CHAPTER 1

INTRODUCTION

1. Statement and significance of the problem

Detection of carriers of thalassemia and hemoglobinopathies is an important strategy for prevention and control of thalassemia and hemoglobinopathies in countries having high frequencies of these disorders including Thailand. This strategy conventionally consists of 2 principal steps, starting with the screening step to search for individuals possibly carrying abnormal globin genes in heterozygous states, followed by the confirmation step that aims to generate the definite diagnosis in those having the positive screening results. The carrier screening tests conventionally comprise a group of simple tests; one-tube osmotic fragility test (OFT) employing either 0.36% NaCl or 0.45% glycerine saline solutions, automatically derived red blood cell indices including mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), and HbE screening test such as the dichlorophenol indolphenol precipitation (DCIP) test and the AMS-HbE Tube test (Fucharoen and Winichagoon 2011, Tatu and Kasinrerk 2011, Wasi 1983, Weatherall and Clegg 2001). The confirmation tests are composed of the tests for hemoglobin studies and the tests for DNA analysis. Hemoglobin study techniques are composed of the manual cellulose acetate electrophoresis (CAE), isoelectric focussing

(IEF), high performance liquid chromatography (HPLC), low pressure liquid chromatography (LPLC), capillary zone electrophoresis (CZE) and immunological detection of hemoglobin (Clarke and Higgins 2000). Although hemoglobin studies provide information enough to obtain diagnosis of β-thalassemia carrier, HbE carrier and, for some extent, α-thalassemia carrier, specific types of β-globin gene mutations as well as co-existence of α-thalassemia in various β-hemoglobinopathies cannot be figured out in this step. The definite diagnosis of these carriers has to be done via DNA analysis. The DNA analysis to identify defects of globin genes causing the thalassemia and hemoglobinopathies currently employed is mostly performed using the polymerase chain reaction (PCR)-based techniques such as PCR with restriction digestion, PCR with allele-specific oligonucleotide (ASO)-hybridization, PCR with reversed dot blot (RDB)-hybridization, nucleotide sequencing, allele-specific PCR such as mutagenically separated (MS)-PCR and amplification refractory mutation system (ARMS)-PCR (Clark and Thein 2004, Weatherall and Clegg 2001). These allele-specific PCRs have been proved to be highly applicable in simultaneously detecting globin gene mutations in several platforms. Thus, this thesis aimed initially to develop the in-house singleplex allele-specific PCR, and subsequently the in-house multiplex allele-specific PCR for detecting both α- and β-thalassemia/hemoglobinopathies endemic in Thailand.

Most PCR protocols currently employed need DNA as the template. Preparation of the target DNA requires several laborious and hazardous steps that prevent the DNA analysis from being the front-line test in thalassemia diagnosis. Those eligible for DNA analysis must have positive results from the screening tests and possess abnormal

hemoglobin patterns. Therefore, this thesis also aimed to search for alternative PCR technique without using genomic DNA as the template, but directly added whole blood into the PCR reaction. It was anticipated that combination of the in-house singleplex and multiplex allele-specific PCR with the use of whole blood instead of purified genomic DNA may highlight the DNA analysis as the front-line test in diagnosis of thalassemia/hemoglobinopathies. Definite diagnosis of these carriers should be successfully done with only screening tests followed by the whole-blood PCR, without the Hb typing step.

2. Literature review

2.1 Human hemoglobin

Hemoglobin is a principal protein in red blood cells that carries oxygen from lungs to the tissues and carbondioxide from tissues to lungs in the body. It has a quaternary structure consisting of four subunits of globular globin chains; two α-like and two β-like. A heme group containing an iron ion held in a heterocyclic ring or porphyrin embeds in each globin subunit of this tetramer. Iron portion of the heme group serves as site for oxygen binding moity of functional hemoglobin molecules (Figure 1.1). The α-like globin chains are composed of 2 functional globin chains; zeta (ζ) and alpha (α)-globin chains. These two α-like globin chains are produced from ζ- and α-globin genes localized in the α-globin gene cluster on the short arm of chromosome 16 (Ch16p13.2). Both ζ and α-globin chains have 141 amino acids. However, 57 of 141 amino acids were found to be different between the α-and the ζ-globin genes (Clegg and

Gagnon 1981). The β-like globin chains are synthesized from their corresponding genes localized in the β-globin gene cluster on the short arm of chromosome 11 (Ch11p15.5). These globin chains consist of 4 functional globin genes; ε, γ, δ and β. Although all these 4 β-like globin chains share identical 146 amino acids, they differ, for some extent, in their amino acid constituents. The γ-globin chains differ by 39 or 40 amino acids, δ-globin chain by 10 amino acids and ε-globin chain by 7 amino acid from the β-globin chain (Steinberg, *et al* 2001, Weatherall and Clegg 2001, Zheng, *et al* 1999).

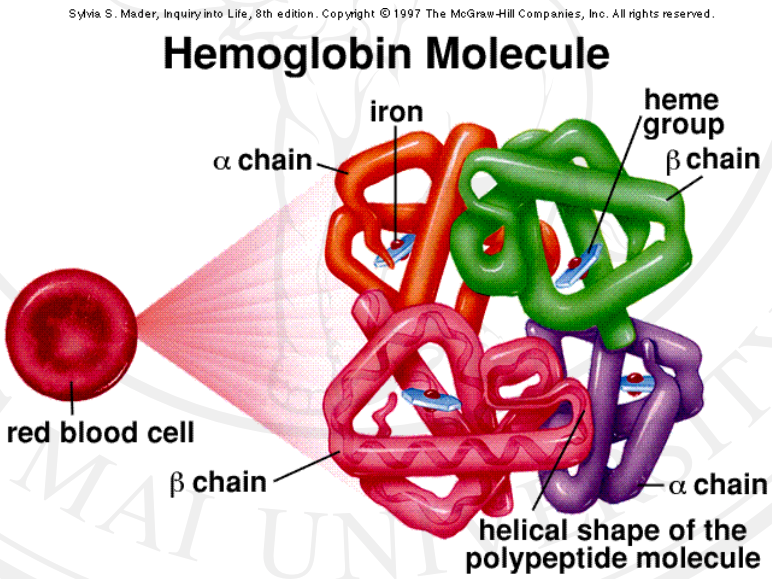

Figure 1.1 Structure of functional human hemoglobin molecule

(http://www.phil-islands.com/the-amazing-design-of-hemoglobin-molecule 6/09/2012)

2.2 Globin gene clusters, globin genes and hemoglobin switching

As stated previously, there are 2 groups of globin chains; the α-like and β-like. The α-like globin chains are synthesized from their corresponding genes localized on the α-globin gene clusters, whereas the β-like globin chains are the products of the globin genes on the β-globin gene cluster.

The α-globin gene cluster is approximately 30-kb long containing 3 functional genes (ζ, α2, α1), 3 pseudogenes (ψζ, ψα2, ψα1) and one newly discovered gene the function of which is still not clear (θ), arranged in the developmental stage specific order; 5'-ζ-ψζ-ψα2-ψα1-α2-α1-θ-3' (Steinberg, *et al* 2001, Waye and Chui 2001)(Figure 1.2). The ζ-globin gene expresses in the embryonic stage and the α-globin genes in the fetal throughout the adult lifes (Figure 1.3). Both ζ- and α-globin genes have 3 exons and 2 introns. These 2 introns interrupt the sequence between codons 31 and 32 and between codons 99 and 100 (Figure 1.4). In addition to the differences of 57 codons, several sequence diversities have been shown at several regions outside coding sequences of these two α-like globin genes (Table 1.1) (Steinberg, *et al* 2001, Weatherall and Clegg 2001). The β-globin gene clusters is approximately 70-kb long and is composed of 5 functional genes (ε,^{G}γ, ^{A}γ, δ, β) and one pseudogene (ψβ1). These genes are also arranged in the developmental stage specific order; 5'-ε-^{G}γ-^{A}γ-ψβ1-δ-β-3' (Figure 1.2). The ε-globin gene expression starts in embryonic state and switches to the γ-globin gene expression very early in gestation. The γ-globin gene expresses predominantly in fetus and switches to β-globin gene expression just before birth (Figure 1.3). All β –like globin

chains have 3 exons and 2 introns. These 2 introns interrupt the sequence between codons 30 and 31 and between codons 104 and 105 (Figure 1.4). Despite having the same numbers of triplet codons, the β-like globin genes have some differences. The γ-globin gene shows 39-40 codons, the δ-globin gene 10 codons and the ε-globin gene 7 codons being different from the β-globin gene. Moreover, other sequence variations outside the coding sequences of the β-like globin genes have also been shown (Table 1.1) (Steinberg, *et al* 2001, Weatherall and Clegg 2001).

As expression of globin genes is developmental stage specific, different sets of hemoglobin are synthesized in embryo, fetus and adult. This phenomenon is termed "hemoglobin switching". In embryonic stage, Hb Gower I ($\zeta_2\varepsilon_2$), Hb Gower II ($\alpha_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$) are produced. In the fetal stage, HbF ($\alpha_2\gamma_2$) and HbA ($\alpha_2\beta_2$) are synthesized, while in adult life, HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) are formed. Table 2 summaries the amount of hemoglobin synthesized in each developmental stage in human (Weatherall and Clegg 2001).

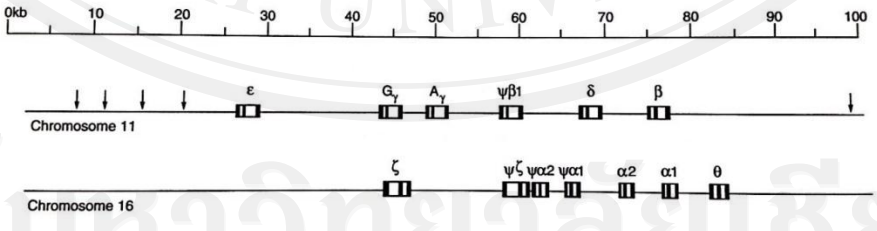

Figure 1.2 Structure of the human α-like globin gene clusters and β-like globin gene clusters (Steinberg, *et al* 2001)

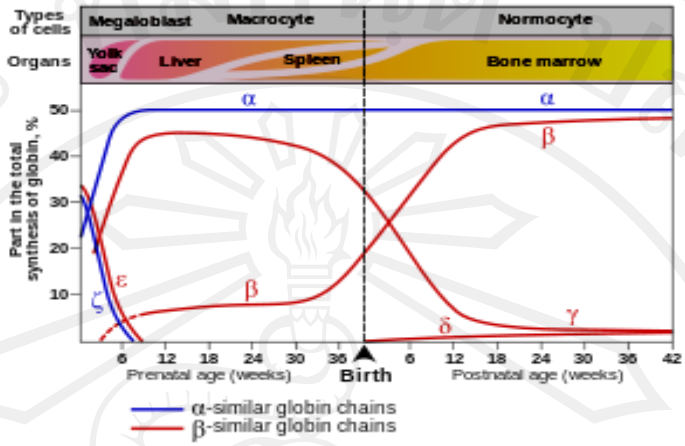

Figure 1.3 Developmental stage specific production of globin genes in human (http://en.wikipedia.org/wiki/Hemoglobin)

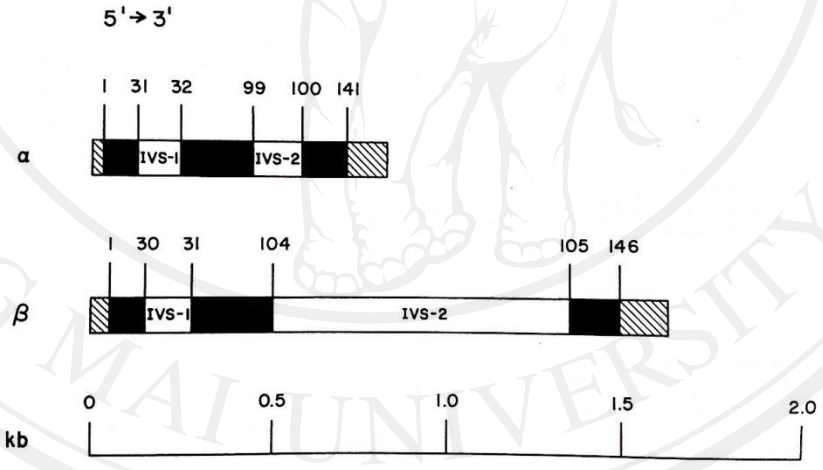

Figure 1.4 Structure of the human α-globin gene and β-globin gene. The black blocks represent the exons, the white blocks represent the introns and the cross-hatched blocks represent the 5' and 3' untranslated sequences (Steinberg, *et al* 2001).

Table 1.1 Differences of length of UTR and IVS of the human globin genes. (modified from (Weatherall and Clegg 2001))

Genes	5' UTR (bp)	IVS 1 (bp)	IVS 2 (bp)	3' UTR (bp)
ζ	58	866	239	108
α2	40	117	142	113
α1	40	117	149	113
ε	53	122	855	99
$^{G}\gamma$	53	121	886-904	66
$^{A}\gamma$	53	121	866-876	66
δ	48	138	898	111
β	50	130	850	113

Table 1.2 Hemoglobin constituents in each developmental stage in human (Weatherall and Clegg 2001)

	Embryo (%)	**Fetus (%)**	**Adults (%)**
Hb Gower I ($\zeta_2\varepsilon_2$)	42	Absent	Absent
Hb Gower II ($\alpha_2\varepsilon_2$)	24	Absent	Absent
Hb Partland ($\zeta_2\gamma_2$)	Present	Absent	Absent
HbF ($\alpha_2\gamma_2$)	Present	90	<1
HbA ($\alpha_2\beta_2$)	Absent	10	97
HbA_2 ($\alpha_2\beta_2$)	Absent	Absent	2.5

2.3 Thalassemia

Thalassemia is a group of inherited anemia caused by reduction or absence of globin chain production due to defects of the globin genes. These genetic defects, which could be either mutations or deletions, lead to reduced or absent synthesis of the globin chains which results in imbalanced-globin chain synthesis. The imbalanced-globin chain production results in excess α-like globin chains and β-like globin chains that may form homotertramer inside the red blood cells (α4, β4, γ4). These homotetramers halm red blood cells, via their own specific mechanisms, leading to erythrocytic break down or hemolysis and anemia (Weatherall and Clegg 2001).

In general, thalassemia is named after the affected globin chains, i.e. α-thalassemia if α-globin chain is affected, β-thalassemia if β-globin chain is affected, γ-thalassemia if γ-globin chain is affected, γ-, δ-, β-thalassemia if γ-, δ- and β-globin chain are affected and so on. However, only α-thalassemia and β-thalassemia are endemic across the world, whereas other types of thalassemia are found sporadically (Flint, *et al* 1998, Weatherall and Clegg 2001).

Thalassemia is inherited in an autosomal recessive fashion. Those heterozygous for the defective globin genes are not affected and may be called thalassemia carriers or thalassemia traits. Those homozygous for the abnormal globin genes are certainly affected by the disease. In addition, if an individual inherited one abnormal α-globin gene and one abnormal β-globin gene, this individual is called double heterozygote for α- and β-thalassemia. This doublely heterozygous individual is clinically asymptomatic and may have milder changes of red blood cell parameters than the single heterozygote.

However, if an individual carries two different mutations of the same globin gene, this individual is named compound heterozygote. This type of heterozygote clinically resembles the homozygous state of any particular globin genes from which the thalassemia disease results (Steinberg, *et al* 2001, Weatherall 1983).

2.4 Alpha (α)-thalassemia

Alpha (α) thalassemia is a blood disorder that the production of α-globin chain is reduced or absent. As a result, hemoglobin level declines leading to anemia and homotetramer of an excess γ-globin chain (γ4) or Hb Bart's as well as that of an excess β-globin chain (β4) or HbH form. Thus, presences of Hb Bart's and HbH should be indicative of the α-thalassemia. This type of thalassemia is predominantly resulted from deletions of the α-globin gene cluster removing one or both α-globin genes (Figure 1.5). These molecular defects then provide the basis of classification of this disorder.

The α-thalassemia consists of two groups; α-thalassemia 1 or α^{O}-thalassemia and α-thalassemia 2 or α^{+}-thalassemia, depending on numbers of deleted α-globin gene. α-thalassemia 1 or α^{O}-thalassemia is characterized by the deletion of two functional α-globin genes *in cis*. The heterozygote (genotype: --/αα) normally does not have health problems, except modest alteration of red blood cell parameters. The homozygote (genotype: --/--), in contrast, is life-threatening and causes the dead-fetus *in-utero* or still born baby (Higgs, *et al* 1989, Weatherall and Clegg 2001). There are in fact several types of α-thalassemia 1 deletion depending on an extent of the deletions of the α-globin gene cluster. The most frequent deletion found in Thailand is the Southeast Asian (SEA) type.

This deletion removes 19.3-kb fragment of the α-globin gene cluster spanning nucleotides 26264 to 45564 according to GenBank access code NG_000006.1 or nucleotides 155395 to 174700 according to GenBank access code AE006462. (Figure 1.5)(Ho, *et al* 2007).

α-thalassemia 2 or α^{+}-thalassemia is resulted from deletion of one of the α-globin gene on the α-globin gene cluster. A reciprocal recombination between Z-boxes of the α-globin gene cluster removes 3.7-kb segment and leaves one functional α-globin gene. This is called the 3.7-kb or rightward deletion ($-\alpha^{3.7}$) (Figure 1.6). Likewise, a reciprocal recombination between mispaired X-boxes on the α-globin gene cluster results in a 4.2-kb deletion of the α-globin cluster and is then called the 4.2-kb or leftward deletion ($-\alpha^{4.2}$) (Figure 1.6). Both heterozygote (-α/αα) and homozygote (-α/-α) show no clinical burdens. Hematologic changes in the heterozygote are very mild and, in most occasion, cannot be differentiated from normal. In homozygote, however, hematologic alterations resemble those observed in the α-thalassemia 1 heterozygote.

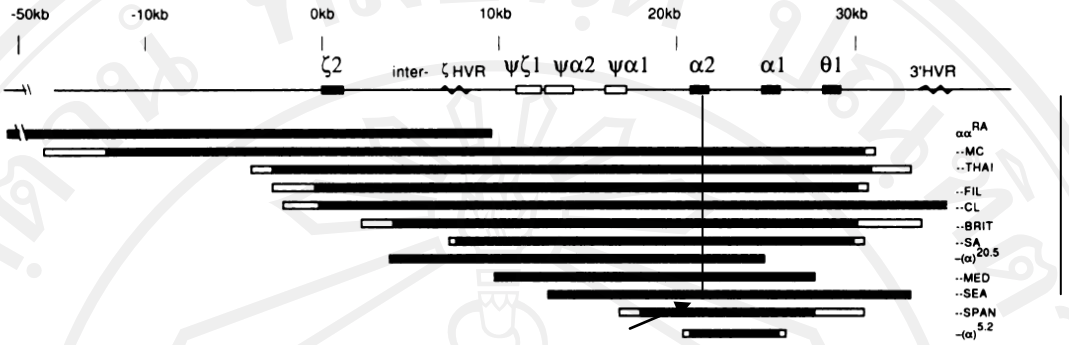

Figure 1.5 Deletions of α-globin gene cluster in α-thalassemia 1. Bars represent extent of the deletions. Vertical lines indicate extent of SEA-deletion. Name of each deletion is placed at the right-hand side of the bar. SEA-type of deletion is arrowed. (modified from (Higgs, *et al* 1989))

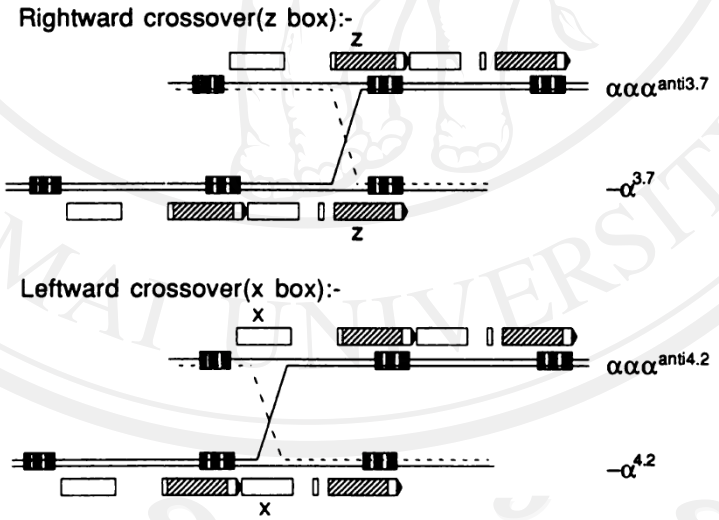

Figure 1.6 Reciprocal crossovers on α-globin cluster causing 3.7-kb (Z-Z boxes) and 4.2-kb (X-X boxes) deletions causing α-thalassemia 2 or α^{+}-thalassemia (modified from (Higgs, *et al* 1989))

2.5 Beta (β)-thalassemia

β-thalassemia is a group of hereditary anemia characterized by a reduction or absence of β-globin chain. It is caused by mutations of the nucleotides residing within and flanking the β-globin gene, i.e. structural and regulatory parts. In contrast to the α-thalassemia in which the numbers of deleted α-globin gene determine severity of disease, locations where the mutations occur determine whether the patients are severely affected or not. Accordingly, two types of the β-thalassemia are categorized; β^{O}-thalassemia and β^{+}-thalassemia.

β^{O}-thalassemia is characterized by completely absent of the β-globin chain production. Point mutations such as base substitutions, base additions and small base deletions within and closed to coding sequences mostly lead to this type of β-thalassemia. Adults homozygous or compound heterozygous for β^{O}-thalassemic genes cannot make normal β-globin chains at all, which results in complete absence of HbA. The β^{+}-thalassemia, in contrast, is characterized by only reduction of the β-globin chain. This type of β-thalassemia is mostly caused by point mutations residing outsite the coding regions, notably in the regulatory sequences; e.g promoter region. Adults homozygous and compound heterozygous for β^{+}-thalassemia are able to produce some β-globin chain and, as a result, HbA. Thus, clinically and hematologically, the β^{O}-thalassemia is more severe than the β^{+}-thalassemia. In Thailand, the most common mutations of the β-globin gene causing the β^{O}-thalassemia are the TTCT deletion at codons 41/42 ($\beta^{41/42(-TTCT)}$) and the A-T substitution at codon 17 ($\beta^{17(A-T)}$). The most prevalent mutations leading to the β+-thalassemia in Thailand is the A-G substitution at the nucleotide -28 to Cap site (β^{-}

$^{28(A-G)}$). However, some other β-globin gene mutations have also been characterized in Thailand. The list of these mutations are shown in Table 1.3 (Fucharoen and Winichagoon 1997, Nopparatana, *et al* 1995).

2.6 Hemoglobinopathies

Hemoglobinopathies comprise abnormal hemoglobins that are formed from abnormal globin chains. They are sometimes called structural or hemoglobin variants. These hemoglobin variants can be either α-like or β-like globin chains. Until recently, a total of 1152 hemoglobin variants originated from γ, δ, β and α-globin chains have been reported (http://globin.bx.psu.edu/cgi-bin/hbvar/query_vars3). In Thailand, HbE; the β-globin structural variant, and Hb Constant Spring (CS); the α-globin structural vatiant, are commonly found across the country. The β-globin chain in HbE contains lysine instead of glutamic acid at codon 26, while the α-globin chain in HbCS contains 31 additional amino acids. Abnomal β-globin chain in HbE and abnormal α-globin chain in HbCS are synthesized at the seemingly low rate compared to their normal counterparts. This phenomenon then makes HbE and HbCS behave phenotypically as β^+ and α^+-thalassemia, respectively. Thus, if HbE gene co-exists with β-thalsssemia gene, the thalassemia disease named HbE/β-thalassemia results. If HbCS is co-inherited with the α-thalassemia 1 deletion, the thalassemia disease named HbH-CS disease forms.

Table 1.3 Mutations of β-globin genes commonly found in Thailand (Fucharoen and Winichagoon 1997, Nopparatana, *et al* 1995)

Types of β-globin gene mutation	South (%)	Central (%)	North (%)	Northeast (%)	Average (%)
$\beta^{41/42\ (-TTCT)}$	30.1	41.6	39.8	37.7	37.3
$\beta^{IVS\ 1\ nt5\ (G-C)}$	18.8	4.3	2.8	0	6.5
$\beta^{19\ (A-G)}$	15.2	2.9	ND	0	6.0
$\beta^{17\ (A-T)}$	11.3	16.5	39.8	29.5	24.3
$\beta^{IVS1\ nt1\ (G-T)}$	6.0	1.3	ND	1.6	3.0
$\beta^{-28\ (A-G)}$	5.7	9.3	3.5	1.6	5.0
$\beta^{3.5\text{-}kb\ del}$	4.3	1.1	ND	ND	2.7
$\beta^{IVS2\ nt654(C-T)}$	2.1	8.0	1.4	9.8	5.3
$\beta^{41(-C)}$	1.4	0.8	ND	0	0.7
$\beta^{8/9\ (+G)}$	0.4	0	0	0	0.1
$\beta^{105\text{-}bp\ del}$	0.4	0	0	0	0.1
$\beta^{15\ (G-A)}$	0.4	0	0	0	0.1
$\beta^{CAP\ (A->C)}$	0.4	0	0	0	0.1
$\beta^{IVS1\ nt1\ (G-A)}$	0.4	0	0	0	0.1
$\beta^{-88\ (C-T)}$	0	0	0	0	0
$\beta^{-86\ (C-G)}$	0	0.5	0	0	0.1
$\beta^{16\ (-C)}$	0	0	0	0	0

$\beta^{35\ (C-A)}$	0	2.7	0	0	0.6
$\beta^{35\ (-C)}$	0	0	0	0	0
$\beta^{71/72\ (+A)}$	0	2.1	0	13.1	3.8
$\beta^{26\ (G-T)}$	0	ND	0	1.6	0.5
$\beta^{619\text{-bp del}}$	0	1.1	0	0	0.3
$\beta^{43\ (G-T)}$	0	0.8	0	0	0.2
$\beta^{15\ (-T)}$	0	0.3	0	0	0.1
$\beta^{14/15(+G)}$	0	0.3	0	0	0.1
Uncharacterized	3.1	5.1	13.3	4.9	6.6
Number of alleles	282	375	113	61	831

Note : ND = not done, del = deletion

2.7 Incidence of thalassemia and hemoglobinopathies in Thailand

Thalassemia and hemoglobinopathies are the most common genetic disorders in Thailand. Approximately 40% of Thai population are carriers of thalassemia and hemoglobinopathies in which 20-30%, 3-9%, 10-60% and 1-6% are carriers of the α-thalassemia, the β-thalassemia, the HbE and the HbCS, respectively (Sanguansermsri, *et al* 1998). These high frequencies of carriers can lead to the generation of new thalassemic patients with the annual risk of about 1,250 newborn of homozygous α-thalassemia-1 (Hb Bart's hydrops fetalis), 625 newborn with homozygous β-thalassemia and 3,250 newborn with HbE/β-thalassemia (Lebnak, *et al* 2005).

2.8 Clinical classification of thalassemia and hemoglobinopathies

The thalassemia and hemoglobinopathies can be classified on the clinical ground. This classification relies on several clinical and hematological aspects and includes 3 groups; thalassemia major, thalassemia intermedia and thalassemia minor. Thalassemia major is the most severe form of thalassemia. On average, this type of thalassemia is characterized by early onset of the disease (less than 2 years old), marked anemia (a steady-state Hb level less than 3-4 g/dL), requirement of frequent blood transfusions (2-3 times/month) and significant organomegaly with several fatal complications. The example of α-thalassemia major is Hb Bart's hydrops fetalis syndrome. However, some homozygotes for β^{O}-thalassemia and some HbE/β^{O}-thalassemia may present clinically as β-thalassemia major. The thalassemia intermedia, in contrast, always presents with a broad spectrum of clinical picture ranging from a condition little milder than the thalassemia major to symptomless disorder that is discovered only by chance on blood examination. The age of presentation of β-thalassemia intermedia tends to be latter than the β-thalassemia major. The clinical features are characterized by varying degree of anemia and splenomegaly and, in the more severe forms, bone changes similar to the major form of the illness. The hemoglobin level settles down to 7-10 g/dL. Blood transfusion is not need to be as frequent as for those with thalassemia major. HbH disease is the α-thalassemia manifesting clinically as α-thalassemia intermedia. For the β-thalassemia/hemoglobinopathies, some homozygous β^{O}, some compound heterozygous β^{+}/β^{O} and some HbE/β-thalassemia may show β-thalassemia intermedia feature. Finally, the thalassemia minor is observed in those heterozygous for thalassemia. Usually they are

characterized by mild anemia and small, poorly hemoglobinized red cells. Hemoglobin values of patients with thalassemia heterozygtes are usually in the range of 8-10 g/dL. The abnormallity is discovered only on performing a routine blood examination (Ho, *et al* 1998, Weatherall and Clegg 2001).

2.9 Laborarory diagnosis of thalassemia and hemoglobinopathies

The conventional strategy for making diagnosis of thalassemia and hemoglobinopathies consists of 2 principal steps including the screening and the confirmation steps.

Screening is the first step that help to identify individuals with increased risk for being the carriers and, ultimately, intends to reduce the new thalassemia cases in the population. This step is composed of the tests that, in theory, must be easy, cheap, rapid, reproducible and accurate. Those positive for the screening tests often requires further confirmation by more specific tests. A set of screening test conventionally employed in Thailand includes OFT and/or red blood cell indices (MCV and MCH) and HbE screening test such as DCIP test (Fucharoen, *et al* 2004, Tujinda, *et al* 2010, Wasi 1983). OFT, MCV and MCH have been shown to be effective in screening for carriers of α- and β-thalassemia with very high sensitivity and specificity (Fucharoen, *et al* 2004, Sirichotiyakul, *et al* 2004, Sirichotiyakul, *et al* 2009b, Tangvarasittichai, *et al* 2004). Most of the β- and α-thalassemia carriers can be screened in by these tests. However, HbE carriers can be either positive or negative for OFT and red cell indices (Fucharoen, *et al* 2004, Sirichotiyakul, *et al* 2009b). Thus, HbE screening must be performed in all

individuals after the initial screening. Recently, at least 2 HbE screening tests have been introduced in Thailand. These include DCIP test and the AMS-HbE Tube test (Chapple, *et al* 2006, Tatu and Kasinrerk 2011). Those individuals obtaining positive results for the initial and HbE screening tests are subjected to further confirmation tests for generating definite diagnosis. The confirmatory laboratory tests presently performed include the hemoglobin studies and DNA analysis.

Hb studies aim to determine types and quantities of hemoglobins in blood samples. Except the α-thalassemia carriers, Hb studies provide enough information to make the definite diagnosis of the thalassemia diseases, the β-thalassemia carriers and carriers of abnormal hemoglobins. At present, for Thailand in particular, Hb studies are performed using several techniques such as high pressure liquid chromatography (HPLC), low pressure liquid chromatography (LPLC), cellulose acetate electrophoresis (CAE) and capillary electrophoresis (CE), most of which are automatically operated (Clarke and Higgins 2000, Cotton, *et al* 1999, Kim, *et al*, Kutlar 2007, Ou-Yang, *et al* 2004, Singsanan, *et al* 2007, Sirichotiyakul, *et al* 2009a).

DNA analysis is the confirmatory step for diagnosis of thalassemia and hemoglobinopathies; i.e. to identify the causal globin gene defects. This step is particularly essential in the carriers seeking the prenatal diagnosis (PND) of the *in-utero* fetuses at risk of severe thalassemia/hemoglobinopathies. DNA analysis also provides information useful for the epidemiological surveys of thalassemia and hemoglobinopathies. Several DNA analysis techniques have been conducted in thalassemia diagnostic field. These techniques include the obsolete gene mapping and

several PCR-based techniques performed in both conventional and real-time PCR (Clark and Thein 2004, Clarke and Higgins 2000, Liu, *et al* 2009, Munkongdee, *et al* 2010, Pornprasert, *et al* 2011, Vrettou, *et al* 2003).

2.10 DNA analysis for diagnosis of thalassemia and hemoglobinopathies

As stated, the thalassemia and hemoglobinopathies are genetic disorder caused by abnormal globin genes resulting in quantitative and qualitative defects of globin chain synthesis, respectively. Thus, detection of specific globin gene mutations should be the ultimate goal for those affected. Todate, Polymerase Chain Reaction (PCR) is the molecular technique principally employed in the field of thalassemia diagnosis. The so-called Gap-PCR, either singleplex or multiplex, has been carried out to diagnose α-thalassemia in several centers. In 1991, Chang, JG. *et al* successfully carried out the singleplex Gap-PCR for detecting the SEA-α thalassemia 1 in prenatal diagnosis of Hb Bart's hydrops fetalis (Chang, *et al* 1991). In addition, Chan, A.Y. *et al* compared the singleplex Gap-PCR and Hb H inclusion body test for the SEA-α thalassemia 1 analysis and correlated results were shown (Chan, *et al* 1996). The singleplex PCR, however, was capable of detecting only one type of mutation at a time. Thus, several attempts have been undertaken to develop the so-called multiplex PCR which was capable of detecting more than one mutation in a single PCR reaction. Foglietta, E. *et al* developed the multiplex Gap-PCR and allele specific-PCR that could simultaneously identify 7 types of α-thalassemia including $-\alpha^{3.7}$, $-\alpha^{4.2}$, $\alpha^{Ncol}\alpha$, $\alpha^{Hphl}\alpha$, $\alpha\alpha\alpha^{3.7}$, $--^{Med}$ and $-\alpha^{20.5}$ (Foglietta, *et al* 1996)**.** In 2000, 8 types of α-thalassemia mutations were simultaneously characterized

by 3 mutiplex PCR developed by Li u, Y.T. *et al*, including $--^{SEA}$, $-\alpha^{20.5}$, $--^{MED}$, $--^{FIL}$ and $--^{THAI}$ in the first reaction, $-\alpha^{3.7}$, $\alpha\alpha\alpha^{3.7}$ in the second reaction and $-\alpha^{4.2}$ in the final reaction (Liu, *et al* 2000). Another multiplex PCR for detecting 6 types of α-thalassemia including $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $-\alpha^{20.5}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$ was developed by Chong, S.S. *et al* (Chong, *et al* 2000). In 2001, Tan, A.S. *et al* developed the mutiplex PCR for 7 common types of α-thalassemia in Southeast Asia, including $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $--^{THAI}$, $-\alpha^{20.5}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$ (Tan, *et al* 2001). In 2003, 3.7/4.2 multiplex PCR was proposed to combine with 7 deletion multiplex PCR for identifying α-globin gene tripication in β-thalassemia patients (Wang, *et al* 2003).

The allele-specific PCR in both singleplex and multiplex protocols have also been employed to identify the β-globin gene mutations. In 1991, 6 common types of β-thalassemia in Taiwan including $\beta^{IVSInt5(G-C)}$, $\beta^{41/42(-TTCT)}$, $\beta^{-28(A-G)}$, $\beta^{17(A-T)}$, $\beta^{-29(A-G)}$ and $\beta^{27/28(+C)}$ were systematically characterized by using PCR, dot-blot hybridization, and direct sequencing of amplified genomic DNA (Lin, *et al* 1991). In 1994, 7 β-thalassaemia mutations in Arab including $\beta^{IVSInt5(G-C)}$, $\beta^{8/9(+G)}$, $\beta^{IVSInt1(G-T)}$, $\beta^{(25\ bp\ del),}$ $\beta^{5(-C)}$, $\beta^{IVSIInt1(G-T)}$, $\beta^{30(G-C)}$ and $\beta^{15(G-A)}$ were characterized by a PCR method based on allele specific priming known as the amplification refractory mutation system (ARMS) (Quaife, *et al* 1994). Bianco, I. *et al* studied the correlation of genotype and phenotype of silent α-thalassemia and silent β-thalassemia by using a complete molecular study of the α- and β-globin genes by means of the ARMS, SSCP, DGGE, PCR and Southern blotting techniques (Bianco, *et al* 1997).

In Thailand, several groups have successfully characterized the SEA-α thalassemia 1 in pregnant women using the singleplex Gap-PCR (Sirichotiyakul, *et al* 2004, Sirichotiyakul, *et al* 2009b, Tangvarasittichai, *et al* 2004). For β-thalassemia, in 2004, Sritong, W. *et al* demonstrated the potential application of the singleplex Mutagenically Separated (MS)-PCR for a rapid and accurate detection of 6 common β-thalassemia including $\beta^{41/42(-TTCT),}$ $\beta^{17(A-T)}$, HbE, $\beta^{71/72(+A),}$ $\beta^{-28(A-G)}$ and $\beta^{IVSIInt654(C-T)}$ (Sritong, *et al* 2004). In addition, the multiplex PCR protocols have also been developed for α-thalassemia and β-thalassemia mutations. The HbE mutation and SEA-α thalassemia 1 deletion were identified by the multiplex allele-specific PCR developed by Siriratmanawong, N. *et a*l in 2001(Siriratmanawong, *et al* 2001). In 2003, Fucharoen, S. *et al* developed a simultaneous system based on multiplex asymmetric allele-specific PCR for detection and differential diagnosis of HbCS and Hb Pakse' in two Cambodian families (Fucharoen, *et al* 2003). In 2004, Panyasai, S. *et al* developed a multiplex allele-specific PCR system for detection of three common DNA deletions causing $(\delta\beta)^0$-thalassemia and HPFH in Southeast Asians (Panyasai, *et al* 2004). In 2007, a multiplex allele-specific PCR for characterization of Hb Queens and Hb Siam was developed and tested by Fucharoen, S. *et al* (Fucharoen, *et al* 2007). Recently, Tatu, T. *et al* also developed the in-house multiplex allele-specific PCR for simultaneous detection of both the SEA-α thalassemia 1 and β-thalassemia mutations commonly encountered in Thailand (Tatu, *et al*). This PCR protocol is currently employed in this laboratory.

2.11 PCR from whole blood without DNA preparation

In general, the PCR reaction utilizes the genomic DNA as template for amplification. The DNA is usually purified from white blood cells via various DNA extraction method such as the standard phenol-chloroform method, Chelex™ resin method and commercial DNA purification kit (Joseph and Russell 2001, Walsh, *et al* 1991). These DNA purification methods are time-consuming as multiple steps are involved. This multi-step procedure has prevented the PCR from being the front-line test in diagnostic platform. More importantly, in the emergency cases or in situations where many samples are in the list, the PCR without DNA extraction is important. Thus, attempts have been made in order to carrying out the PCR without using the purified DNA, but whole blood instead. In 1990, Mercier, B. *et al* proposed the direct PCR from whole blood by using 2-µl whole blood in the 100-µl PCR reaction mixture and adding heat-cool steps just prior to the typical PCR cycles (Mercier, *et al* 1990). In 1994, Burckhard, J. *et al* presented the direct PCR from whole blood, also, by modifying PCR cycles resembling that described by Mercier, B. *et al*, except more heat-cool steps were performed (Burckhardt 1994). In 2005, Castley, A. *et al* described an innovative approach that combined PCR amplification directly on whole blood and real-time detection PCR technology (WB-RTD PCR). In this protocol, the whole blood was treated with formamide solution prior to adding to the amplification reaction (Castley, *et al* 2005). In 2009, Jadaon, M.M. *et al* presented a simple method for DNA extraction from white blood cells in whole blood. DNA was released from white blood cells by rupturing

the cells using freeze/thaw and then isolated by microwave at 700 Watt for 7 minutes (Jadaon, *et al* 2009).

The full potential of direct PCR from whole blood is limited, in part, by the presence of inhibitory substances in samples such as heme, immunoglobulin G and lactoferrin which may inactivate the thermostable DNA polymerase. To overcome this problem, various additives have been included in the PCR reaction to relieve this inhibition. In 1996, Kreader, C.A. *et al* optimized concentration of PCR facilitators such as bovine serum albumin (BSA) and T4 gene 32 protein (gp32) and found that 400 ng/μL BSA or 150 ng/μL gp32 were optimal (Kreader 1996). Abu Al-Sold, W.A. *et al*, in 2000, also presented the abilities of 16 amplification facilitators in conventional PCR. They found that addition of 0.6% (w/v) BSA or 11.7% (w/v) betaine to reaction mixtures significantly reduced the inhibitory effect of blood (Al-Soud and Radstrom 2000). In the next year, Abu Al-Sold, W.A. *et al* also found that addition of 0.4% (w/v) BSA allowed AmpliTaq Gold to amplify DNA and adding 0.02% (w/v) gp32 reduced the inhibitory effects of hemoglobin and lactoferrin (Al-Soud and Radstrom 2001). In 2007, "AnyDirect buffer" was developed for effective use in direct PCR from whole blood under various conditions (Yang, *et al* 2007). The ingradient of this buffer, however, was confidential. In 2008, Bu, Y. *et al* described a novel approach to directly amplify genomic DNA from whole blood and dried blood spotted on filter paper without any DNA isolation by using the PCR buffer with a higher pH (9.1-9.6) (Bu, *et al* 2008).

3. Objectives

3.1 To develop and evaluate the in-house mutiplex allele-specific PCR for detecting α- and β-gene mutations commonly found in Thailand

3.2 To develop and evaluate the direct PCR from whole blood without DNA isolation for identification of globin gene mutations commonly encountered in Thailand