# CHAPTER IV

# DISCUSSION

In the north of Thailand, the four common of β-thalassemia mutations were characterized by standard nucleotide sequencing comprising 47% codons 41/42, 32% codon 17, 10% IVS I-nt 1 G-T and 6% codons 71/42 (Channarong 2003). This means that a chance of those having these mutations to match each other is high. Thus the invention of a simple and rapid molecular diagnostic technique to detect these four mutations would be useful both in clinical setting and population screening. As it had been realized that the ARMS-PCR is easy to handle with high accuracy of mutations identification, this thesis aimed mainly to optimized the ARMS-PCR with the purpose to apply this technique for routine use in most laboratories.

As the ARMS-PCR has just been introduced in the Thalassemia Research Unit, Faculty of Medicine, Chiang Mai University, optimization of ARMS-PCR had to be carried out in several aspects including annealing temperature, amount of primers, amount of dNTPs, amount of $MgCl_2$ and amount of common primer.

In the search for optimal annealing temperature for the ARMS-PCR, although the temperature ranging from $59^0$C to $69^0$C was found to be suitable for ARMS-PCR, the temperature of $65^0$C was originally chosen as this temperature was in the midway of this range. In addition, it was suggested by Old in 1990 that this temperature was optimal for the length of the primers used of about 30 bp to generate robust amplified products (Old, *et al* 1990).

The ARMS-PCR was generally performed in two separate reaction i.e. normal reaction which used the common primer in combination with the normal primer; and mutant reaction which used the common primer in combination with the mutant primer. The optimal amount of these primers was investigated in this study and all of them were successfully optimized. However, for the IVSI-nt 1, the optimal condition in the normal tube was not achieved as many nonspecific amplified products with low signal intensity were seen. It was populated that the normal primer for this mutation had low affinity to the specific binding site as well as other sites that it could also bind. Another postulation was that the normal primer was slipped in a very low concentration and the annealing temperature set up was too low comparing to its annealing temperature. This ultimately gave rise to multiple non-specific PCR bands with low signal intensity. In the author's opinion, to design a new normal primer would be able to correct this problems. Although the normal reaction was problematic in the IVS I-nt 1, the optimization of the mutant and common primers in the mutant tubes was successful. This optimized condition could at least be useful to identify the IVS I- nt 1 mutation in the clinical samples.

In addition to the single ARMS-PCR, this study also set up and optimized the multiplex ARMS-PCR in which more than one mutation were identified simultaneously. However, the ARMS-PCR product signaling for detect the wild type of codons 71/72 had low intensity with the both single and multiplex ARMS-PCR. Increasing of this primer concentration would be able to correct this problem. The multiplex ARMS-PCR would be very helpful tool in detection of β-thalassemia mutations especially in the screening among general population. Again some parameters in this multiplex ARMS-PCR were required to get optimized for the best result. These parameters included dNTPs concentration, amount of $MgCl_2$ and common primers quantities. The four combinations of primers including 1) codon 17 (A-T) + codons 41/42 (-TTCT), 2) codon 17 (A-T) + Hb E, 3) codons 41/42 (-TTCT) + Hb E and 4) codons 41/42 (-TTCT) + codons 71/72 (+A) + codon 17 (A-T) were successfully optimized in both reaction tubes in the multiplex ARMS-PCR. This success would encourage the use of this multiplex ARMS-PCR

technique in β-thalassemia heterozygote screening, Hb E screening and prenatal diagnosis.

In both the single ARMS-PCR and the multiplex ARMS-PCR, the α-globin specific primers were added in the reactions to serve as the internal control. In this analysis, the absence of internal controls could conceivably give rise to incorrect diagnoses and hybridization to blots of the reaction mixtures was required. The internal control would make the performers confused between the real negative results or failure of the PCR reaction. The amplified of uncandidate gene were suggested for the internal control. The use of internal control would overcome this trouble.

The evaluation of application potential of ARMS-PCR for β-thalassemia heterozygote screening was performed in four groups of subject. In the first three groups in which the existence of β-thalassemia heterozygote was less likely, ARMS-PCR were used to exclude common β-thalassemia mutations which might be overlooked due to the normal findings of the screening tests in these individuals. As suspected, no discordant results were found. Moreover, by doing this, the potential of ARMS-PCR would simply be evaluated simultaneously. The last group, however, a chance to the existence of β-thalassemia heterozygote was apparent as indicated by the screening results. ARMS-PCR were undoubtedly able to characterized the types of β-thalassemia mutations in 28 from 30 individuals of this last group and 4 mutations were identified comprising 53% (16/30) codons 41/42, 30% (9/30) codon 17, 7% (2/30) IVS I-nt 1 and 3% (1/30) codons 71/72, all of which accounting for 93% of those preliminarily identified as β-thalassemia heterozygotes and leaving only 2 cases unidentified. This would emphasize the application potential of this ARMS-PCR in β-thalassemia detection. This was also the case for HbE detection.

The application of ARMS-PCR in PND case was reliable. The findings from HPLC did totally agree with that obtained by the ARMS-PCR in 4 fetal blood samples: non β-thalassemia disease by HPLC and β-thalassemia heterozygote by the ARMS-PCR. The concordant results were strengthened by those generated by the standard nucleotide

sequencing. This results further emphasize the application potential of the ARMS-PCR, especially in PND work. The discordant result obtained by HPLC and by ARMS-PCR was, however, observed in one fetal blood sample. This sample was diagnosed as β-thalassemia/Hb E disease by HPLC as no Hb A peak was seen. The ARMS-PCR, however, showed negative results for all four analyzed β-thalassemia mutations. The confirmation by direct nucleotide sequencing could identify the compound heterozygote for Hb E/Hb Tak in this case. In PND, the fetal blood Hb identification only might be incorrect diagnoses. Thus, detection of β-thalassemia mutations should be carried out in all PND samples.

Contamination of the fetal sample by maternal tissues or blood is one of the main problems in prenatal diagnosis. In this study, the fetal blood and chorionic villi sampling was performed. The Hb F staining were used to excluded the contaminated fetal blood and the Southern blot for detect individuals genes (paternity test) were used to excluded the contaminated CVS.

The aim of PND is to provide an accurate and rapid results as early in the pregnancy as possible. The comparison of ARMS-PCR and HPLC method indicated the advantage of ARMS-PCR over HPLC in performing the PND for the β-thalassemia and β-hemoglobinopathies. The ARMS-PCR is the DNA-based technique able to early diagnose in PND case. This is because the CVS were obtained at between 10-12 weeks of gestation while the fetal blood sampling were obtained at between 18-22 weeks of gestation.

Besides ARMS-PCR optimization, the WBC numbers were also in the process of optimization. This study was carried out as the DNA was simply prepared from the whole blood by using the Chelex™ technique. Thus, the WBC amount must be enough to yield sufficient DNA template to the ARMS-PCR. The lowest WBC numbers of 21,000 to 33,000 cells for the single ARMS-PCR; using 5 μl of genomic DNA and 10,500 to 16,500 cells for multiplex ARMS-PCR; using 7 μl of genomic DNA that still produced the

amplified products easily visualized on 2.0% agarose gel electrophoresis would suggest the highly applicability of the ARMS-PCR in varieties of blood samples.

The comparison of single ARMS-PCR and multiplex ARMS-PCR for detection of the four β-thalassemia mutations indicated the multiplex ARMS-PCR inexpensive than single ARMS-PCR. The condition of multiplex ARMS-PCR set up in this thesis could be further optimized by adding more than 2 specific primers in a single reaction (i.e. duplex and triplex). If these further optimizations are successfully performed, it would be very useful for the β-thalassemia mutation detection in the north of Thailand.