CHAPTER I

INTRODUCTION

1.1 Statement and Significance of the Problem

α-thalassemia is a genetic disease that is transferred to offspring in an autosomal recessive manner. The incidence of α-thalassemia in Thailand is high, it can be estimated that about 1,250 fetuses will have Hb Bart's hydrops fetalis and about 7,000 fetuses with Hb H disease per year (Fucharoen *et al.*, 1991), which based on a total birth rate of about 1 million per year. The method to treatment of the thalassemia in present is not appropriate. The only form of treatment available for thalassemia children are regular blood transfusion, iron-chelation therapy in an attempt to prevent iron overload (Weatherall *et al.*, 1991). But, these treatments are expensive and the government has to use a high budget to treat this single disease. For this reason, it seems reasonable to develop preventive strategies to avoid these serious genetic diseases.

Thalassemia control programs by public education, mass carrier detection, genetic counseling, prenatal diagnosis and termination of pregnancies with affected fetuses are important in order to reduce the incidence of severe thalassemia syndromes (Fucharoen *et al.*, 1992). The genetic counseling that is screening total population when they are still in school and warning carriers about the potential risk of marriage

to another carrier. Especially, prenatal diagnosis for the prevention of thalassemia entails screening of mothers at their first prenatal visit, screening the father in case in which the mother is a thalassemia carrier, and offering the couple the possibility of prenatal diagnosis and therapeutic abortion if they are both carriers for a severe thalassemia.

In the present, many methods have been developed for screening α-thalassemia such as High Performance Liquid Chromatography (HPLC) for detect type of hemoglobin such as Hb F (Bannerman *et al.*, 1983). DNA diagnosis is rapidly be developed like slot blot analysis (Rubin *et al.*, 1985), the polymerase chain reaction (Saiki *et al.*, 1985) and allele specific oligonucleotide probes (Wu *et al.*, 1989). These more recent approaches significantly decrease the amount of material needed for analysis and increase the speed of the diagnostic procedure.

In this study, polymerase chain reaction (PCR) and erythrocyte osmotic fragility test coupled with Hb A_2 determinant were applied to detect α-thalassemia 1 in pregnancies. PCR is an exquisitely sensitive method for detection α-thalassemia 1 in university hospitals. However, in small hospitals which with a low budget for supplies these equipments and lack of the trained personnel, erythrocyte osmotic fragility test (EOFT) and Hb A_2 determinantion are appropriated for pre-screen α-thalassemia 1.

1.2 Literature Review

1.2.1 Hemoglobin Function

The major functions of red blood cell is the transportation of oxygen from capillaries of the lungs to the body tissues; an associated function is the binding of carbon dioxide and protons by deoxyhemoglobin, thereby serving to buffer the blood on the venous side of the circulation (Honig *et al.*, 1986). This function is dependent upon hemoglobin which normally constitutes over 90% of the soluble protein in the red cell. Normal human hemoglobin molecules are composed of four subunit, each containing a protein globin chain and an iron-porphyrin heme moiety. The properties of individual hemoglobin are consequences of their quaternary structure of globin protein. Two different types of protein globin chain, with a pair of each of the chains being represented in the complete tetramer molecule. Hemoglobin contains two α-like globin protein subunit and two β-like globin protein subunit. The α-like globin protein subdivided into 2 types are α-globin protein and ζ-globin protein which each contains amino acid 141 residues and similar in primary structure. The β-like globin protein subdivided into 4 types are ε, γ, δ and β-globin which each contains amino acids 146 residues and similar in primary structure like in α-globin. Numerous bonds are formed between α and β-like subunits, however to produce tightly coupled αβ dimers, these bonds referred to

as $\alpha_1\beta_1$ contacts. The pair of α subunit have no interconnecting bonds between them, as is also the case for the β subunits. Allosteric interactions among the four globin chain result in physiologically appropriate sigmoid oxygen binding curve and adjustments in the oxygen binding affinity to local tissue pH (Bohr effect), temperature, pCO_2 and level of erythrocyte organic phosphates (2,3 DPG).

1.2.2 Hemoglobin Production in Humans

The normal pattern of human hemoglobin production during early development involves a succession of "switches" in which the synthesis of a set of globin subunits is activated for a specific period during development, and then is "switched off" and replaced by a different set of globin polypeptides. This process begins in the early embryo and is largely completed by about six months of age postnatally.

The earliest recognizable hemoglobin-containing cells in the human embryo appear during the fourth week of gestation, arising from mesenchyme of yolk sac origin in embryos of less than 6 weeks gestation hemoglobins Hb Gower1 ($\zeta_2\varepsilon_2$) and Hb Gower2 ($\alpha_2\varepsilon_2$) predominate, and may account for as much as 66% of the total hemoglobin with Hb Portland ($\zeta_2\gamma_2$) representing as much as 20%. HbF ($\alpha_2\gamma_2$) has also been identified in the earliest embryos that have been examined; the synthesis of HbF increases rapidly and by 8 weeks of

gestation HbF accounts for at least 90% of the hemoglobin in the erythrocytes and remains the predominant hemoglobin form through fetal life and in the neonatal period. HbA ($\alpha_2\beta_2$) is undetectable before 8-10 weeks of gestation and thereafter accounts for 4-13% of total hemoglobin synthesis. The major switch from HbF to HbA takes place in the first few weeks of life, with HbF falling to less than 3% of the total by 6 months of age and reaching levels of less than 2% by one year. HbA_2 ($\alpha_2\delta_2$) is present in the blood only in trace amounts at birth and increases to 2-3% of the total hemoglobin by one year of age (Karlsson *et al.*, 1985)

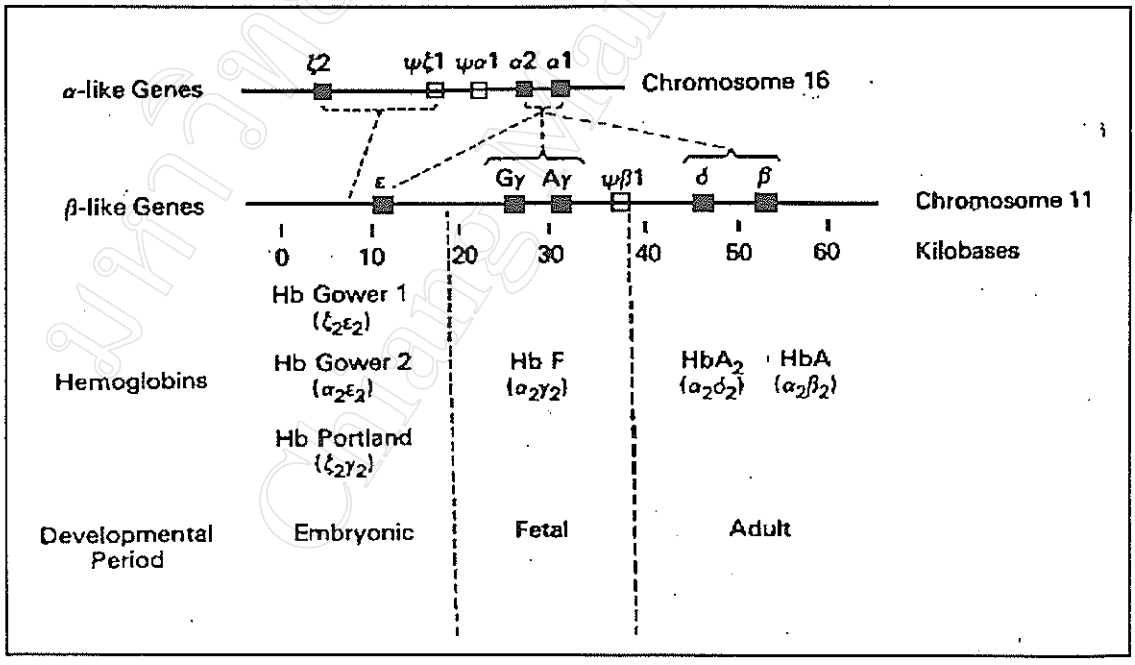

Figure 1 Organization of the human globin genes and hemoglobins production in each stage of human development (Marks *et al.*,1986).

Of interest is the potential physiological advantage of hemoglobin switching during development. Red cells containing HbF exhibit a higher oxygen affinity than cells containing HbA. The higher oxygen affinity of fetal blood compared with maternal blood may facilitate oxygen transport across the placenta (Parer, 1970).

1.2.3 The α-Globin Gene Expression

The α-globin like gene cluster is located adjacent to the 16 p telomere (16p13.3-pter) (Buckle *et al.*, 1988). The α-globin gene like cluster on chromosome 16 spans 30 kb and contains the duplicated α genes (α1 and α2), an embryonic α-like gene (ζ2), three pseudogenes (ψζ1, ψα2, ψα1), and a gene of undetermined function (θ) arranged in the order 5'-ζ2-ψζ1-ψα2-ψα1-α2-α1-θ-3' (Lauer *et al.*, 1980). The ζ-globin gene that codes for ζ-globin protein is expressed during the first five weeks of *in utero* development by the primitive erythroblasts of the yolk sac (Gale *et al.*, 1979). This gene shows only 58% homology in 141 amino acid with α-globin gene. The α1 and α2 globin genes differ slightly in sequence, especially in intron2 and exon3. The α-globin genes are composed of three exons interrupted by two short introns. These genes are highly similar with structural divergence limited to intron2 and exon3. However their protein coding regions are identical (Liebhaber *et al.*, 1981). In addition, three pseudogenes (ψζ, ψα2,

ψα1) that are found in the α-globin gene cluster are defective in some essential components of gene structure and thus cannot express a globin product despite their sequence homology to functional gene. For example, the ψζ differs from the functional ζ gene by only three base substitutions in the protein coding region (Proudfoot *et al.*, 1982). The θ gene that is located 4 kb 3' to the α1 gene has been discovered only recently. It is highly similar in structure to the α-globin gene and appears to be expressed in early fetal life but its function is unknown (Higgs *et al.*, 1989). There are five separate regions within or adjacent to the α-globin gene cluster which are structurally hypervariable (hypervariable regions, HVRs). Each of these regions is composed of multiple tandem repeats of a short GC rich sequence ranging in size from 17 (3' HVR) to 57 bp (5' HVR). They appear to be highly polymorphic in most populations, they can be used as a marker for the short arm of chromosome 16.

Throughout development a balance between α-like and β-like globin gene expression must be maintained so that functional hemoglobin tetramers can be assembled. The defect in the thalassemias is the loss of this balance (Weatherall *et al.*,1965).

1.2.4 Molecular Defects in α-Thalassemia

α-thalassemia is a hemolytic anemia resulting from the deficient synthesis of α-globin chains. The imbalanced globin synthesis gives rise to an excess of γ-chain or β-chains which form the nonfunctional, unstable tetrameric hemoglobins Hb Bart's (γ_4) and Hb H (β_4). Anemia results from a combination of the underproduction of hemoglobin and haemolysis due to intracellular precipitation of Hb Bart's and Hb H, which ultimately damages the red cell membrane. α-thalassemia can be inherited or acquired and can result from defects in, or deletion of one or more of the four α-globin genes. The severity of the anemia and the amount of abnormal hemoglobin produced is directly related to the degree of α -chain synthesis deficiency.

1.2.4.1 Gene Deletion α-Thalassemia

The great majority of α-thalassemias result from deletion of one or both of the α-globin gene. The size and position of at least 14 deletions are now defined in partial or full detail (Gonzalez-Redondo *et al.*, 1988). Normal individuals have two α-genes on each chromosome 16 (αα/αα), carriers for α-thalassemia are classified by the number of genes that are deleted. The most common gene deletions causing α-thalassemia and some of their features are shown in table 1.

1.2.4.2 Non-Gene Deletion α-Thalassemia

Point mutations in vital areas of the α-globin gene may produce α-thalassemia by a number of mechanisms. Point mutations may totally nullify or merely reduce gene expression. The phenotype is also influenced by whether the mutation occurs in the dominant α_2-gene or the α_1-gene. Two types of point mutations are of special interest because they result in thalassemic hemoglobinopathies: First, several mutations in the third exon of the α_2-gene produce hyper-unstable variants that fail to accumulate or partake in stable tetramer formation (Liebhaber *et al.*,1987). Second, a group of mutations abolish the normal termination codon that occurs following the carboxyl-terminal amino acid residue. Another amino acid is inserted and translation proceeds until the next termination codon is encountered. The new α-chain, is able to form hemoglobin tetramers but is unstable and associated with an unstable mRNA. Examples of some of these mutation, along with the mechanism by which they impair gene expression are shown in table 2.

Table 1 Features of Some Gene Deletions Causing α-Thalassemia

(Martin *et al.*, 1991)

Deletion	Prevalence	Phenotype in heterozygotes	Comments
$\alpha^{-3.7}$	wide distribution Blacks (0.30); Asians (0.01-0.98) Pacific Islanders (0.30-0.90) Mediterranean (0.04-0.18)	no disease: virtually indetectable hematologically	remaining α-gene expression-enhanced
$\alpha^{-4.2}$	Asians; present, but rare in other groups	mild microcytosis	no enhancement of remaining α-gene expression
SE Asia	Asians (0.03): Mediterranean	microcytosis	ζ-genes intact: accounts for presence of hydrops fetalis& HbH disease in Asian and Mediterraneans
British/ blacks	British Caucasians, blacks (rare)	microcytosis	ζ-genes intact ; accounts for presence of hydrops fetalis & rarity of Hb H disease in Blacks

Table 2 Non-Gene Deletion Forms of α-Thalassemia

(Martin *et al.*, 1991)

Mutation site	α-Gene	Mechanism	Features
5-bp deletion; donor splice site IVS-1	α2	abnormal splicing and decrease of α2 mRNA	Mediterranean
poly (A) signal; mutation	α2	abnormal mRNA processing	Mediterranean
initiation codon; various single base changes	α2 α1 $-\alpha^{3.7}$	decreased translation	Mediterranean Blacks
termination codon	α2	elongated unstable globin and mRNA	Hb Constant spring common in SE Asians
nonsense mutation	α2	premature termination; unstable globin	Blacks
frameshift: α30/31-2 base deletion	$-\alpha^{3.7}$		Blacks
Exon3 Hb Quong Sze Hb Suan Dok	α2	highly unstable globin	SE Asian

1.2.5 Deletion of a Single α-Globin Gene

Deletions of a single α-globin gene are called α-thalassemia2. There are two major subtypes of the single α-globin gene deletion. The most common results in the loss of 3.7 kb of DNA and is commonly noted as the $-\alpha^{3.7}$ deletion. A somewhat less common deletion results from the loss of 4.2 kb of DNA and is noted as the $-\alpha^{4.2}$ deletion (Embury, 1980). Both of these deletions result from an unequal homologous recombination within the α-globin like cluster which is shown in figure 2. Because of the relative positioning of the crossovers, $-\alpha^{3.7}$ is often refered to as the rightward deletion and the $-\alpha^{4.2}$ as the leftward deletion. The $-\alpha^{3.7}$ deletion is extremely widespread, being found in all populations studied and is the most common form in Thailand (Higgs *et al.*, 1984). The $-\alpha^{4.2}$ is most frequently found in Asian populations (Oppenheimer *et al.*, 1984).

Normally, the deletion of a single α-globin gene is phenotypically silent (Bowden *et al.*, 1987). The haematologic parameters such as hemoglobin concentration, red cell indices, and number of red cells are within normal limits. It is not possible to distinguish these individuals from normal, except by DNA analysis.

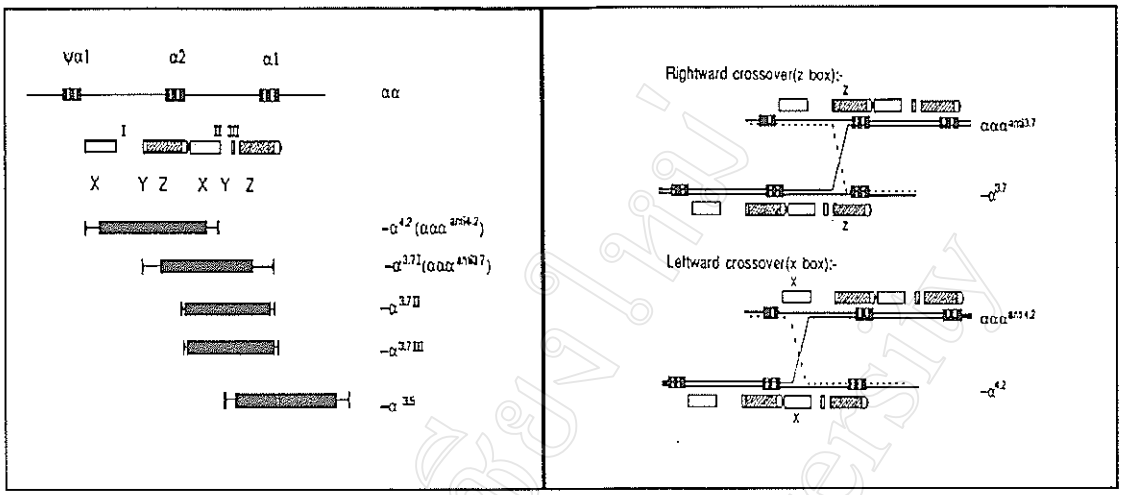

Figure 2 The major deletions in α-thalassemia 2 (Higgs *et al.*, 1989)

1.2.6 Deletion of Both α-Globin Genes

Deletion of both α-globin genes are called α-thalassemia1. These deletions range in size from a 5.2 kb segment of DNA in which a part of α-globin gene is still intact to those which remove the entire α-globin gene cluster (Fischel-Ghodsian *et al.*, 1988). The two most common deletions are the Southeast Asia type ($--^{SEA}$) and Mediterranean type ($--^{Med}$) that occur in Southeast Asia and the Mediterranean Basin respectively. In the Southeast Asia type, the deletion is approximately 20 kb in length and removes both α-globin genes but spares the functional ζ2 gene (Nicholls *et al.*, 1987). The 5′ start of the deletion is within the first exon of the $\psi\alpha_2$-gene (Hardison *et* al., 1986) and the 3′ end of the deletion terminates close to the hypervariable region locate at the 3′ end of the α-globin gene complex. The northern part of Thailand

appears to have the highest reported frequencies of α-thalassemia1 ($--^{SEA}$) (Fucharoen *et al.*, 1991).

This genotype is usually associated with a significant microcytosis, an elevated red cell count, and an appreciable imbalance in the α/β synthetic ratio (Maude *et al.*, 1985). The carrier may perhaps show a mild anemia.

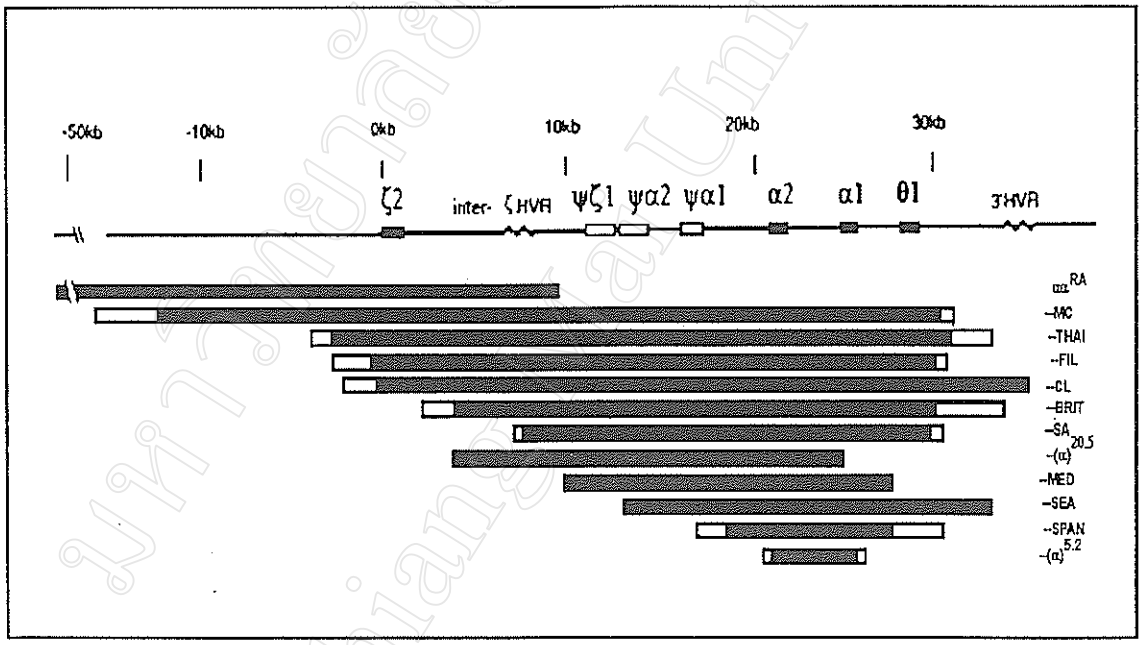

Figure 3 The major deletions in α-thalassemia 1 (Liebhaber, 1989)

1.2.7 Hemoglobin H Disease

The most common genotype associated with Hb H disease (--/-α), most frequently results from the interaction of α-thalassemia1 and α-thalassemia2 determinants. In Thailand, more than 400,000 people are

affected (Winichagoon *et al.*, 1988). Other less frequent genotypes can also result in Hb H disease including coinheritance of deletion and nondeletion defects such as α-thalassemia1 with Hb Constant Springs ($--/\alpha\alpha^{cs}$) (Wasi *et al.*, 1974) or homozygosity for nondeletion defects (Kattamis *et al.*, 1988). This syndrome occurs most commonly in Southeast Asia and in the Mediterranean. An excess of β-globin chains are produced in Hb H disease and form the β-globin tetramer (β_4), which is unstable and precipitates in the cell. Hb H precipitation in red cells results in red cell damage and premature clearance by the reticuloendothelial system, thus shortening the red cell life span (Shchory *et al.*, 1972).

Hb H disease is usually that of thalassemia intermedia although there is considerable variability in the clinical severity (Higgs, 1993a). The predominant feature are anemia with jaundice and hepatosplenomegaly. The red cell morphology is usually abnormal with basophilic stippling, target cells, and teardrop forms. Probably the most common complication of Hb H disease is the development of severe splenomegaly with hypersplenism. Hb H disease is usually not severe enough to interfere with the activities of daily living, longevity, or reproductive function. However, severe exacerbations of the anemia may occur coincident with pregnancy, infections, and exposure to oxidant drugs.

1.2.8 Hemoglobin Bart's Hydrops Fetalis Syndrome

This syndrome results from the coinheritance of two α-thalassemia1 determinants (--/--). Therefore it has only been reported in individuals of Southeast Asia or Mediterranean. An excess of γ-globin chains in the newborn assembles into γ_4 (Hb Bart's). Hb Bart's (γ_4) is nonphysiologic because it lacks any Bohr effect, has no heme-heme interaction, and is functionally useless for O_2 transfer (Horton *et al.*, 1962) which results in erythrocyte damage and dysfunction. In Thailand, About 1,250 such fetuses are born each year with this disease.

This disorder is a frequent cause of stillborn babies in Southeast Asia. Affected infants either are stillborn or are born alive but die within a few hours. The predominant physical findings in the fetuses are generalized and massive edema, ascites, gross enlargement of the liver with a spleen which may be normal or only slightly englarged, and a large friable placenta. The peripheral blood demonstrates severe erythroblastosis with accompanying reticulocytosis, hyperchromia with fragmentation and decreased osmotic fragility. This condition is associated with a high incidence of maternal toxemia in pregnancy and difficulties at the time of birth because of the massive placenta (Wasi *et al.*, 1980) possibly reflects severe intrauterine hypoxia.

Table 3 Frequencies of thalassemias and abnormal hemoglobins in different regions of Thailand (Panich *et al.*, 1992)

Region	α-thal 1	α-thal 2	α-thal	Hb E	Hb CS
Central (Bangkok)	0.0185	0.0837	0.0128	0.0696	0.008
North (Chaing Mai)	0.0236 0.058	0.0991	0.0264	0.044	0.0331
Northeast (Khon Kaen)	0.0234	0.1719	0.0119	0.263	0.55
Northeast (Sakon Nakhon)	0.017	0.356	0.0127	0.4958	0.153
South (Songkhla)	0.0217	0.065			0.0292

1.3 Principle and Rationale

1.3.1 Introduction to Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is an in *vitro* method for DNA synthesis. This method is a relatively simple technique by which a DNA or cDNA template is amplified many thousand or million-fold quickly and reliably. A great advantage of the PCR method is that the target DNA does not need to be particularly pure or abundant. PCR is extremly useful not only in basic research, but also in commercial uses including genetic identity testing, forensics, industrial quality control and in vitro diagnostics.

Normally, the initiation of DNA replication begins with the separation of its two complementary DNA strands. Each strand then acts as a template for the formation of a new DNA molecule. A short RNA molecule is required for priming. The primer is then extended from the 3′-end by a DNA polymerase which catalyzes the addition of a deoxyribonucleotide to the DNA chain. Each nucleotide added to the chain is a deoxyribonucleoside triphosphate; the release of pyrophosphate from this activated nucleotide and its subsequent hydrolysis provide the energy for the DNA synthesis and make it effectively irreversible. The nucleotide to be added at each step is selected by a process that requires it to form a complementary base pair with the next nucleotide in the parental template strand, thereby

generating a new DNA strand that is complementary in sequence to the template strand.

1.3.2 Principle of the PCR Method

The PCR method is based on the repetitive cycling of three reactions. The first step in the procedure is the heat denaturation of native double-stranded DNA.

In the second step of the cycle, performed at lower temperatures, two short DNA primers are annealed to complementary sequences on opposite strands of the target DNA. These primers are chosen to encompass the target sequence, they define the two ends of the amplified DNA. The specificity of PCR derives from the precise annealing of the primers to their complementary DNA sequence.

The cycle's third step is the extension of each annealed primer by a heat-stable DNA polymerase in the presence of excess deoxyribonucleoside triphosphates. A new single stranded of DNA is synthesized for each annealed primer. An essential feature of the PCR procedure is that all previously synthesized products act as templates for new primer-extension reactions in each ensuing cycle. After extension of the primers the cycle is repeated, first by raising the temperature so that all double-stranded DNA is converted to single stranded DNA, thus aborting any ongoing polymerization, and then by lowering the

temperature to allow annealing and extension step. Each PCR cycle theoretically doubles the amount of target template sequence in the reaction. Ten cycle theoretical multiply the amplicon by a factor of about one thousand, 20 cycles by a factor of more than a millon in a matter of hours (Vosberg, 1989, Jane *et al.*, 1990).

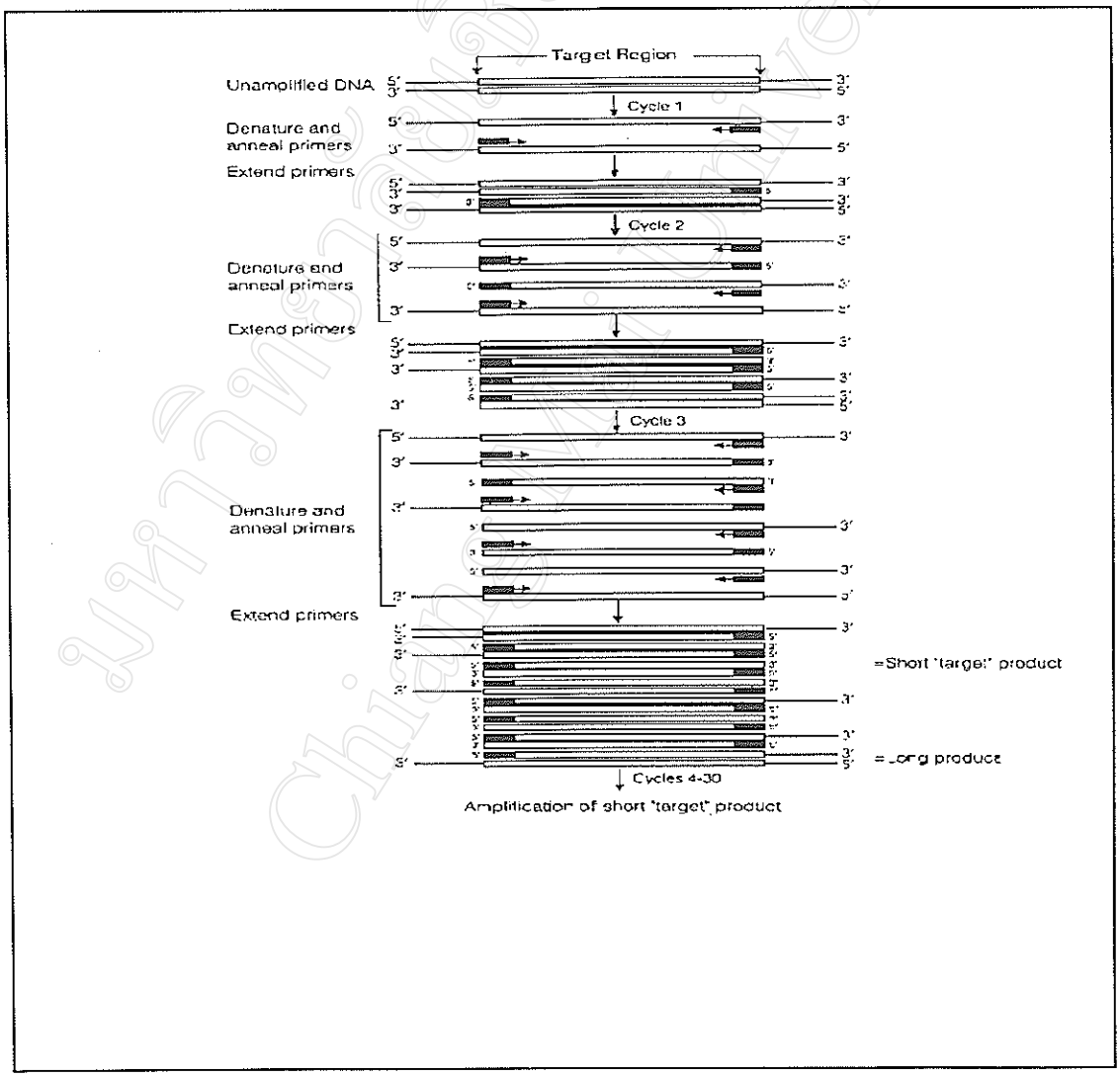

Figure 4 Schematic diagram of the PCR process (Promega, 1996)

1.3.3 Standard Method of PCR

The stardard composition of a PCR is presented below.

- the target DNA
- 1 unit of thermostable DNA polymerase/ 50 µl reaction
- 0.2-1 µM each of oligonucleotide primer
- 200 µM each of deoxynucleotide triphosphates (dNTP)
- 20 mM tris-HCl buffer pH 8.3
- 100 µg/ml gelatin or nuclease-free bovine serum albumin
- 1.5 mM magnesiumchloride ($MgCl_2$)
- 50 mM potassiumchloride (KCl)
- 0.01% (v/v) Tween 20

The components of the reaction are mixed and placed in an automated thermal cycler. The standard PCR protocol works for most applications but some DNAs can only be successfully amplified with a modified protocol.

Denaturation	90-95°C	30-60 s
Annealing	40-60°C	30-60 s
Extension	72°C	1-2 min

1.3.4 PCR Optimization

An ideal PCR would be the one with high specificity, efficiency, and fidelity. Studies indicate that each of these three parameters is influenced by numerous components of the PCR, including the buffer conditions, the PCR cycling regime, and the type of DNA polymerase. Unfortunately, adjusting conditions for maximum specificity may not be compatible with high yield; likewise optimizing for the fidelity of PCR may result in reduced efficiency. Thus, when setting up a PCR, one should know which of the three parameters is the most important for its intended application and optimize PCR accordingly.

Template : Successful amplification of the region of interest is dependent on the amount and quality of the template DNA. The amount of template required is dependent upon the complexity of DNA sample. For example 0.1-1 μg of mammalian genomic DNA is utilized per PCR (Scharf *et al.*, 1986). Conversely, in bacterial genomic DNA or plasmid DNA that represent much less complex genome, picograme to nanograme quantities are used per reaction.

Magnesium Concentration : Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA polymerase. A low magnesium concentration reduces enzyme fidelity (Eckert *et al.*, 1990) and may increase the level of nonspecific amplification (Ellsworth *et al.*, 1993). For these reason, it is important to empirically determine the

optimal $MgCl_2$ concentration for each reaction. The concentration of $MgCl_2$ is commonly between 1.5-3.0 mM.

Enzyme : The choice of the correct enzymes for use in the PCR is determined by several factors. *Taq* DNA polymerase, the first heat-stable enzyme used for PCR, still is used commonly. This polymerase possesses a relatively high processivity. An excess of enzyme does not increase product yield, but results in increased unspecific background. The optimal emzyme concentration has to be found by titration.

dNTPs : The fraction of free dNTPs not only depends on the number of target sequences generated but also on the size of the target sequence. The concentration of dGTP, dCTP, dATP, and dTTP should be balanced. The optimal concentrations of dNTPs are between 50 and 200 μM. An excess of dNTPs may promote base misincorporation (Petruska *et al.*, 1988).

Cycle parameters : In general, a higher annealing temperature and a shorter time for annealing and extension improves the specificity of PCR. Alternatively, unspecific bands may results from overamplification (Witter *et al.*, 1991). Converesly, it is necessary to increase the duration of each step for efficient amplification of fragments larger than 1 kb (Kowk *et al.*, 1990).

1.3.5 Principle and Application of Erythrocyte Osmotic Fragility Test

The erythrocyte osmotic fragility test is a simple means of estimating the surface area/volume ratio of erythrocytes. As this ratio falls the cell becomes more sensitive to osmotic lysis. Its greatest usefulness is in the diagnosis of hereditary spherocytosis, but it has also been used in screening for thalassemia.

When red cells are placed in a hypotonic solution, water is drawn into the erythrocyte osmotically. The cell swells and becomes spherical. After a critical volume in the cell is reached, the membrane first leaks small molecules such as potassium. Subsequently, as membrane pores increase in diameter, large molecules such as hemoglobin leave the cell. The amount of hemoglobin in the supernatant is measured colorimetrically and is compared with a sample of completely lysed cells.

Increased resistance to hemolysis is a characteristic of thalassemia, in both the homozygous and heterozygous forms, in iron deficiency, and in any other condition in which an increase of the surface area/volume ratio of the red cell is present, for example, in some forms of liver disease.

1.3.8 Rationale

The α-thalassemia syndromes are an important health problem in Thailand, it can be estimated that about 1,250 fetuses will be born with Hb Bart's hydrops fetalis caused by homozygous α-thalassemia-1 (Panich *et al.*, 1992). The absence of any medical treatment is an important problem of α-thalassemia. Thus, a management of this disorder is required for its control. Thalassemia control programs by public education, mass carrier detection, genetic counseling, prenatal diagnosis and termination of pregnancies with affected fetuses have been applied successfully in many country. This program can provide families with full medical information to help them have healthy children. Especially, the prevention of further thalassemic offspring in the case of couples at risk can be planned by prenatal diagnosis. Follow by genetic counseling can help the carrier make plans about future families.

In α-thalassemia, a prenatal diagnosis of Hb Bart's hydrops fetalis is important that allows the option for early termination of pregnancy which may decrease the risk to the mother of perinatal complications (Liang *et al.*, 1985). In addition, the accurate diagnosis allows the physicians formulate the most appropriate actions in the immediate postpartum period. Couples at risk are usually identified by a prior hydrops fetalis pregnancy. In racial groups in which the deletion of

α-thalassemia 1 (Southeast Asia) is frequent screening for couples with α-thalassemia 1 ($--^{SEA}$) genotype can also be effective (Chan *et al.*, 1988). Many methods are applied to screen for α-thalassemia1 such as gene mapping (Goosens *et al.*, 1983), slot blot analysis (Rubin *et al.*, 1985), allele specific oligonucleotide probe (Wu *et al.*, 1989), radioimmunoassay (Tang *et al.*, 1992), and PCR. In this study, a PCR method is applied for detection of α-thalassemia1 because PCR can use a small amount of sample, rapid, cheap, simple, and sensitive which have many literature about that :

Chehab et al. (1987) : They detect sickle cell anemia and α-thalassemia 1 by simple and rapid technique which is PCR.

Chang et al. (1991) and Ko et al. (1992) : They diagnose α-thalassemia 1 of Southeast Asia type by PCR. Three oligonucleotide primers were used as a rapid tool for carriers detection and for prenatal deagnosis.

So in this study, PCR was used to detect α-thalassemia 1 carriers among pregnant women for genetic counseling. PCR method can be used for prenatal diagnosis of α-thalassemia1 carrier and Hb Bart's hydrops fetalis in a large hospital similar with Maharaj Nakorn Chiang Mai hospital.

However, in small hospital that do not have specialists and the instruments of a DNA laboratory. PCR method is not appropriate for

small hospital. First, erythrocyte osmotic fragility test has been used to screen for β-thalassemia in 1980 by Flatz and Flatz. This method is simple, highly reliable, specific and inexpensive by lysis erythrocyte in glycerine-saline milieu and record by photometrically. In 1974 Efremov, detected abnormal levels of HbA_2 in β-thalassemia traits, sickle cell anemia and Hb-S-β-thalassemia by microcolumn chromatography. This method is equally applicable to hemolysate and whole blood which provide the rapid and accurate determinant. In this study, the erythrocyte osmotic fragility test and HbA_2 determination are applied to screen for α thalassemia1 carrier by study the correlation between α-thalassemia1 carrier with the level of erythrocyte osmotic fragility test couple with HbA_2 determinant. These method are simple and inexpensive which do not use specialist and expensive instrument. Small hospital can use these method for screening α-thalassemia1 carrier before confirm some case in large hospital. The PCR method in this study can be used to decrease the incident of Hb Bart's hydrops fetalis.

1.4 Objective

1. To study the prevalence of α-thalassemia 1 of the Southeast Asia type among pregnant women at Maharaj Nakorn Chiang Mai hospital by PCR technique.

2. To study the correlation of erythrocyte osmotic fragility test with HbA_2 determination and PCR technique for diagnostic value.

3. To study the usefulness of this PCR method for routine detection of carriers for α-thalassemia 1 of the Southeast Asia type.