CHAPTER V
DISCUSSION AND CONCLUSION

Genomic DNA extraction from whole blood using the Chelex method was practical and yields enough genomic DNA for amplification of the β-globin gene exons by PCR.

The amplification of the β-globin gene exons by PCR was simple for because the size of each amplicons was just 300-400 bp and there was no high GC-content. For optimization, the glycerol was used for enhanced efficiency and specificity of PCR amplification (Cheng *et al.*, 1994 and Nagai *et al*, 1998). For exon 3 glycerol was not necessary because new primers for amplification were designed by computer software, Primer Detective. A good quality of PCR products of is necessary for use as template in chain-termination cycle sequencing.

Purification of the amplicons of each exon was done using the QIA quick PCR purification kit (QIAGEN, Germany). DNA yields and concentration depends on the three factors: the volume of the elution buffer, how the buffer is applied to the column and the incubation time of the buffer on the column. 100-200 µl of elution buffer is sufficient to ensure a maximum yield. Elution with ≤50 µl of buffer requires the buffer to be added directly to the center of the column. If elution is done with the minimum recommended volume of 30 µl, an additional 1 min incubation is required for maximum yield and gives DNA yields similar to 50 µl without incubation.

For sequencing, of the three exons of the β-globin gene the dRhodamin Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Cetus, USA) was used and the sequencing primers used were the same as the PCR primers. The kit contains sequncing enzyme Ampli*Taq*®DNA polymerase, FS. This enzyme is a variant of *Thermus aquaticus* DNA polymerase. It lacks of any 5'- to 3' exonuclease activity. The dye terminator is dRhodamine to give better signal, more even peak heights. The dyes have a narrow emission spectrum, less spectral overlap and less noise. This kit did not facilitate the optimization of any constitutions.

From the sequencing data, a single base substitution, A→T, occurred at the nucleotide number 1631 (GeneBank, HUMHBB 221) in exon 1 of the β-globin gene and with corresponding of glutamic acid being replaced by valine (Glu→Val) at the amino acid residue. This amino acid substitution from an acidic to a neutral residue caused the separation of the abnormal hemoglobin from the normal Hb A in cellulose acetate gel electrophoresis. This finding agrees with Hb S (β6(A3)Glu→Val) that was first reported by Ingram(1956). Furthermore, another single base substitution, G→T, occurred at the nucleotide number 1704 (GeneBank, HUMHBB 221) in the first position of intron 1 of the β-globin gene, which resulted to change this site at the splice junction (IVS-1nt1) and caused β^{o}-thalassemia.

The HPLC use on the Bio-Rad Variant automated analyzer with the "β-thalassemia Short" program to separate normal and abnormal hemoglobin: Hbs A, S, C and F (Papadea and Cate, 1996). The most commonly occurring variants are Hb S (β6Glu→Val), Hb C (β6Glu→Lys), Hb E (β26Glu→Lys) and Hb D-Punjab (β121Glu→Gln). Using the retention time window makes presumptive identification of these abnormal Hbs. The retention times of these commons are variants. The range of elution time of Hb S about 4.12 - 4.42 min (Riou *et al*, 1997) In this study, the elution time of Hb S is 4.44 min.

The origin of Hb S is in Africa. The structural regions of the Hb S gene are identical. Substitutions in the flanking regions of the gene show that Hb S arose separately at least four times in Africa and once in the Near East (Nagle and Fleming, 1992). The four African haplotypes show broad trends in disease severity. Hb S is common in some areas of the Mediterranean basin, including regions of Italy, Greece, Albania and Turkey (Boletini *et al.*, 1994)(Schiliro *et al.*, 1990). Haplotype analysis shows that the Hb S in these areas originated in Africa. The genes probably moved along ancient trading routes between wealthy kingdoms in western Africa and the trade centers in the Mediterranean basin. The high levels of Hb S attained in some areas may in part have resulted from protection against malaria. The fact that the Hb S mutation apparently arose in response to malaria on the Arabian peninsula supports the " malaria

explanation" of the prevalence of the gene. The Arabian haplotype is found in regions of India (Ramasamy *et al.*, 1994)(Kar *et al.*, 1986). The pockets of Hb S in India probably resulted from migration of people from the Middle East along trade routes. This Near East variety of Hb S may on average produce fewer complications than its African counterparts (Perrine *et al.*, 1978). Today Hb S is found in America, Southern Italy, Northern Greece, Southern Turkey, the Middle East, Saudi Arabia, the Eastern of central India and Africa. In currently believed there are four haplotypes: Senegal, Benin, Bantu/Central African Repubic and Asian haplotype.

The β^{o}-thalassemia (IVS-1nt1) was found common mutation in South of Thailand about 6% (Nopparatana *et al.*, 1995: Fucharone and Winichagoon, 1997)(see Table 2).

This study is the first report in Thailand on Hb S with β^{o}-thalassemia (IVS-1nt1). From the results it can be concluded that the patient's father is Hb S trait and the mother is β^{o}-thalassemia (IVS-1nt1) trait. The inheritance of mutated hemoglobin genes from parents caused the patient to be Hb S/β^{o}-thal (IVS-1nt1).

The molecular biological diagnosis of hemoglobin variants generally proceeds in two steps: the detection of the variants by a screening method, usually cellulose acetate electrophoresis (pH 8.5) or HPLC followed by confirmation and identification by various methods. Sequencing and PCR techniques were used to study structural analysis of the β-globin gene cluster and data of sequencing can be approach structural information.

In a further study that addresses the origin of the Hb S found here in Thailand a haplotype analysis of the Hb S gene would be very helpful.