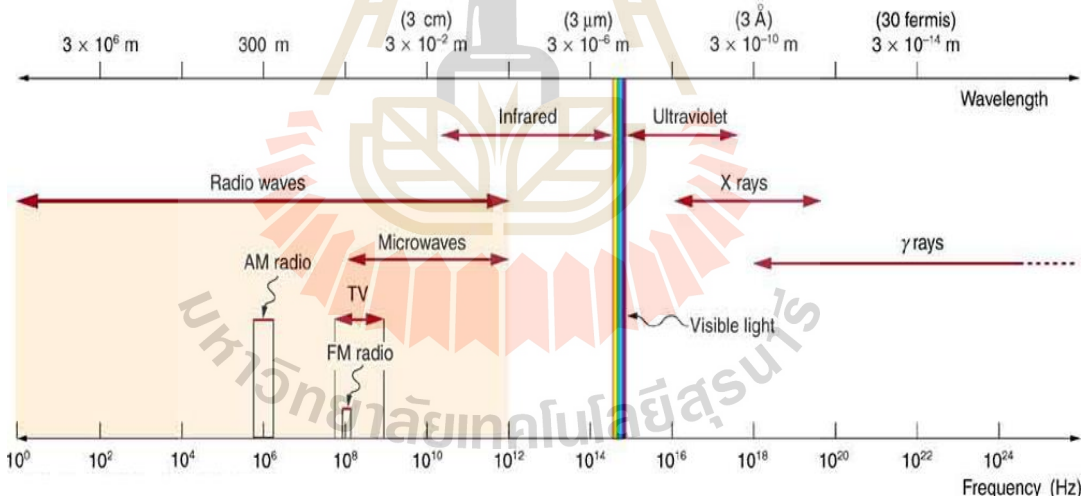


Figure 2.14 Electromagnetic spectrums (http://cnx.org/content/m42444/latest/?collection=col11406/1.7).

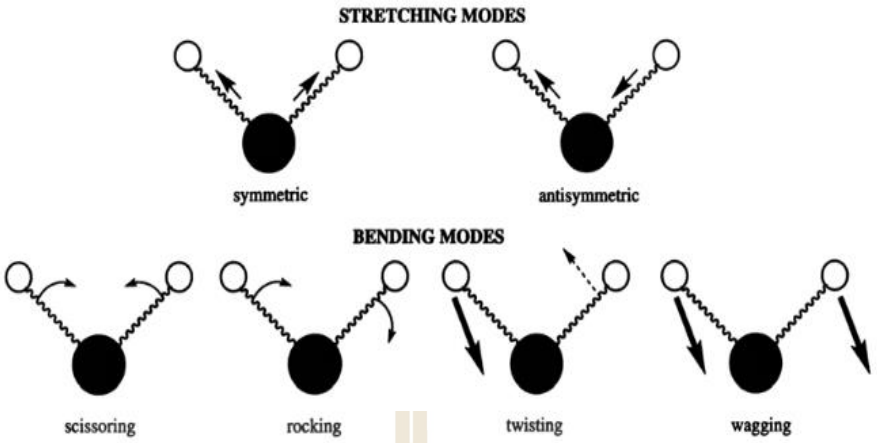


Figure 2.15 Vibration pattern of methylene bonds (http://e-book.ram.edu/e-book/c/CM328/CM328-10.pdf).

Bonds between different atoms such as C=H, C=O, O-H, C≡N absorb energy from different frequencies of infrared. It depends on the types of bond and masses of the atom. Furthermore, same bond in the molecule such as O-H bond may absorb energy from more than one frequency, such as at frequency ~ 3300 cm^{-1} (~3.0 μm), it passes an O-H bond and gives the stretching vibration and another frequency lower than 1250 cm^{-1} (8.0 μm), it absorbs energy with the bending vibration. Oscillation of this differentiation is the mode of vibration which are shown in Figure 2.16 and 2.17 (Pavia, 2009).

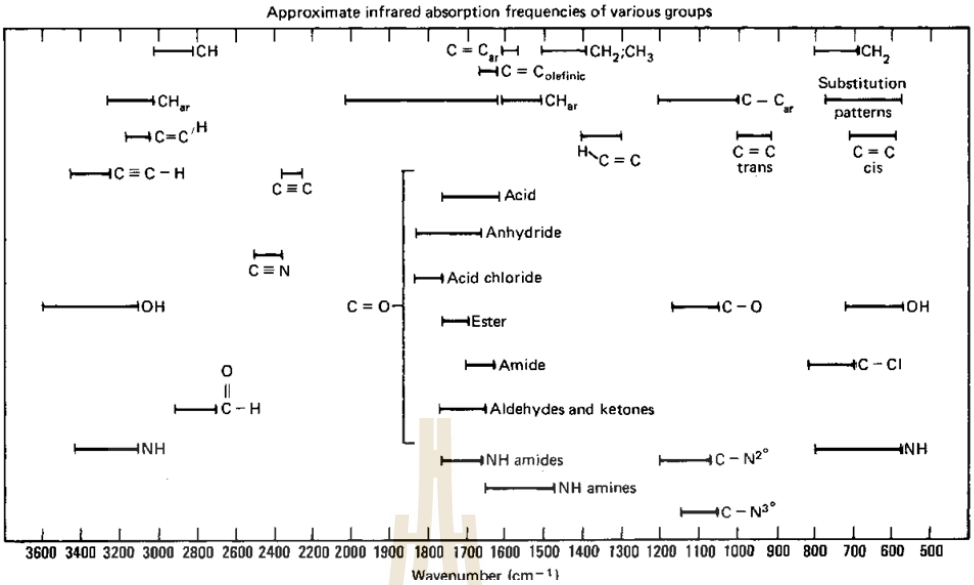


Figure 2.16 Infrared absorption frequencies of various groups (http://what-when-how.com/organic-chemistry-laboratory-survival-manual/infrared-spectroscopy-part-1-laboratory-manual/).

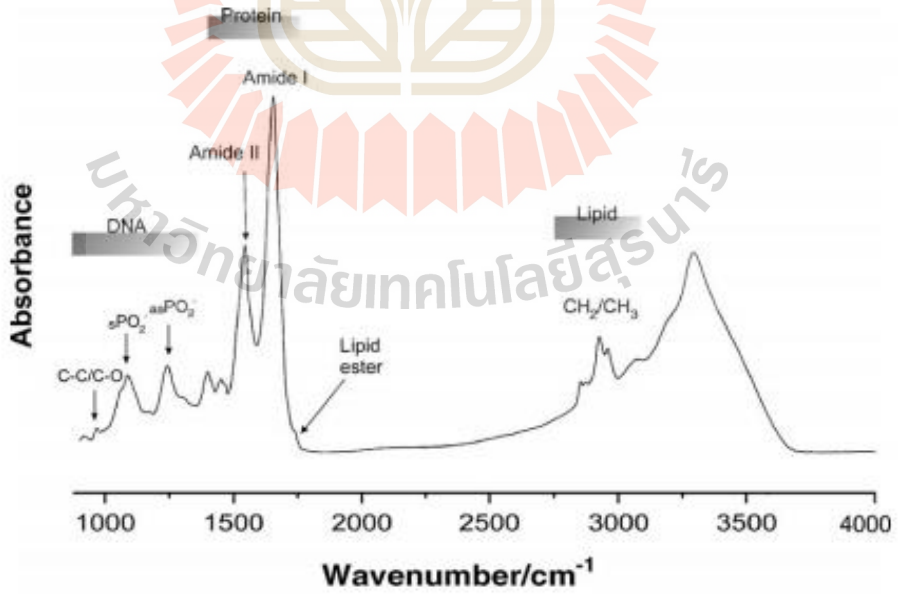


Figure 2.17 Representation of IR spectrum, revealing basic cellular molecules such as protein, lipid and DNA as marked (Liu et al., 2005).

The IR spectroscopy consists of 2 parts, an IR spectrometer and a microscope. The IR spectrometer is the IR spectrum recorder which has an IR light source (Globar source) with the energy in the mid-infrared. The light from Globar source proceeds to the beam splitter (scattering radiation). Half of the light is refracted towards the stationary mirror and the remaining is transmitted towards the moving mirror. Light from these two mirrors reflects back to the beam splitter and 50% of light pass the sample which lies on the microscope. Thereafter, the light is refocused on the detector (Figure 2.18). The result has many data points, so it is necessary to use the computer to analyze. All the spectra are scanned within less than one second. Repeated scans can be done rapidly to make the real peak and remove the interfere peak. The result of the spectrum is clear and corrected by comparing with the reference peak.

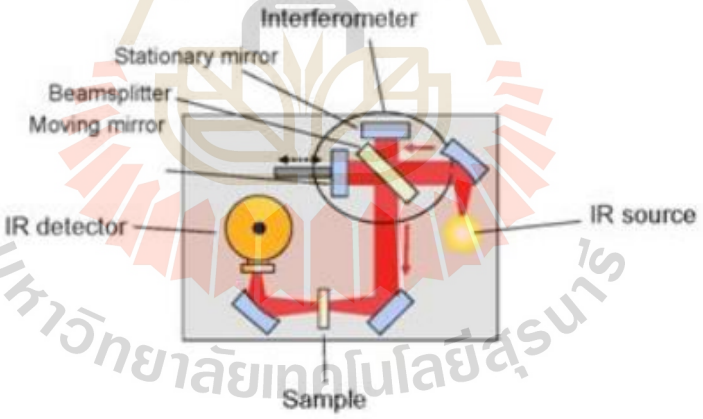


Figure 2.18 Infrared spectrometer (http://www.slri.or.th/th/index).

For the microscope, It includes all optical, mechanical, and electronic components, provides high stability and reliability. Featuring with many contrast enhancement tools, it gives a wide variety of dedicated objectives, and chemical imaging. The lateral resolution power is only limited by diffraction of the incident light.

Due to the high light-throughput, a very high sensitivity is reached even at the highest lateral resolution. The infrared beam path in it is confocal. Apertures can be placed in conjugate image planes individually before and after test the sample in transmission as well as in reflection.

In transmission mode, the IR radiation onto the sample is focused by an objective len and a condenser accumulates the transmitted radiation before leading to the detector. In standard reflection mode, the half of the light is reflected from the sample is collected and focused by the same objective len. Then, it is forwarded to the mirror and a half of light is directed to the detector. The other reflection mode is a beamsplitter which represents the mirror. It contrasts the standard reflection mode as the whole light onto the sample goes to a beamsplitter and a half of light is collected to the detector (Figure 2.19) (Bechtel et al., 2009).

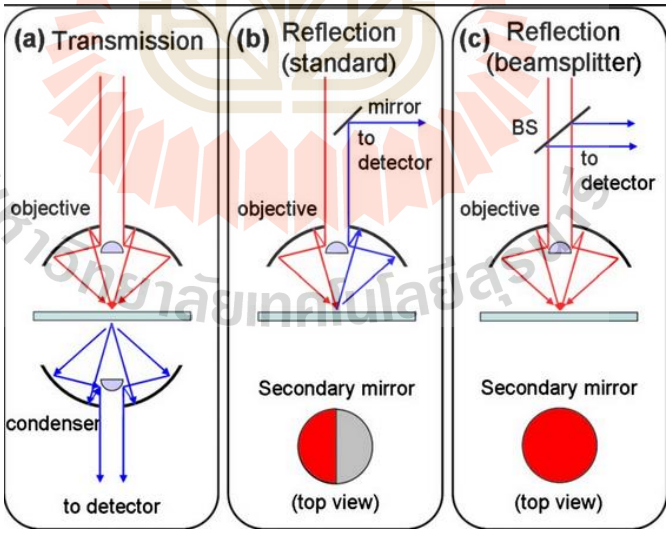


Figure 2.19 Schematic diagram of three different modes of infrared microscopy: a) transmission, b) reflection with the standard mirror design, and c) reflection with the beamsplitter design (Bechtel et al., 2009).

CHAPTER III

MATERIALS AND METHODS

3.1 Samples

The 100 samples of thalassemic patients were obtained from the Health Promoting Hospital in Nakhon Ratchasima and the Genome Molecule Laboratory Company in Bangkok, where confirmed the thalassemic types by DNA analysis. The sample size is calculated to be at least 20 samples per group as shown below (Cochran, 1977). These samples were divided into 5 groups as α^0 thalassemia, α^+ thalassemia, β^0 thalassemia (included the combination of HbE: β^0/HbE 2 samples and with α^0 thalassemia: β^0/α^0 2 samples), β^+ thalassemia (included the combination of HbE: β^+/ HbE 3 samples) and HbE (included the combination of α^0 thalassemia: HbE/α^0 1 sample). For normal group, 24 samples were obtained from the blood donors of Regional Blood Center in Nakhon Ratchasima and also were confirmed by DNA analysis. The aforementioned samples in this study had been reviewed and approved as were minimal risk review by the Ethics Committee for Researches Involving Human Subjects, based on the Declaration of Helsinki with project code EC-56-28 of Suranaree University of Technology.

$$N = \left[\frac{(Z_\alpha + Z_\beta)\sigma}{\overline{x}_1 - \overline{x}_0} \right]^2 \qquad N = \left[\frac{(1.96 + 1.28)2.70}{98 - 100} \right]^2 = 19.13$$

N	=	sample size
Z_α	=	1.96 ($\alpha = 0.05$)
Z_β	=	1.28 ($\beta = 0.10$)
Σ	=	standard deviation
$\overline{x}_1$	=	study sample mean (pilot study)
$\overline{x}_0$	=	standard sample mean

From the research of Liu et al. (2003), they investigate the potential of IR spectroscopy technique to diagnose and screen β thalassemia. This result was 98% accuracy for partitioning normal and β thalassemia samples and found that 2.7% false negative.

3.2 Preparation of hemoglobin lysates

To prepare hemolysates, 3 mL of peripheral blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) were centrifuged at 1,800 g for 15 minutes by the KOKUSAN model H-30. Plasma was discarded, and RBCs were washed three times in normal saline solution (NSS, 0.9% NaCl) lot 4031113 of Thai Nakorn Patana companies. The RBCs were lysed by the addition of two volumes of distilled water to the washed RBCs and centrifuged at 1,800 g for 30 min. Then the supernatant was collected to do the further experiments using the techniques modified from Kan-Zhi Liu et al. (2003) and Jun-Hui Wan et al. (2013).

3.3 Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a modern infrared spectroscopy. The Fourier transform is a mathematical process that converts the raw spectrum (or interferogram) to an actual spectrum. It is performed by two Fourier transformations: one by the interferometer and one by the computer. The interferometer originates the Fourier transform of the spectrum when a monochromatic source is regarded (Figure 3.1). This study used a Vertex 70 FTIR spectrometer coupled to an IR microscope (Bruker Hyperion 2000) equipped with a KBr beamsplitter and the MCT detector cooled with liquid nitrogen with a measurement range of 4000-200 cm^{-1} which is a mid-IR.

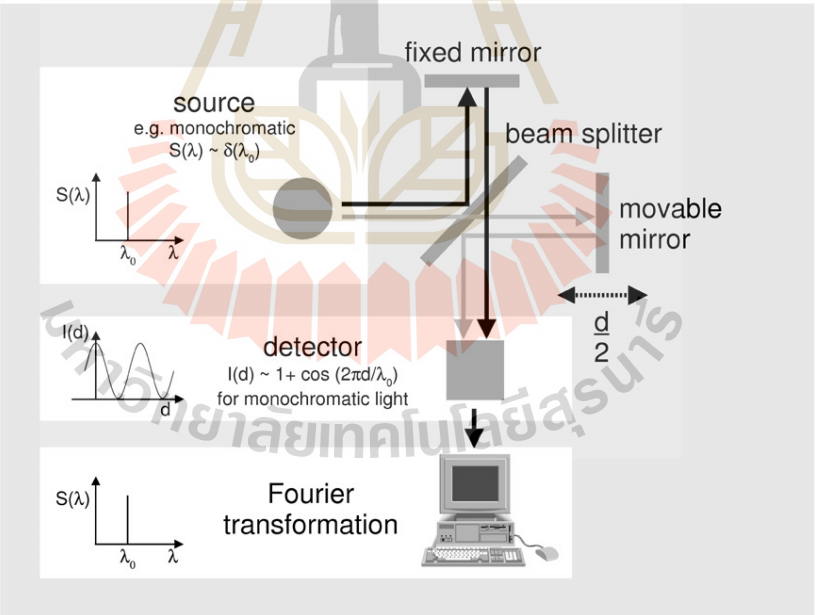


Figure 3.1 Scheme of a Fourier transform infrared spectrometer (© 2006 Nova Science Publishers).

3.4 IR measurement

Each specimen was tested by Kan-Zhi Liu and Jun-Hui Wan applicative technique using 2 μL aliquot of the hemolysates which was evenly spread on an IR reflective slide (Mirr-IR Corner Frosted, Kevley Technologies, USA) with 3 millimetre diameter, it was dried to produce glassy film and was stored in a desiccator until spectra was required. An IR absorption spectra acquires an IR spectrometer couple with an IR microscope and connects to the detector cooled with liquid nitrogen over the wavenumber range from 4000 cm^{-1} to 200 cm^{-1}. A spectral acquisition was made in a reflection mode with a focal plane aperture size of 5 μm × 5 μm at 4 cm^{-1} a spectral resolution with 64 co-added scans at 36 × objectives (Bruker Optics, Ettlingen, Germany) measured background 1 time every 5 times of specimen measurement and controlled the temperature and the humidity lower than 27ºC and 20%, respectively. All interferograms were co-added and converted to absorb spectra using the OPUS 7.2 software.

3.5 Spectra manipulation

The recorded IR spectra with a Vertex 70 FTIR spectrometer were transformed with an OPUS 7.2 software. They were done to the second derivative spectra and vector normalization over the region 1800-1400 cm^{-1} and 3400-2800 cm^{-1}. All spectra were qualitative selected with a weak absorbance (an absorbance of 1684-1656 cm^{-1} region < 0.30 units) or a high absorbance (an absorbance of 1684-1656 cm^{-1} region > 1.2 units) (Thumanu et al., 2014). Then, these areas were integrated for clustering and classification trials with Uncrambler® X program.

3.6 Statistical analysis

Various spectral features differed between the spectra of Hb from normal controls and each Hb from 4 groups: α^0, α^+, β^0, β^+ thalassemic patients, as well as among the individual spectra acquired for HbE. In next step, therefore, applied a separating analysis using OPUS 7.2 software and the Uncrambler® X version 10.3 software package (CAMO, Germany) to discriminate whether the individual IR spectra can be classified according to their clinical diagnosis. This method uses a statistical approach to classify spectra into groups according to subtle spectral differences. The outcome of discrimination can be visualized with a dendrogram in which the discriminant formation is plotted as a horizontal, interconnecting line located at the distance at which the discriminant is being formed. Spectra are presented to the algorithm without diagnostic labels, and the algorithm separates them into subgroups based on spectral similarities. In the ideal scenario, Hb spectra from all thalassemic patients form a discriminant separate from that for Hb specimens from normal controls; however, it is only after this analysis that each spectrum is marked retrospectively as Hb from normal controls or from patients with thalassemia.

CHAPTER IV

RESULTS

The outcome of measurement from IR spectroscopy was 652 spectra. It was divided into the normal, β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia group, which were 114, 166, 70, 105, 89 and 108 spectra, respectively (Figure 4.1a). Later, it was performed on second derivative spectra using the spectral region from 3400-2800 cm^{-1} and 1800-1000 cm^{-1} (Figure 4.1b). The characteristic functional groups contributed to the formation of the protein side chain and backbone vibration. The average second derivative spectra after 7 points of smoothing and normalization with Extended Multiplicative Signal Correction (EMSC) over the spectral range of 3400-2800 cm^{-1} are shown in Figure 4.2 and 1800-1000 cm^{-1} are shown in Figure 4.3. There were 7 major bands of NH stretching, CH_2 stretching, CH_2 stretching, CH_3 stretching, CH_3 stretching, C=O stretching (amide I) and NH bending (amide II) derived from around 3300, 2960, 2929, 2871, 2852, 1650 and 1540 cm^{-1}, respectively (Rahmelow et al., 1998; Barth, 2007).

Principal Component Analysis (PCA) has proven to be useful in analysis, providing two types of information which were visualization of similar spectral clustering and identification of variables (spectral bands representing various molecular groups within the samples). The major variability between the spectra can be concentrated into a smaller set of values called principal components (PCs). To define the major variability between normal and abnormal of β^+ thalassemia,

β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia, they were applied to the pre-processed spectra recorded in the 3400-2800 cm^{-1} and 1800-1000 cm^{-1} regions.

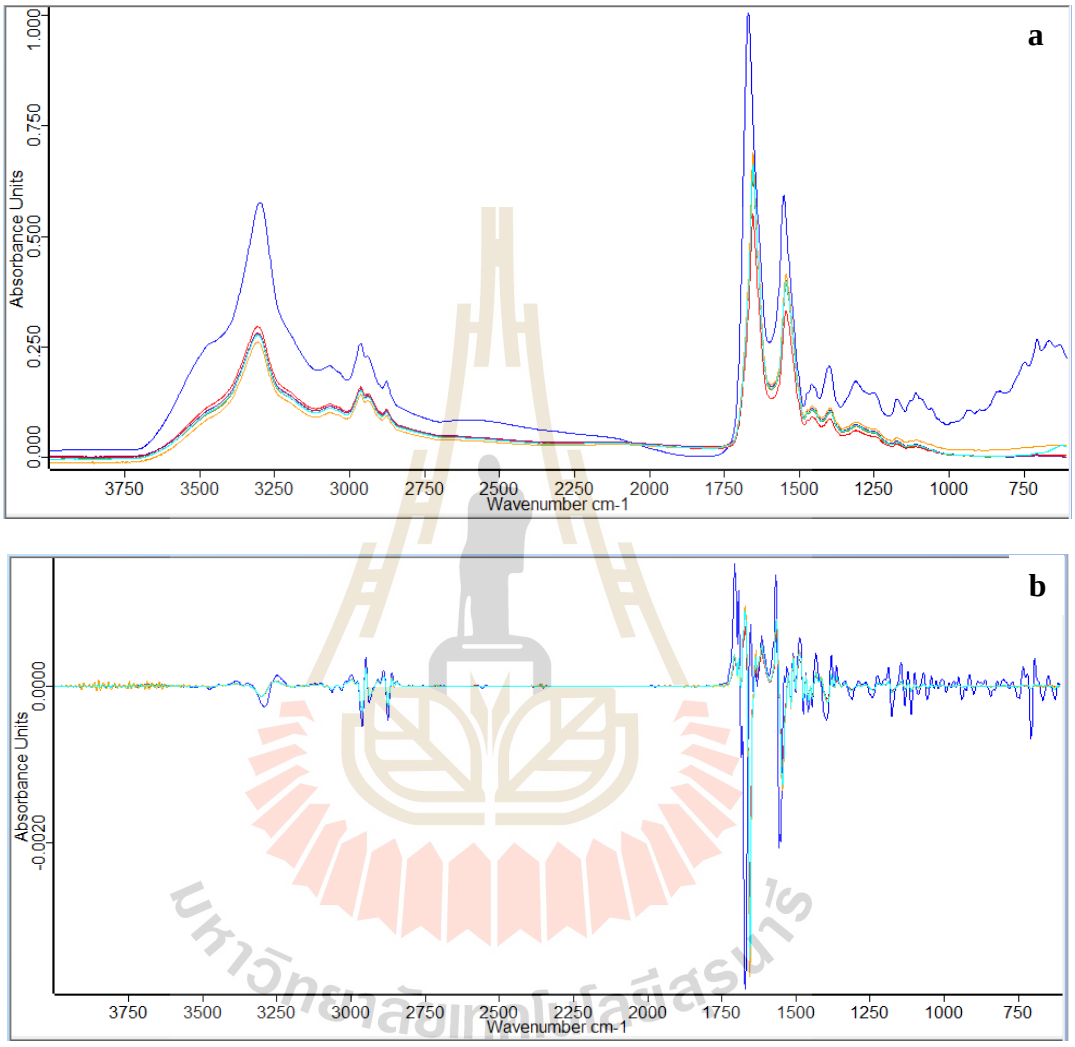


Figure 4.1 The average IR spectrum of Hb (a) and the second derivative spectrum (b) from normal 24 samples: 114 spectra (navy blue line)**,** β^+ thalassemia 20 samples: 166 spectra (violet line), β^0 thalassemia 20 samples: 70 spectra (orange line), HbE 20 samples: 105 spectra (blue line), α^0 thalassemia 20 samples: 89 spectra (red line) and α^+ thalassemia 20 samples: 108 spectra (green line).

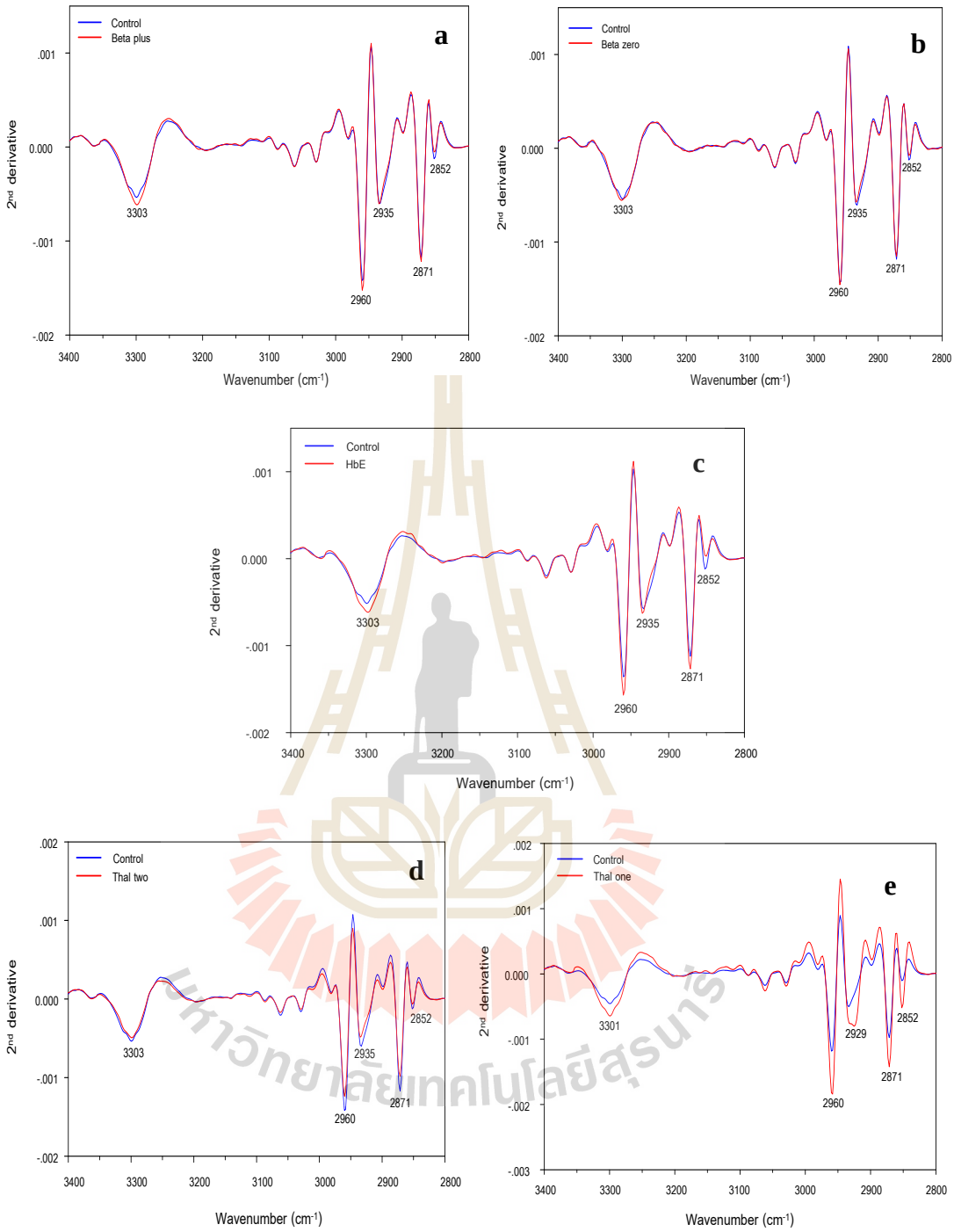


Figure 4.2 The average second derivative spectra after 5 points of EMSC over the spectral range of 3400-2800 cm^{-1} compare between normal and abnormal Hb of β^+ thalassemia (a), β^0 thalassemia (b), HbE (c), α^+ thalassemia (d) and α^0 thalassemia (e).

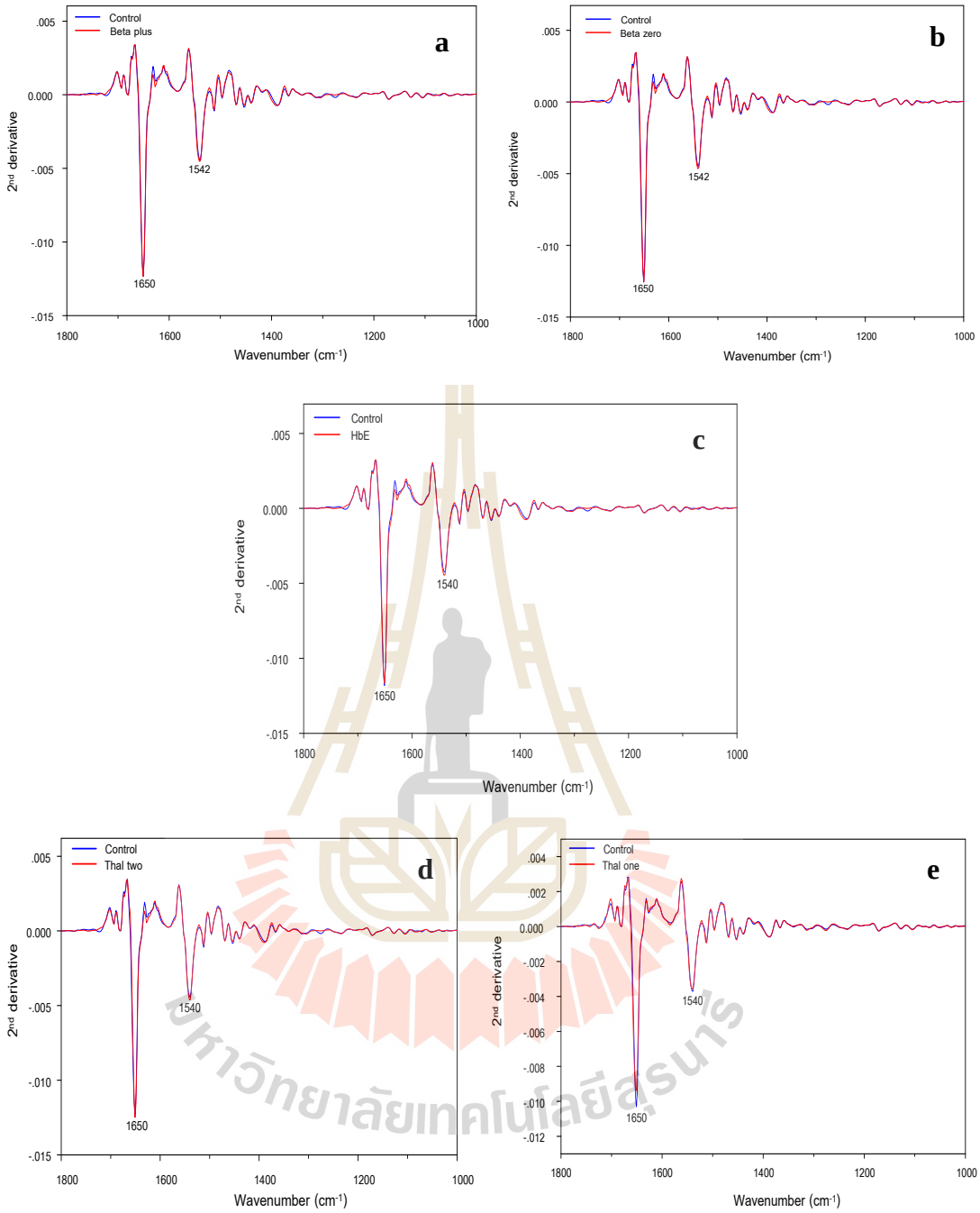


Figure 4.3 The average second derivative spectra after 2 points of EMSC over the spectral range of 1800-1000 cm^{-1} compare between normal and abnormal Hb of β^+ thalassemia (a), β^0 thalassemia (b), HbE (c), α^+ thalassemia (d) and α^0 thalassemia (e).

The comparison of PCA scores plot between normal and abnormal Hb of β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia can be clustered separately along PC1, PC2 and PC3. Scores and loading plots from PCA of the spectra data for normal Hb (114 spectra from 24 samples) and β^+ thalassemia Hb (166 spectra from 20 samples) explained 64% of total variance in the data set (58% for PC1 and 6% for PC3) (Figure 4.4).

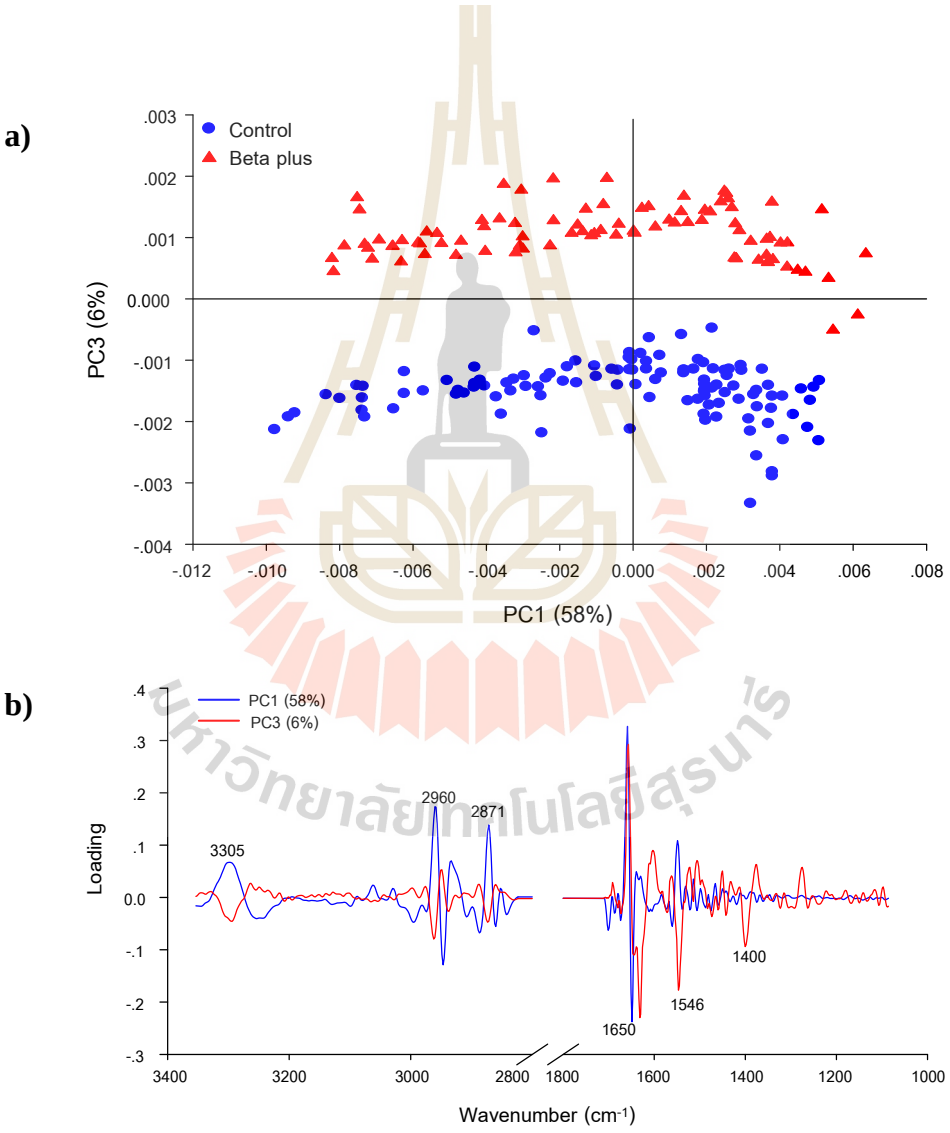


Figure 4.4 Scores (a) and loading plots (b) from PCA of the spectra data for normal and β^+ thalassemia.

Fifty-one percent variance of PC1 and PC3 were explained by 5% for group between normal and β^0 thalassemia (70 spectra from 20 samples) as shown in Figure 4.5. A perfect separated between normal and HbE (105 spectra from 20 samples) was 60% of total variance (52% of PC1 and 8% of PC3) (Figure 4.6) as same as the group between normal and α^+ thalassemia (108 spectra from 20 samples) (Figure 4.7). The last group between normal and α^0 thalassemia (89 spectra from 20 samples) was clearly separation which explained by 33% variance of PC2 and 2% of PC3 (Figure 4.8).

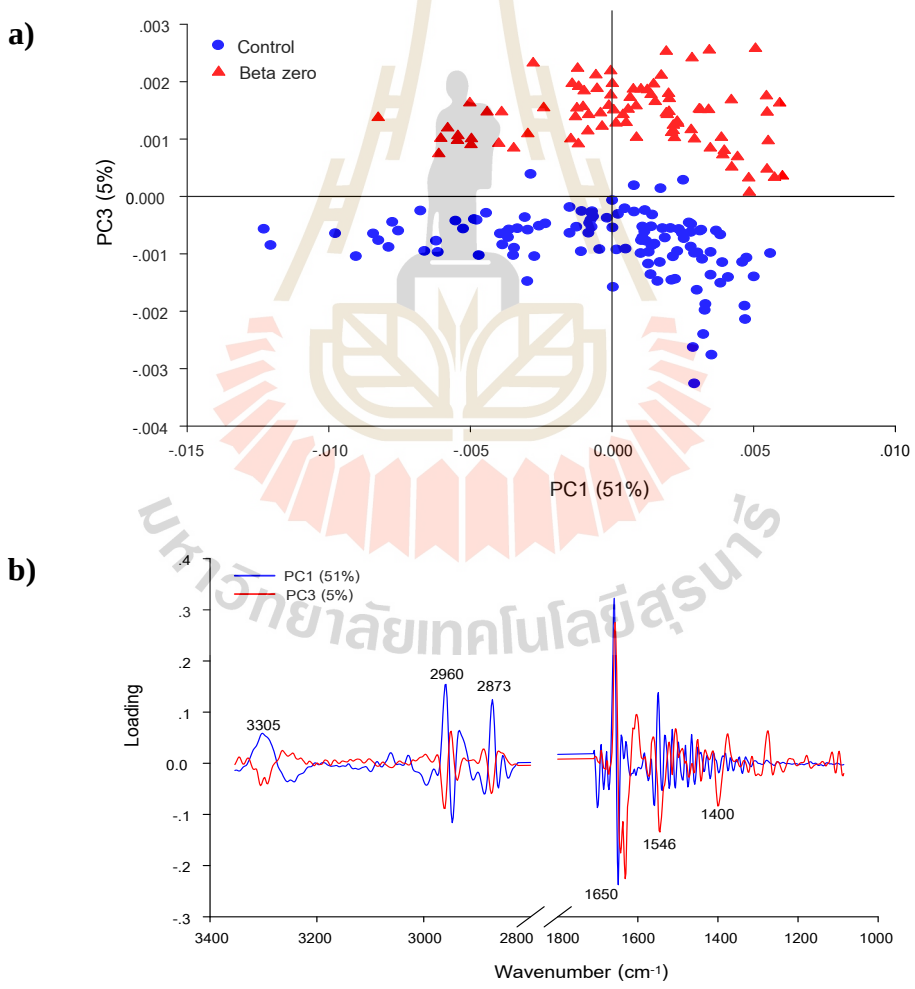


Figure 4.5 Scores (a) and loading plots (b) from PCA of the spectra data for normal and β^0 thalassemia.

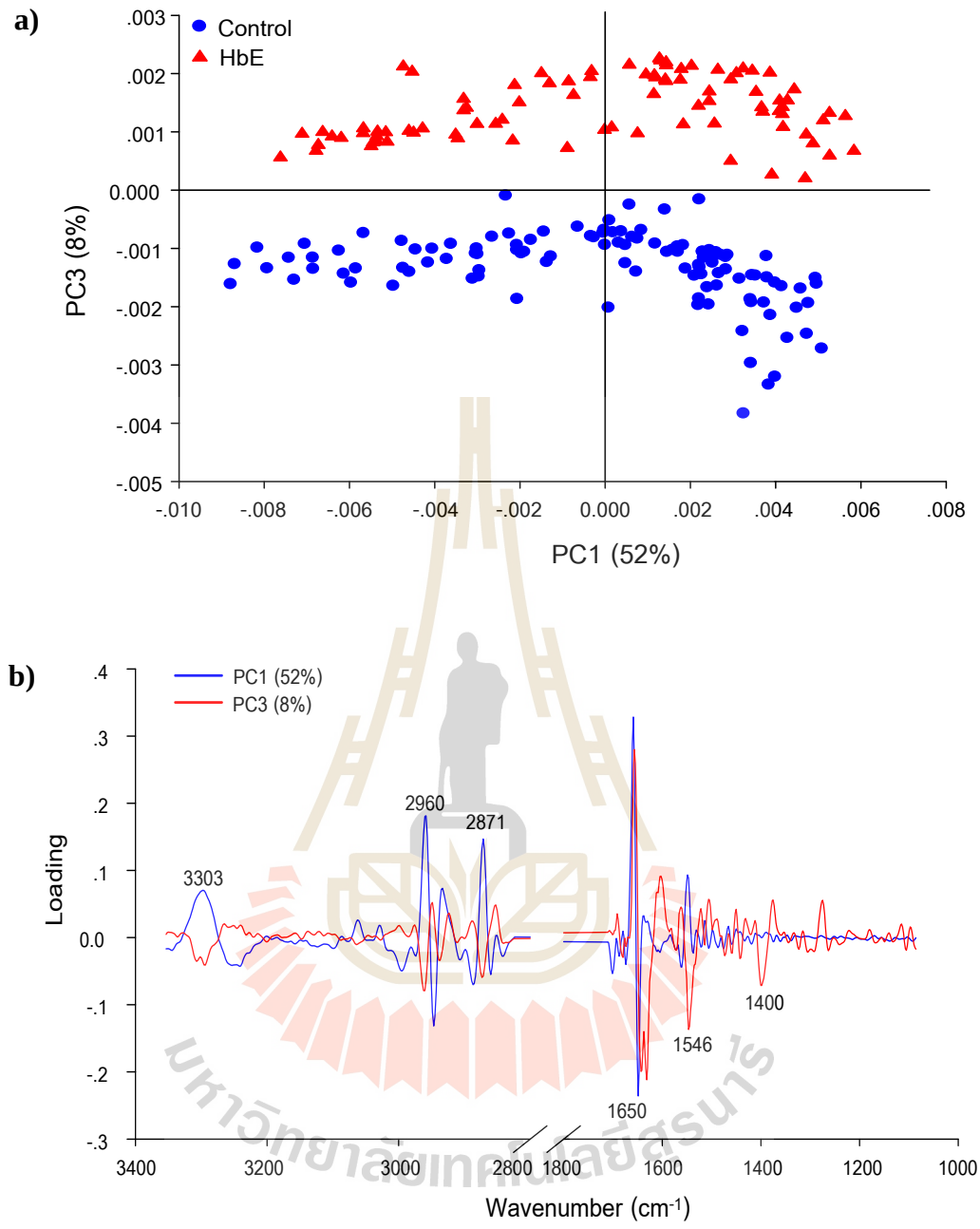


Figure 4.6 Scores (a) and loading plots (b) from PCA of the spectra data for normal and HbE.

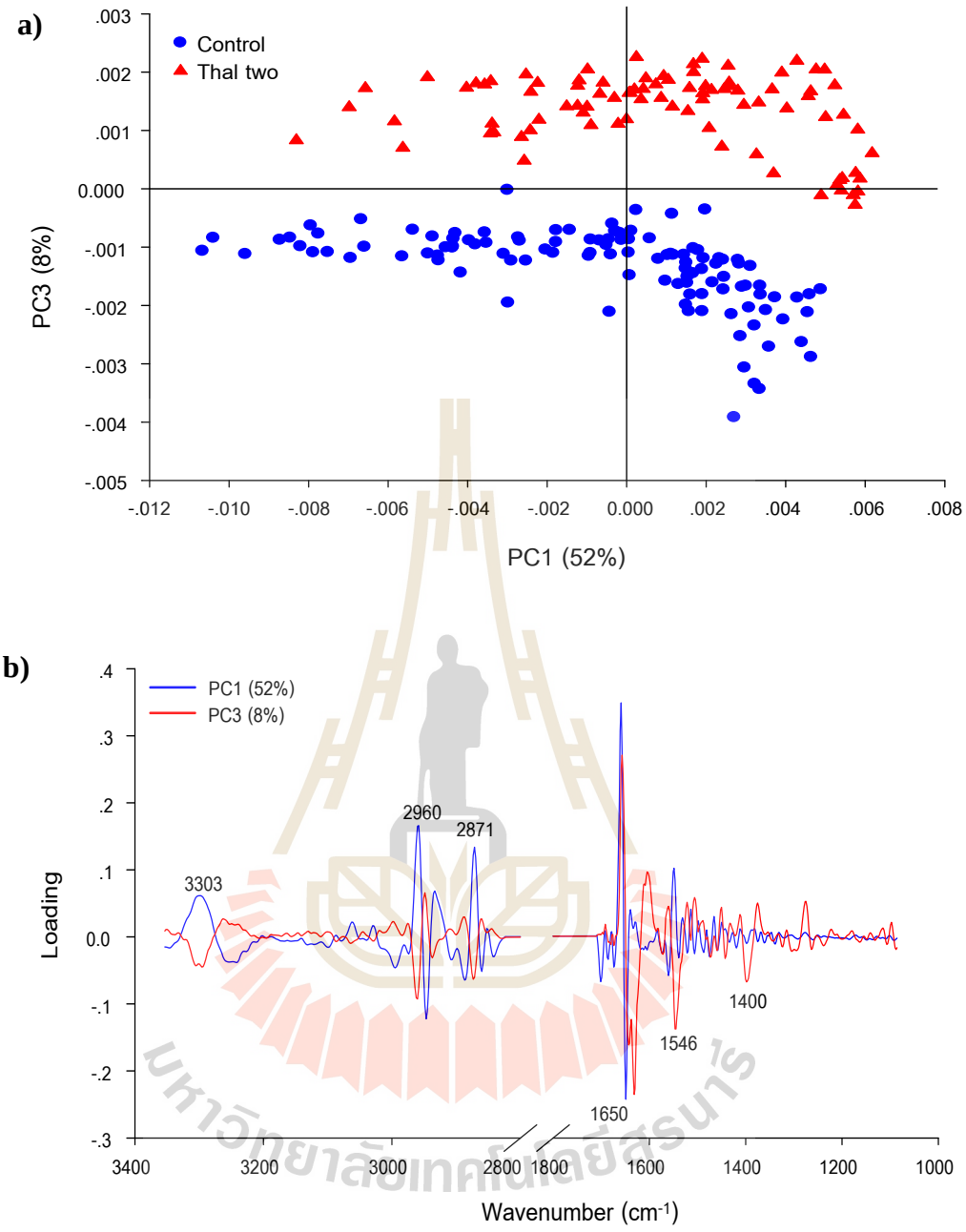


Figure 4.7 Scores (a) and loading plots (b) from PCA of the spectra data for normal and α^{+} thalassemia.

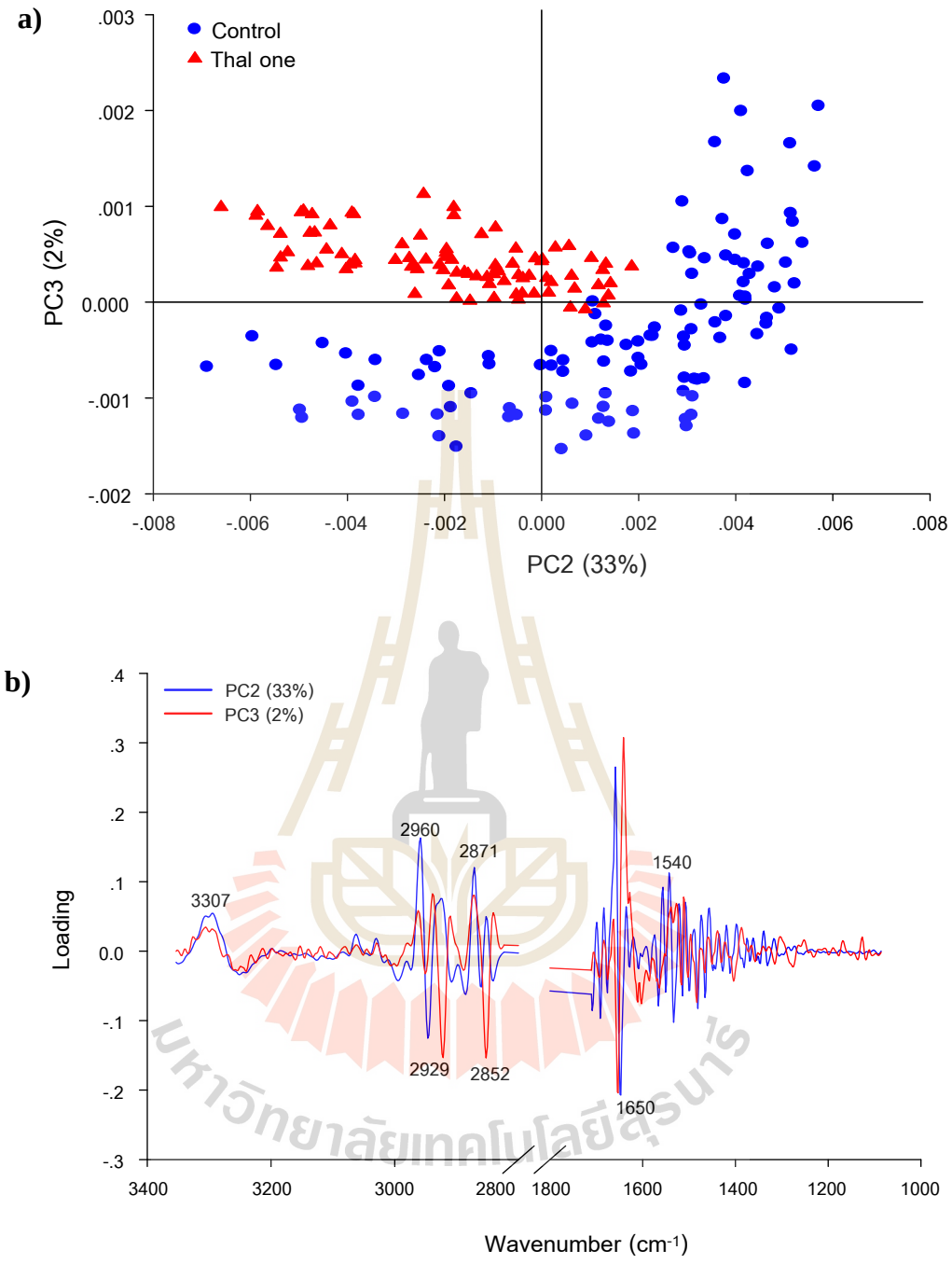
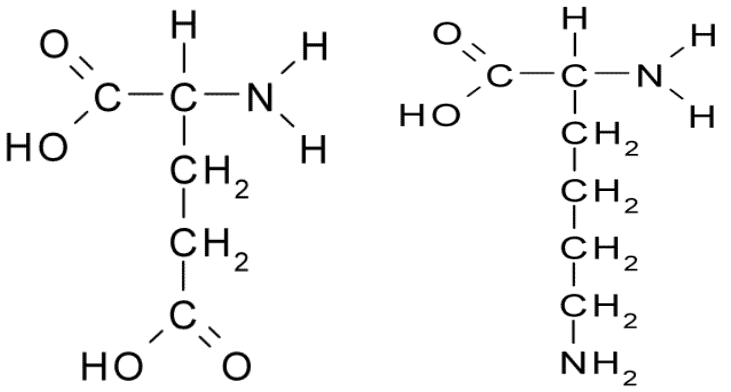


Figure 4.8 Scores (a) and loading plots (b) from PCA of the spectra data for normal and α^0 thalassemia.

The loading plot of PC1 and PC2 exhibited C=O stretching, NH bending and NH stretching which effected on the amide I, amide II and amines as a negative peak at 1650, 1540 and 3300 cm^{-1}. These showed the change in the level of helix secondary structure, according to thalassemic Hb which mutated as marked changes in heme environment resulted in distinctly different spectral bands for CO bound to α and β subunits. The PC3 was focused to obtain the variations in minor components which were the bonds of the protein side chains.

The α^0 thalassemic spectra had clearly high intensity in the CH bands at 2960, 2929, 2871 and 2852 cm^{-1} which were assigned more protein side chains. Since α^0 thalassemic Hbs had no α globin chain, so it had excess β globin chains and became to be free β globin chains. These free β globin chains were not stable, therefore the anemia was happening. The CH bands of α^+ thalassemic spectra were different from α^0 thalassemic spectra and showed low level, because these thalassemias had less protein side chains than normal spectra (synthesis of α globin chains were low).

For HbE, the phenomenon was the same as α^0 thalassemic spectra with high CH bands. According to HbE Hbs which mutated from glutamic acid to lysine at position 26 of β globin chain and had lower CH bands than lysine (Figure 4.9), but the C=O had equal normal spectra which was different from α^0 thalassemic spectra. This performed that these Hbs were functionality. The β^0 and β^+ thalassemic spectra had low intensity in the CH bands at 2852 cm^{-1} same as the HbE spectra. The β thalassemia had defect at β globin chains, but δ and γ globin chains were combined with α globin chains instant of β globin chains which were defect. However, the occurrences were as same as the normal spectra in many bands that were C=O, NH and other CH bands.



Glutamic acid Lysine

Figure 4.9 Glutamic acid and lysine structure (https://commons.wikimedia.org/wiki).

Unsupervised hierarchical cluster analysis (UHCA) was applied to examine similarities and differences between spectra of normal and abnormal Hb of β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia using spectral information in the range of 3400-1000 cm^{-1}. The average spectra from α^0 thalassemia samples in branch A was clearly separated from 5 groups (normal, β^+ thalassemia, β^0 thalassemia, HbE and α^+ thalassemia) in branch B1 and B2. Branch B21 and B22 of β^+ thalassemia, β^0 thalassemia, HbE, and α^+ thalassemia were classified from branch B1 of normal (Figure 4.10). These results were used to confirm the PCA analysis.

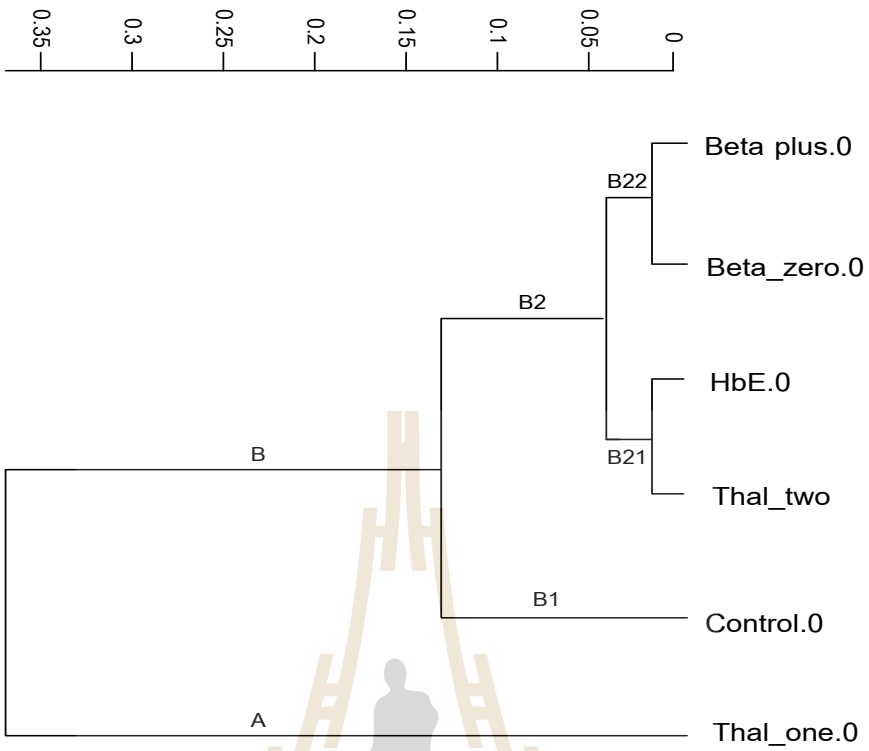


Figure 4.10 Dendogram obtained by UHCA of average spectra from normal and abnormal Hb of β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia.

CHAPTER V

DISCUSSION AND CONCLUSION

Thalassemias and hemoglobinopathies are the most common single-gene disorders in human being all over the world on a worldwide basis (Clarke et al., 2000). It has been found that since 2007, 1% of Thai population has got this illness (Ministry of Public Health, 2007). Thalassemia is characterized by quantitative defects in the α and/or β-globin chains, one of the backbone protein of the Hb molecule. It is estimated by WHO, World Bank and other international agencies that there is a massive increase in the number of patients with different forms of thalassemia in many emerging countries and it is imperative to establish more surveys about the frequency of the disease and set up centers for its control and management.

More than 150 globin gene mutations cause thalassemia, usually single-base substitutions or small insertions/deletions of the globin gene (Huisman, 1992). Although the complete blood count can give clues for the diagnosis and screening of some thalassemia but not all. First, the screening tests by CBC using MCV, MCH or OF test can detect thalassemias but cannot screen Hb variants. Confirmation requires additional analytical tests, such as electrophoresis, isoelectric focusing and HPLC, to assay Hb derivatives and to detect the presence of any globin chain tetramers. Recently, molecular analysis of DNA has emerged as an alternative method for early detection of mutant globin genes and alleles (Wild and Bain, 2006). These additional confirmation tests are usually complex and labor-intensive, thus it may be a burden for countries with limited resources.

In this study, the IR spectra of hemolysates derived from a cohort of patients with HbE, α and β thalassemia, revealed the important alterations in the protein secondary structures. The prominent spectral changes in Hb of patients with thalassemia were as follows: decreased α helix content; increased parallel and antiparallel β sheet content (amide I) and decreased in the intensities of tyrosine absorption bands ($-CH_2$). Similar changes were observed in the hemolysate derived from a patient with HbE disease. It should be that the major IR features, indicated that the reduction of α helix content and the increasing of β structure likely arised from the denatured unbound α chains characteristic of thalassemia.

These results explained that the amount of Hbs within RBCs of thalassemic patients had lower amount than non-thalassemic patients (amide I was the point of protein quantity). They related to MCV and MCH index which were lower than 80 fL and 27 pg, respectively for thalassemic patients. The peak of α^0 thalassemic absorbance had clearly different CH_2 stretching around 2929 cm^{-1} from normal blood sample because these Hbs presented the combination of more β chain which found an increase amino acid side chain. For β thalassemia, γ and δ chains increased, and HbE had more glutamic acid in β chain. This study extended the previous findings by using more samples for each type of thalassemia and using the PCA and UHCA program analysis to designate individual spectra of Hb from normal controls and thalassemic patients and found that this technique could classify and differentiate Hbs of HbE and all thalassemia (found in Thailand) from normal controls. This is the first report to classify between normal Hb and Hb of β^+ thalassemia, β^0 thalassemia, HbE, α^0 thalassemia and α^+ thalassemia using only FTIR. It will be very useful to use this technique to screen for the carriers which can reduce the risk of the children from married couples who are

both carriers, because 30-50% of Thai population are thalassemic carriers, which they do not know themselves and unaware to check before marriages. Since the routine laboratory investigation cannot detected thalassemic carrier in one step, it require 4 steps which were CBC, OF test, DCIP and HPLC techniques. To complete these 4 techniques are expensive, take time and require the experienced personnels to handle especially HPLC technique.

There are several potential advantages and benefits in using the IR-based method to screen and discriminate thalassemia: (*a*) it is reagent-free, the IR "color patterns" of the species of interest provide the basis for detection and quantification; (*b*) it uses a small sample volume (2 μL), leaving sample material for other clinical tests, this is particular importance in the fetus, for which collecting blood sampling is difficult and limited; (*c*) it can be automated and (*d*) it is simple (the expertise can be acquired after minimal training); (*e*) it can be used to detect abnormal Hb from all types of thalassemia which found in Thailand. To simplify the method, it is not necessary to centrifuge after preparing hemolysates, which will shorten the turnaround time for testing because other possible influences from cell membrane and other cellular components have less than 3%. In the future, more sample sizes should be analyzed to confirm that this technique can classify each type of thalassemia. Therefore, it will reduce the expenses significantly and also helps the clinicians to advise the couples before get pregnancy. Since the babies from thalassemic carrier couples are risk to be severe thalassemia and will be the public health problems in the future.

REFERENCES

REFERENCES

Abdullah, F., Ling, S.K., Man, S., Tan, A.L., Tan, H.P. and Abdullah, Z. (2012). Characterization and identification of labisia pumila by multi-steps infrared spectroscopy. **Vibrational Spectroscopy**. 62(1): 200-206.

Abou-Diwan, C., Young, A.N. and Molinaro, R.J. (2009). Hemoglobinopathies and clinical laboratory testing. **Medical Laboratory Observes**. 41(1): 8-18.

Adiana, M.A. and Mazura, M.P. (2011). Study on senna alata and its different extracts by Fourier transform infrared spectroscopy and two-dimensional correlation infrared spectroscopy. **Journal of Molecular Structure**. 991(1-3): 84-91.

Al-Mustafa, J.I. (2002). FTIR investigation of the conformational properties of the cyanide bound human hemoglobin. **Vibrational Spectroscopy**. 30(2): 139-146.

Asakura, T., Adachi, K., Shapiro, M., Friedman, S. and Schwartz, E. (1975). Mechanical precipitation of hemoglobin Köln. **Biochimica et Biophysica Acta (BBA) - Protein Structure**. 412(2): 197-201.

Aster, J.C., Pozdnyakova, O. and Kutok, J.L. (2012). **β-Thalassemia**. **Hematopathology: High-Yield (Expert Consult-Online and Print)**. Elsevier Health Sciences.

Bain, B.J. (2011). Haemoglobinopathy diagnosis: Algorithms, lessons and pitfalls. **Blood Reviews**. 25(5): 205-213.

Bain, B.J., Wild, B.J., Stephens, A.D. and Phelan, L.A. (2010). **Globin genes and haemoglobin**. **Variant haemoglobins: A guide to identification**. Blackwell Publishing Ltd.

Bank, A. (1978). The thalassemia syndromes. **Blood**. 51(3): 369-384.

Barth, A. (2007). Infrared spectroscopy of proteins. **Biochimica et Biophysica Acta (BBA)-Bioenergetics**. 1767(9): 1073-1101.

Bechtel, H.A., Martin, M.C., May, T. and Lerch, P. (2009). Improved spatial resolution for reflection mode infrared microscopy. **Review of Scientific Instruments**. 80(12): 1-3.

Boiret, M., Meunier, L. and Ginot, Y.M. (2011). Tablet potency of Tianeptine in coated tablets by near infrared spectroscopy: Model optimisation, calibration transfer and confidence intervals. **Journal of Pharmaceutical and Biomedical Analysis**. 54(3): 510-516.

Bunn, H., Forget, B., Bunn, H. and Forget, B. (1986). **Hemoglobin: Molecular, genetic and clinical aspects**. Philadelphia: WB Saunders.

Bunn, H.F. (2012). **Approach to the anemias**. **Goldman's cecil medicine** (24th ed.) Philadelphia: WB Saunders. 1031-1039 pp.

Camitta, B.M. and Jean Slye, R. (2012). Optimizing use of the complete blood count. **Pediatria Polska**. 87(1): 72-77.

Chen, R. and Spiro, T.G. (2002). Monitoring the allosteric transition and CO rebinding in hemoglobin with time-resolved FTIR spectroscopy. **Journal of Physical Chemistry A**. 106(14): 3413-3419.

Chow, J., Phelan, L. and Bain, B.J. (2005). Evaluation of single-tube osmotic fragility as a screening test for thalassemia. **American Journal of Hematology**. 79(3): 198-201.

Clarke, G.M. and Trefor, N.H. (2000). Laboratory investigation of hemoglobinopathies and thalassemias: Review and update. **Clinical Chemistry**. 46(8): 1284-1290.

Cochran, W. (1977). **Sampling techniques**. (3rd ed.). New York: Wiley and Sons. 259-261 pp.

Colthup, N.B., Daly, L.H. and Wiberley, S.E. (1990). Vibrational and rotational spectra. **Introduction to infrared and raman spectroscopy**. (3rd ed.) Oxford: Academic Press. 1-74 pp.

Dailey, H.A. and Meissner, P.N. (2013). Erythroid heme biosynthesis and its disorders. **Cold Spring Harbor Perspectives in Medicine**. 3(4): 1-18.

Dalibalta, S., Ellory, J.C., Browning, J.A., Wilkins, R.J., Rees, D.C. and Gibson, J.S. (2010). Novel permeability characteristics of red blood cells from sickle cell patients heterozygous for HbS and HbC (HbSC genotype). **Blood Cells, Molecules, and Diseases**. 45(1): 46-52.

de Oliveira, I.K., de Carvalho Rocha, W.F. and Poppi, R.J. (2009). Application of near infrared spectroscopy and multivariate control charts for monitoring biodiesel blends. **Analytica Chimica Acta**. 642(1-2): 217-221.

Department of Medical Science, Clinical Research Center. (2010a). **Hemoglobin typing and quantitation**. **Manual of laboratory for diagnosis thalassemia and hemoglobinopathy**. (3rd ed.). Nonthaburi: Knockoutblow Press.

Department of Medical Science, Clinical Research Center. (2010b). **Hemoglobin typing and quantitation**. **Manual of laboratory for diagnosis thalassemia and hemoglobinopathy**. (3rd ed.). Nonthaburi: Knockoutblow Press.

Dong, A. and Caughey, W.S. (1994). Infrared methods for study of hemoglobin reactions and structures. **Methods in Enzymology**. 232(1): 139-175.

Dörr, S., Schade, U. and Hellwig, P. (2008). Far infrared spectroscopy on hemoproteins: A model compound study from 1800-100 cm^{-1}. **Vibrational Spectroscopy**. 47(1): 59-65.

Fucharoen, G., Sanchaisuriya, K., Sae-ung, N., Dangwibul, S. and Fucharoen, S. (2004a). A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in south-east Asia. **Bulletin of the World Health Organization**. 82: 364-372.

Fucharoen, G., Sanchaisuriya, K., Sae-ung, N., Dangwibul, S. and Fucharoen, S. (2004b). A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in South-East Asia. **Bulletin of the World Health Organization**. 82: 364-372.

Fucharoen, S. and Weatherall, D.J. (2012). The hemoglobin E thalassemias. **Cold Spring Harbor Perspectives in Medicine**. 2(8): 1-15.

Fucharoen, S. and Winichagoon, P. (2010). Prevention and control of thalassemia in Asia. **Asian Biomedicine**. 1(1): 1-6.

Fucharoen, S. and Winichagoon, P. (2012). New updating into hemoglobinopathies. **International Journal of Laboratory Hematology**. 34(6): 559-565.

Fukumaki, Y., Fucharoen, S., Fucharoen, G., Okamoto, N., Ichinose, M., Jetsrisuparb, A., Sriroongrueng, W., Nopparatana, C., Laosombat, V. and Panich, V. (1991). Molecular heterogeneity of beta-thalassemia in Thailand. **Southeast Asian Journal of Tropical Medicine and Public Health**. 23(2): 14-21.

Galanello, R. and Origa, R. (2010). Review: Beta-thalassemia. **Orphanet Journal Rare Diseases**. 5(11): 1-15.

Gao, X., Butler, I.S. and Kremer, R. (2005). A near-infrared Fourier transform Raman spectroscopy of epidermal keratinocytes: Changes in the protein-DNA

structure following malignant transformation. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy**. 61(1-2): 27-35.

Giardine, B., van Baal, S., Kaimakis, P., Riemer, C., Miller, W., Samara, M., Kollia, P., Anagnou, N.P., Chui, D.H., Wajcman, H., Hardison, R.C. and Patrinos, G.P. (2007). HbVar database of human hemoglobin variants and thalassemia mutations: 2007 update. **Human Mutation**. 28(2): 206.

Gregoriou, V.G., Jayaraman, V., Hu, X. and Spiro, T.G. (1995). FT-IR difference spectroscopy of hemoglobins A and Kempsey: Evidence that a key quaternary interaction induces protonation of Asp. beta. 99. **Biochemistry**. 34(20): 6876-6882.

Hagh, M.F., Fard, A.D., Saki, N., Shahjahani, M. and Kaviani, S. (2011). Molecular mechanisms of hemoglobin F induction. **International Journal of Hematology-Oncology and Stem Cell Research**. 5(4): 5-9.

Hartwell, S.K., Srisawang, B., Kongtawelert, P., Christian, G.D. and Grudpan, K. (2005). Review on screening and analysis techniques for hemoglobin variants and thalassemia. **Talanta**. 65(5): 1149-1161.

Higgs, D.R., Engel, J.D. and Stamatoyannopoulos, G. (2012). Thalassaemia. **The Lancet**. 379(9813): 373-383.

Hoyer, J.D., Penz, C.S., Fairbanks, V.F., Hanson, C.A. and Katzmann, J.A. (2002). Flow cytometric measurement of hemoglobin F in RBCs: Diagnostic usefulness in the distinction of hereditary persistence of fetal hemoglobin (HPFH) and hemoglobin S-HPFH from other conditions with elevated levels of hemoglobin F. **American Journal of Clinical Pathology**. 117(6): 857-863.

Hughes, T.L., Methven, C.M., Jones, T.G.J., Pelham, S.E., Fletcher, P. and Hall, C. (1995). Determining cement composition by Fourier transform infrared spectroscopy. **Advanced Cement Based Materials**. 2(3): 91-104.

Huisman, T.H.J. (1972). Normal and abnormal human hemoglobins. In B. Oscar and A.L. Latner (eds.). **Advances in clinical chemistry** (Vol. 15, pp. 149-253). Elsevier.

Huisman, T.H.J. (1992). The β-and δ-thalassemia repository. **Hemoglobin**. 17(5): 479-499.

Jung, C. (2000). Insight into protein structure and protein-ligand recognition by Fourier transform infrared spectroscopy. **Journal of Molecular Recognition**. 13(6): 325-351.

Karimi, M. and Rasekhi, A.R. (2002). Efficiency of premarital screening of beta-thalassemia trait using MCH rather than MCV in the population of Fars Province, Iran. **Haematologia**. 32(2): 129-133.

Karnpean, R., Pansuwan, A., Fucharoen, G. and Fucharoen, S. (2011). Evaluation of the URIT-2900 automated hematology analyzer for screening of thalassemia and hemoglobinopathies in Southeast Asian populations. **Clinical Biochemistry**. 44(10-11): 889-893.

Kato, G.J. and Gladwin, M.T. (2009). Mechanisms and clinical complications of hemolysis in sickle cell disease and thalassemia. In G.J. Kato and M.T. Gladwin (eds.). **Disorders of hemoglobin: Genetics, pathophysiology and clinical management** (2nd ed., pp. 239-434). Cambridge University Press.

Kaul, D.K. (2008). Sickle cell disease. In Ronald, F.T. (eds.). **Microcirculation** (2nd ed., pp. 769-793). San Diego: Academic Press.

Khanmohammadi, M., Garmarudi, A.B., Garmarudi, A.B. and de la Guardia, M. (2012). Characterization of petroleum-based products by infrared spectroscopy and chemometrics. **TrAC Trends in Analytical Chemistry**. 35: 135-149.

Klaessens, J.H., Hopman, J.C., van Wijk, M.C., Djien Liem, K. and Thijssen, J.M. (2005). Assessment of local changes of cerebral perfusion and blood concentration by near infrared spectroscopy and ultrasound contrast densitometry. **Brain and Development**. 27(6): 406-414.

Klecka, W.R. (1980). **Discriminant analysis**. California: Sage.

Lamprell, H., Mazerolles, G., Kodjo, A., Chamba, J.F., Noël, Y. and Beuvier, E. (2006). Discrimination of *Staphylococcus aureus* strains from different species of *Staphylococcus* using Fourier transform infrared (FTIR) spectroscopy. **International Journal of Food Microbiology**. 108(1): 125-129.

Liu, H., Sun, S., Lv, G. and Liang, X. (2006). Discrimination of extracted lipophilic constituents of Angelica with multi-steps infrared macro-fingerprint method. **Vibrational Spectroscopy**. 40(2): 202-208.

Liu, J.J., Xu, H., Cai, W.S. and Shao, X.G. (2011). Discrimination of industrial products by on-line near infrared spectroscopy with an improved dendrogram. **Chinese Chemical Letters**. 22(10): 1241-1244.

Liu, K.Z., Tsang, K.S., Li, C.K., Shaw, R.A. and Mantsch, H.H. (2003). Infrared spectroscopic identification of β-thalassemia. **Clinical Chemistry**. 49(7): 1125-1132.

Liu, K.Z., Shi, M.H. and Mantsch, H.H. (2005). Molecular and chemical characterization of blood cells by infrared spectroscopy: a new optical tool in hematology. **Blood Cells, Molecules and Diseases**. 35(3): 404-412

Liu, X.h., Xu, C.H., Sun, S.Q., Huang, J., Zhang, K., Li, G.Y., Zhu, Y., Zhou, Q., Zhang, Z.C. and Wang, J.H. (2012). Discrimination of different genuine Danshen and their extracts by Fourier transform infrared spectroscopy combined with two-dimensional correlation infrared spectroscopy. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy**. 97(1): 290-296.

Mietke, H., Beer, W., Schleif, J., Schabert, G. and Reissbrodt, R. (2010). Differentiation between probiotic and wild-type Bacillus cereus isolates by antibiotic susceptibility test and Fourier transform infrared spectroscopy (FT-IR). **International Journal of Food Microbiology**. 140(1): 57-60.

Miller, S., Dykes, D. and Polesky, H. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. **Nucleic Acids Research**. 16(3): 1215.

Ministry of Public Health, Bureau of Policy and Strategy. (2007). **Number and rate of in-patients (NHSO and CS) according to 298 causes of diseases by all diagnosis, sex and region per 100,000 population, 2007** [On-line]. Available: http://bps.ops.moph.go.th/E-book/statistic/statistic50.

Modell, B. and Darlison, M. (2008). Global epidemiology of haemoglobin disorders and derived service indicators. **Bulletin of the World Health Organization**. 86: 480-487.

Moh, P.P., Fiamingo, F.G. and Alben, J.O. (1987). Conformational sensitivity of β-93 cysteine SH to ligation of hemoglobin observed by FT-IR spectroscopy. **Biochemistry**. 26(1): 6243-6249.

Mounts, J., Clingenpeel, J., White, N. and Villella, A. (2010). Apparent desaturation on pulse oximetry because of hemoglobinopathy. **Pediatric Emergency Care**. 26(10): 748-749.

Nadolny, G., Kempf, I. and Zundel, G. (1993). Specific interactions of the allosteric effector 2, 3-bisphosphoglycerate with human hemoglobin-A difference FTIR study. **Biological Chemistry Hoppe-Seyler**. 374(1-6): 403-407.

Nagel, R.L., Fabry, M.E. and Steinberg, M.H. (2003). The paradox of hemoglobin SC disease. **Blood Reviews**. 17(3): 167-178.

Nienhuis, A.W. (1987). Hemoglobin: Molecular, genetic and clinical aspects: By H. F. Bunn and B. G. Forget. Philadelphia: W. B. Saunders Company. (1986). **Cell**. 48(5): 731.

Nnaji, G.A., Ezeagwuna, D.A., Nnaji, I., Osakwe, J.O., Nwigwe, A.C. and Onwurah, O.W. (2013). Prevalence and pattern of sickle cell disease in premarital couples in Southeastern Nigeria. **Nigerian Journal of Clinical Practice**. 16(3): 309-314.

Nuntakarn, L., Fucharoen, S., Fucharoen, G., Sanchaisuriya, K., Jetsrisuparb, A. and Wiangnon, S. (2009). Molecular, hematological and clinical aspects of thalassemia major and thalassemia intermedia associated with Hb E-β-thalassemia in Northeast Thailand. **Blood Cells, Molecules, and Diseases**. 42(1): 32-35.

Old, J. (2013). Hemoglobinopathies and thalassemias. In L.R. David P. Reed and K. Bruce (eds.). **Emery and rimoin's principles and practice of medical genetics** (6th ed., pp. 1-44). Oxford: Academic Press.

Olivieri, N.F. and Weatherall, D.J. (2007). Thalassemias. In J.A. Robert M.H. Ian and P.S. Owen (eds.). **Pediatric hematology** (3rd ed., pp. 281-301). Blackwell Publishing Ltd.

Orsini, J.J., Yeman, J., Bodamer, O.A., Mühl, A. and Caggana, M. (2010). Semi-quantitative method for determination of hematocrit in dried blood spots, using data collected in HPLC hemoglobin variant testing. **Clinica Chimica Acta**. 411(11): 894-895.

Pasquini, C. and Bueno, A.F. (2007). Characterization of petroleum using near-infrared spectroscopy: Quantitative modeling for the true boiling point curve and specific gravity. **Fuel**. 86(12-13): 1927-1934.

Pavia, L. and Kriz, V. (2009). Infrared spectroscopy. In L.P. Donald M.L. Gary S.K. George and A.V. James (eds.). **Introduction to spectroscopy** (4th ed., pp. 15-104). CengageBrain.com.

Potter, W.T., Hazzard, J.H., Kawanishi, S. and Caughey, W.S. (1983). Direct measurement of carbon monoxide bound to different subunits of hemoglobin A in solution and in red cells by infrared spectroscopy. **Biochemical and Biophysical Research Communications**. 116(1): 719-725.

Rahmelow, K., Hübner, W. and Ackermann, T. (1998). Infrared absorbances of protein side chains. **Analytical Biochemistry**. 257(1): 1-11.

Ratanasiri, T. (2013). Prevention and control of thalassemia in obstetric practice. **Srinagarind Medical Journal**. 22(4): 471-476.

Rees, D.C., Williams, T.N. and Gladwin, M.T. (2010). Sickle-cell disease. **The Lancet**. 376(9757): 2018-2031.

Ring, E. (2000). The discovery of infrared radiation in 1800. **Imaging Science Journal**. 48(1): 1-8.

Roggo, Y., Jent, N., Edmond, A., Chalus, P. and Ulmschneider, M. (2005). Characterizing process effects on pharmaceutical solid forms using near-infrared spectroscopy and infrared imaging. **European Journal of Pharmaceutics and Biopharmaceutics**. 61(1-2): 100-110.

Rosenberg, L.E. and Rosenberg, D.D. (2012). **Single-gene defects**. **Human genes and genomes**. San Diego: Academic Press. 169-196 pp.

Ryan, K., Bain, B.J., Worthington, D., James, J., Plews, D., Mason, A., Roper, D., Rees, D.C., de la Salle, B., Streetly, A. and British Committee for Standards in Haematology. (2010). Significant haemoglobinopathies: Guidelines for screening and diagnosis. **British Journal of Haematology**. 149(1): 35-49.

Sampath, V., Zhao, X.J. and Caughey, W.S. (1994). Characterization of interactions of nitric oxide with human hemoglobin A by infrared spectroscopy. **Biochemical and Biophysical Research Communications**. 198(1): 281-287.

Scott, A.W., Lutty, G.A. and Goldberg, M.F. (2013). **Hemoglobinopathies. Retina** (5th ed., pp. 1071-1082). London: W.B. Saunders.

Seaman, G., Knox, R., Nordt, F. and Regan, D. (1977). Red cell agins. I. Surface charge density and sialic acid content of density-fractionated human erythrocytes. **Blood**. 50(6): 1001-1011.

Shemin, D. and Rittenberg, D. (1946). The life span of the human red blood cell. **Journal of Biological Chemistry**. 166(2): 627-636.

Singer, S.T. (2009). Variable clinical phenotypes of alpha-thalassemia syndromes. **Scientific World Journal**. 9: 615-625.

Strug, I., Utzat, C. and Nadler, T. (2012). IR-based protein & peptide quantitation. Assay Tutorials [On-line serial]. Available: http://www. genengnews.com/gen-articles/ir-based-protein-peptide-quantitation/4586.

Thom, C.S., Dickson, C.F., Gell, D.A. and Weiss, M.J. (2013a). Hemoglobin variants: Biochemical properties and clinical correlates. **Cold Spring Harbor Perspectives in Medicine**. 3(3): 1-22.

Thom, C.S., Dickson, C.F., Gell, D.A. and Weiss, M.J. (2013b). Hemoglobin variants: Biochemical properties and clinical correlates. **Cold Spring Harbor Perspectives in Medicine**. 3(3): 1-22.

Thumanu, K., Sangrajrang, S., Khuhaprema, T., Kalalak, A., Tanthanuch, W., Pongpiachan, S. and Heraud, P. (2014). Diagnosis of liver cancer from blood sera using FTIR microspectroscopy: A preliminary study. **Journal of Biophotonics**. 7(3-4): 222-231.

Tosqui, P. (2010). O papel das argininas alfa-92 e alfa-141 na regulação das propriedades funcionais de hemoglobinas por íons cloreto. **Revista Brasileira de Hematologia e Hemoterapia**. 32: 427-428.

Vályi-Nagy, I., Kaffka, K.J., Jákó, J.M., Gönczöl, É. and Domján, G. (1997). Application of near infrared spectroscopy to the determination of haemoglobin. **Clinica Chimica Acta**. 264(1): 117-125.

Vichinsky, E.P. (2013). Clinical manifestations of alpha-thalassemia. **Cold Spring Harbor Perspectives in Medicine**. 3(5): 1-11.

Wallace, W.J., Volpe, J.A., Maxwell, J.C., Caughey, W.S. and Charache, S. (1976) Properties of hemoglobin A and hemoglobin Zurich (β< sub> 63</sub> histidine→ arginine): Quantitative evaluation of functional abnormalities in hemoglobins. **Biochemical and Biophysical Research Communications**. 68(4): 1379-1386.

Wan, J.H., Tian, P.L., Yin, H., Han, Y., Wei, X.C. and Pan, T. (2013a). A preliminary evaluation of attenuated total reflection Fourier transform infrared

spectroscopy for the hematological analysis of thalassemias. **Clinical Biochemistry**. 46(1-2): 128-132.

Wan, J.H., Tian, P.L., Yin, H., Han, Y., Wei, X.C. and Pan, T. (2013b). A preliminary evaluation of attenuated total reflection Fourier transform infrared spectroscopy for the hematological analysis of thalassemias. **Clinical Biochemistry**. 46(1-2): 128-132.

Wasi, P., Pootrakul, S., Pootrakul, P., Pravatmuang, P., Winichagoon, P. and Fucharoen, S. (1980). Thalassemia in Thailand. **Annals of the New York Academy of Sciences**. 344(1): 352-363.

Weatherall, D.J. and Clegg, J.B. (2001). Inherited haemoglobin disorders: an increasing global health problem. **Bulletin of the World Health Organization**. 79(8): 704-712.

Weed, R.I., Reed, C.F. and Berg, G. (1963). Is hemoglobin an essential structural component of human erythrocyte membranes. **Journal of Clinical Investigation**. 42: 581-588.

Weissbluth, M. (1974). Hemoglobin. In W. Mitchel (ed.). **Hemoglobin: Cooperativity and electronic properties** (Vol. 15). Springer Berlin Heidelberg.

Wibulpolprasert, S. (2011). Public health statistics of number and rate of in-patients (NHSO and CS) according to 298 causes of diseases by all diagnosis ,sex and region per 100,000 population. **Thailand Health Profile 2008-2010**. Nonthaburi: Bureau of Health Policy and Strategy, Ministry of Public Health Press.

Wild, B. and Bain, B.J. (2006). Chapter 12 - Investigation of abnormal haemoglobins and thalassaemia. Dacie and Lewis practical haematology (10th ed., pp. 271-310). Philadelphia: Churchill Livingstone.

Williams, T.N. and Weatherall, D.J. (2012). World distribution, population genetics, and health burden of the hemoglobinopathies. **Cold Spring Harbor Perspectives in Medicine**. 2(9): 1-14.

Winichagoon, P., Thitivichianlert, A., Lebnak, T., Piankijagum, A. and Fucharoen, S. (2002). Screening for the carriers of thalassemias and abnormal hemoglobins at the community level. **Southeast Asian Journal of Tropical Medicine and Public Health**. 33(3): 145-150.

Winslow, R. (2006). Hemoglobin. In J.L. Geoffrey and D.S. Steven (eds.). **Encyclopedia of respiratory medicine** (pp. 263-267). Oxford: Academic Press.

Wood, W.G., Pearce, K., Clegg, J.B., Weatherall, D.J., Robinson, J.S., Thorburn, G.D. and Dawes, G.S. (1976). Switch from foetal to adult haemoglobin synthesis in normal and hypophysectomised sheep. **Nature**. 264(5588): 799-801.

Yamsri, S., Sanchaisuriya, K., Fucharoen, G., Sae-ung, N. and Fucharoen, S. (2011). Genotype and phenotype characterizations in a large cohort of β-thalassemia heterozygote with different forms of α-thalassemia in northeast Thailand. **Blood Cells, Molecules and Diseases**. 47(2): 120-124.

Yokose, N., Sakatani, K., Murata, Y., Awano, T., Igarashi, T., Nakamura, S., Hoshino, T. and Katayama, Y. (2010). Bedside monitoring of cerebral blood oxygenation and hemodynamics after aneurysmal subarachnoid hemorrhage by quantitative time-resolved near-infrared spectroscopy. **World Neurosurgery**. 73(5): 508-513.

APPENDIX

Ethics Committee

For

Researches Involving Human Subjects (Minimal Risk Review)

Suranaree University of Technology

.....

Title of Project : Application of Infared Spectroscopy for Screening Thalassemia and Hemoglobin E

Project Code : EC-56-28

Principal Investigator : Miss Somsamorn Sukpong

Department : School of Pharmacycology, Institute of Science

The aforementioned project has been reviewed and approved by Ethics Committee for Researches Involving Human Subjects, based on the Declaration of Helsinki.

Tongraar A. ..... Chairman

(Assoc. Prof. Dr. Anan Tongraar)

Ethics Committee for Researches Involving Human Subjects,

Suranaree University of Technology

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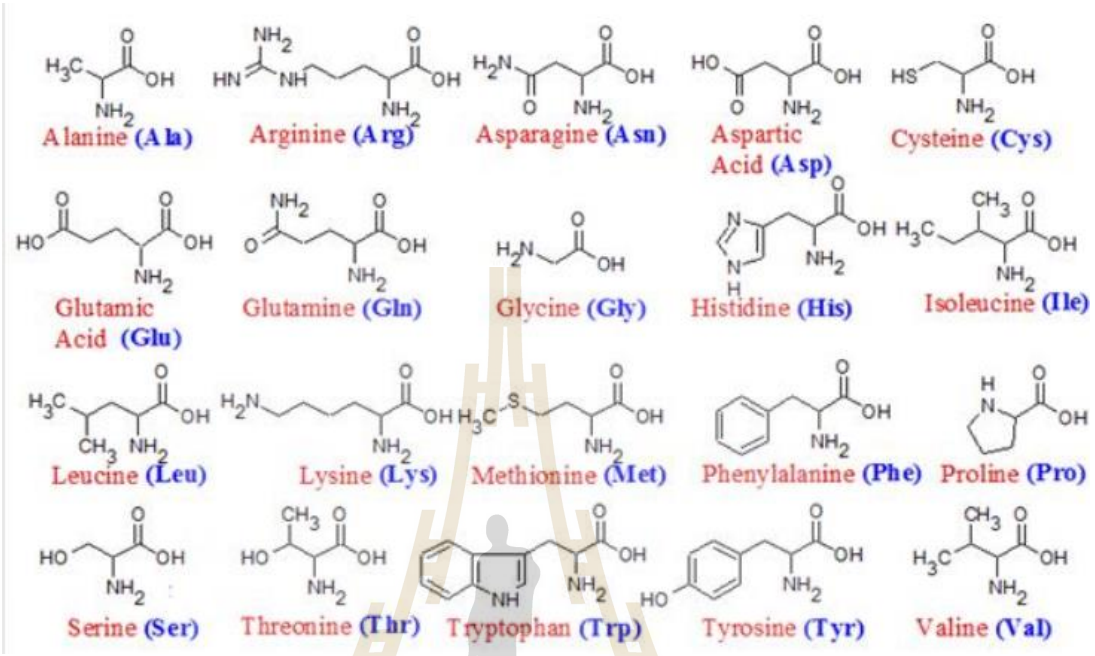
Abbreviations for amino acids

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

From: http://www.carolguze.com/images/biomolecules/AminoAcidJargonStryerBio3

Amino acids structure



From: http://biochemistrycourse.blogspot.com

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