CHAPTER IV
DISCUSSION

There is a common distribution of α-thalassemia throughout Southeast Asia. In Thailand between 15% and 30% of the population are carriers. The severe α-thalassemia syndromes (Hb H disease and Hb Bart's hydrops fetalis) both occur in Thailand, the most frequently encountered defects are the $--^{SEA}$ and $-\alpha^{3.7}$ determinants (68). Due to the incidence of a-thalassemia in Thailand is high, the economic burden placed on society by thalassemia is immense. It was estimated that if regular blood transfusion and iron chelating therapy treat all the thalassemic children who are born in Thailand, the total cost is about 5,000-6,000 million baht per year (69). Clearly, this approach is not always feasible, and hence there is considerable effort toward the development of programs for prevention of the different forms of thalassemia.

There are two options in which this can be achieved for prevention. The first is by prospective genetic counseling, that is, screening total populations while still at school and warning carriers about the potential risks of marriage to another carrier. Because it is felt that this approach is unlikely to be very successful in many populations, considerable effort has been directed toward developing prenatal diagnosis programs. Prenatal diagnosis for the prevention of thalassemia entails screening mothers at the first prenatal visit, screening the father in cases in which the mother is a thalassemia carrier, and offering the couple the possibility of prenatal diagnosis and termination of pregnancy if they are both carriers of a gene for a severe form of thalassemia.

Prenatal diagnosis of α-thalassemia-1 has evolved during the last 10 years (70). Southern blotting of genomic DNA using probes specific for the ζ- and α-globin genes carried out the earliest prenatal diagnoses. In addition, some cases were

investigated using probes located upstream (5' α-HVR, L0, L1) or downstream (3' α-HVR) of the ζ-α gene cluster (7,71-73). Although, this method is specific but it is both labors intensive and expensive. Moreover, its success depends heavily on the quality of the genomic DNA under test and the quantity available (74).

Later, after the development of the PCR, the identification of α-thalassemia-1 was greatly facilitated(65,75-76). These more recent approaches significantly decrease the amount of material needed for analysis and increase the speed of the diagnosis procedure, because PCR produces so much DNA, these fragments can be detected by either ethidium bromide or silver staining of DNA bands on gels; no radioactive probes are required. Although PCR has been proposed as a diagnostic tool in rapid diagnosis of the α-thalassemia-1. If there were maternal cell contamination in the fetal cell samples, it would make the results difficult to interpret. Because of maternal cell contamination was likely to be the cause of the false negative PCR results.

Although, maternal cell contamination could be responsible for the artifacts in the PCR results, but the influence due to the contamination is minimal in Southern blotting technique(77-78). It was concluded that PCR is suitable for screening of carrier adults with α-thalassemia-1, and Southern blotting is ideal for early prenatal diagnosis of the α-thalassemia-1 (71).

Furthermore, the determination of hemoglobin fractions from hemolysates using high performance liquid chromatography (HPLC) is a precise method for the prenatal diagnosis of homozygous α-thalassemia-1 (79-80). Important advantages of the HPLC method are the economy and the rapidity in comparison with DNA analysis. The result of the test is available within 30 minutes after receiving the cordocentesis blood sample in the laboratory (80).

Since its first description in 1988, multiplex PCR (a variant of PCR in which two or more loci are simultaneously amplified in the same reaction) has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse-transcription PCR. Especially, by simultaneously amplifying more than one target DNA in the same reaction, multiplex

PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Because most α-thalassemia mutations are due to deletions of one or more α-loci on chromosome 16, the sensitivity of multiplex PCR techniques to detect these deletions would be useful (65,74,81-83).

Nevertheless there have been several reports of Thai individuals with α-thalassemia-1 in whom there is a complete deletion of the ζ-α complex on one chromosome. They are called the $--^{THAI}$ and $--^{FIL}$ determinants. They cannot be positively identified in heterozygotes (--/αα) by routine PCR of the $--^{SEA}$ determinant and may therefore be missed during genetic counseling and prenatal diagnosis of the couples who are at risk for having child with Hb H disease or Hb Bart's hydrops fetalis.

In this study, we have optimized various parameters of the multiplex PCR capable to detect the α-thalassemia-1 ($--^{SEA}$, $--^{THAI}$, and $--^{FIL}$) deletions. Especially important for a successful multiplex PCR assay are the cycling, the concentration of the PCR buffer, and the balance between the dNTPs and $MgCl_2$ concentrations were tritrated to provide the best result. A clear single band of each specially sized PCR products were obtained for the normal (314-bp), $--^{SEA}$ (188-bp), $--^{THAI}$ (480-bp), and $--^{FIL}$ (597-bp) genotypes had been observed without any complications neither nonspecific background bands nor the formation of primer-dimer.

Modification of the annealing time from 10-sec to 40-sec did not alter the amplification efficiency. But the annealing temperature was one of the most important parameters. In this study, annealing temperatures were varied from 58°C to 64°C (Figure 22); the best result was found at 60°C. The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of the amplification primers.

The extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extension temperature was performed at 72°C because this temperature was optimal for extending primers on genomic DNA template. The extension time was varied for 0.5, 1 and 2 minutes. The expect band was present in all of these time but the best had been observed in 2 minutes without non-specific

bands and higher amount of product. Although extension times of 1 minutes at 72°C is considered sufficient for products up to 2-kb in length, but this protocol required more than the recommendation. Moreover, in multiplex PCR, as more targets DNA are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products. In this study, visibly higher yields of PCR products were obtained for all reactions when a longer extension time was used.

In this study, $MgCl_2$ concentration was kept constant (1.5-mM), while the dNTP concentration was increased stepwise from 100-400-μM each. At dNTP concentrations between 100 and 400-μM each, the results showed no significant difference. Generally, the best results were at 200 and 400-μM each dNTP, values above which the amplification was rapidly inhibited and lower dNTP concentration (50-μM each) allowed PCR amplification but with visible lower amounts of products.

No major differences due to the $MgCl_2$ concentration were seen when keeping dNTP concentration at 200-μM each and gradually increasing $MgCl_2$ from 1.5-3-mM. Generally, a recommended $MgCl_2$ concentration in a standard PCR is 1.5-mM at dNTP concentrations of around 200-μM each (40). Successful multiplex PCR assays are the relative balance between the $MgCl_2$ and dNTP concentrations. To work properly, *Taq* DNA polymerase requires free magnesium (besides the magnesium bound by the dNTP and the DNA) (45). This is probably why increases in the dNTP concentrations can rapidly inhibit the PCR, whereas increases in $MgCl_2$ concentration often have positive effects.

Various PCR additives or adjuvants such as DMSO (2%-10%), PEG 6000 (5%-15%), glycerol (5%-15%), nonionic detergents, and formamide (5%) can also be incorporated into the presence to increase specificity (45,84). Some reactions may amplify only in the presence of such adjuvants, whereas some reactions were decreased the amount of products (56). Therefore, the usefulness of these adjuvants needs to be tested in each case. In this study, the best adjuvant for multiplex PCR is Q-solution, facilitates amplification of difficult templates by modifying the melting behavior

of DNA. Unlike DMSO and other adjuvants, Q-solution is used at a defined working concentration with any primer-template system and is not toxic.

In addition, we have overcome the inherent problem of PCR amplification of the GC-rich α-globin locus by using a modified form of *Taq* DNA polymerase, HotStarTaq DNA polymerase (83), is supplied in an inactive state that has no polymerase activity at ambient temperatures. This prevents extension of nonspecifically annealed primers and primer-dimers formed at low temperatures during PCR setup and the initial PCR cycle.

This study not only identified the deletion types of α-thalassemia-1 by multiplex PCR but also characterized the deletion breakpoints of these deletions by DNA sequencing. The $--^{FIL}$ deletion was characterized using primers FIL-F and FIL-R were used to amplify a 597-bp fragment that spans the $--^{FIL}$ deletion junction. The nucleotide sequence of the junction fragment was compared to the normal 5' and 3' sequences, and the cross-over was determined to be within a region of 21-bp which is identical between the 5' and 3' sequences (Figure 38). The 5' breakpoint lies approximately 1.3-kb upstream of the initiation codon of the ζ_2-globin gene and the 3' breakpoint lies approximately 5.6-kb downstream of the initiation codon of the α_1-globin gene. The total length of the deletion is 30.7-kb.

Both the 5' and 3' breakpoints of the $--^{FIL}$ deletion lie within *Alu* repetitive elements that share 88% sequence homology and are oriented in the same direction. Accordingly, the $--^{FIL}$ deletion probably resulted from homologous recombination between two *Alu* sequences. The ζ-α globin gene cluster contains a disproportionately high concentration of *Alu* repeat elements, and *Alu-Alu* recombination events have been suggested as a mechanism for the formation of other deletions within the cluster (6,85).

The $--^{THAI}$ deletion was characterized using primers THAI-F and THAI-R to amplify a 480-bp fragment that spans the deletion junction. The sequence of this fragment was then used to deduce the 5' and 3' deletion breakpoints (Figure 37). The 5' deletion breakpoint lies approximately 3.0-kb upstream of the initiation codon of the ζ_2-globin gene and the 3' breakpoint lies approximately 6.6-kb downstream of the initiation codon of the α_1-globin gene(7). The total length of the deletion is 33.4-kb, in good agreement

with the original mapping studies. Unlike the $--^{FIL}$ deletion, the 5' and 3' breakpoints of the $--^{THAI}$ deletion do not share significant sequence homology. The 5' deletion breakpoint lies within a partial *Alu* repeat element that shares only 32% sequence homology with the 3' breakpoint sequence.

From the pilot studies, the incidence of non-Southeast Asian deletion type of α-thalassemia-1 ($--^{THAI}$ and $--^{FIL}$ deletions) in normal population should be further determined to increase the importance of the non-Southeast Asian deletion type of α-thalassemia-1. In application, the further DNA-based planning and preventive program of α-thalassemia should be more concerned in this type to facilitate the improvement of medical services such as carrier screening, genetic counseling, and prenatal diagnosis for pregnancies at risk for Hb Bart's hydrop fetalis.