CHAPTER II

MATERIALS AND METHODS

2.1 Subjects

2.1.1 Optimization of Amplification Refractory Mutation System (ARMS)-PCR technique

The subjects in this study were divided into 2 groups, the first group composed of 10 hematologically normal individuals and the second groups 5 subjects with known β-thalassemia mutations [codons 41/42 (-TTCT), codon 17 (A-T), IVS I nt 1 (G-T), codons 71/72 (+A) and codon 26 (G-A) or Hb E]. The subjects in the first group were recruited to evaluate the optimal numbers of white blood cells for the ARMS-PCR. The second group of subjects was for the optimization of ARMS-PCR technique.

2.1.2 Assessment of application potential of ARMS-PCR technique in the β-thalassemia heterozygote screenning

The subjects for this study were pregnant women attending the antenatal care (ANC) clinic, Maharaj Nakorn Chiang Mai Hospital. In this study, the subjects were classified into four groups according to the Osmotic Fragility Test (OFT) results, HbA_2 levels and α-thalassemia 1 (SEA) genotype. The subjects in the first group comprised of fifteen pregnant women with negative OFT, normal Hb A_2 level (1.5-3.5%) and negative α-thalassemia-1 (SEA-type), while 15 of those with positive OFT, normal Hb A_2 level and negative α-thalassemia-1 (SEA-type) were classified into the second group. The third group of subjects comprised fifteen pregnant women whose OFT was positive with normal Hb A_2 levels and positive α-thalassemia-1 (SEA-type). Another thirty

pregnant women with positive OFT and increased Hb A_2 levels (4.0-9.0%) were categorized into the fourth group.

2.1.3 Assessment of application potential of ARMS-PCR technique in HbE screenning

The subjects recruited for this study comprised fifty pregnant women with HbE (Hb A_2 peak from HPLC with level greater than 10%).

2.1.4 Assessment of application potential of ARMS-PCR technique in prenatal diagnosis of β-thalassemia

In this studies, the subjects were classified into 2 groups based on the sources of DNA. The first group of subjects comprised of 12 families at risk for β-thalassemia major and 14 families at risk for β-thalassemia/Hb E disease. In this group, fetal DNA was obtained from fetal blood samples collected by cordocentesis. The second group of subjects was composed of 5 chorionic villi samples obtains by chorionic villi sampling (CVS) under ultrasound-guidance (transcervical or transabdominal) and parents. The identification of high risk couples was carried out by the criterions described in the teble 1.3 .

2.2 Hematological studies

2.2.1 Two-minute Erythrocyte Osmotic Fragility Test (2-min OF)

This technique bases on the limit of hypotonicity which the red cell can withstand. As modified by Sanguansermsri in 2000 (Chamrasratanokorn, *et al* 1998), the "2-min OF" were using spectrophotometry and computerization to measure the optical density and, then, calculate the percentage of hypotonically resistant red blood cells. Practically, 20 μl of whole blood was mixed with 2 ml of 0.45 % glycerine saline solution (0.45% GSS), followed by measurement of optical density (OD) at 620 nm at 15 seconds and 120 seconds. The percentage of hypotonically resistant red blood cells were calculated by the following formula ;

$$\% \text{ 2-min OF} = \frac{\text{OD at time 15 sec.} - \text{OD at time 120 sec}}{\text{OD at time 15 sec}} \times 100$$

Interpretation ; 2-min OF < 60 % indicates "positive OFT"

; 2-min OF > 60 % indicates "negative OFT"

2.2.2 Hemoglobin identification by high performance liquid chromatography (HPLC)

Hemoglobin typing in blood samples can be determined by passing the hemoglobin solution through a weak cation-exchange high performance liquid chromatography or HPLC. The hemoglobin fractions including Hb F are automatically computed. This task was accomplished using the Bio-Rad VARIANT™ Hemoglobin Testing System (Bio-Red Laboratories, 1994) in which The β-Thal Short™ operating software was employed to control the separation profile of the machine. The hemoglobin separation lasts 6 min. The Hb F was eluted first, followed by Hb A and HbA_2/E, respectively. The percentages of the different hemoglobins were then calculated automatically by comparing the area under each hemoglobin peak.

2.3 DNA analysis

2.3.1 Genomic DNA extraction from whole blood

DNA was extracted from whole blood using the Chelex method® with some modification (Sanguansermsri, *et al* 1999). Chelex resin is a chelating agent that removes divalent cation including Mg^{++} which could act as cofacter of Dnase enzyme. In practice, 40 µl of EDTA blood was mixed with 1 ml of 0.5 % Tritron X-100 in 1.5 ml tubes, vortexed and centrifuged at 14,000 rpm for 1 min. The supernatant was removed, 1 ml of water added, centrifuged as above and supernatant removed again. A Chelex-100 suspension was then added to the pellet until a 1-2 mm-thick layer was obtained, followed by 110 µl distilled water. Then the mixture was incubated at 56°C at least 2 hours or overnight prior to heating at 100°C boiling water for 7 min. Incubation at 56°C

was to activate the Chelex-100 resin while heating aimed to destroy the cell and cellular components, such as proteins, to liberated the genomic DNA. The extracts were then stored at 4^0C until use.

2.3.2 Genomic DNA extraction from CVS

DNA was extracted from chorionic villi (collected by the obtetric technique called chorionic villi sampling (CVS)) by using QIAamp DNA Minikit (QIAGEN GmbH, Hiden, Germany). In practice, approximately 25 ng of chorionic villi was placed in a 1.5-ml microcentrifuge tube, 180 µl of ATL buffer and 20 µl Proteinase K added and incubated at 56^0C until the chorionic villi was completely dissolved. The solution was then added with 200 µl of AL buffer and incubated at 70^0C for 10 min before precipitating the DNA with 200 µl absolute ethanol. The mixture was then applied into the QIAamp spin column, centrifuged at 6000 g for 1 min to remove other residues leaving the DNA attached to the membrane in the column. The column was then washed twice with 500 µl of AW1 and AW2 buffer, respectively, followed by the addition of 200 µl AE buffer to elute the membrane-bound DNA into the new clean microcentrifuge tube. The DNA was then stored at 4^0C until use.

2.2.3 Determination of α-thalassemia-1 (SEA) genotype

The Southeast Asian (SEA) deletional type of α-thalassemia-1 involves 19.304 kb fragment of α-globin gene cluster spanning from $\psi\zeta_2$-gene to the sequences 3' to the θ-globin gene. By using 3 primers (P_1, P_2, P_3) that bind at the different sites inside and flanking the SEA deletion, one can easily determine the α-thalassemia-1 of this type by visualizing the amplified products generated from each of primer pairs (Figure 2.1).

The Gap-PCR was performed in a total volume of 8 μl containing 50 ng genomic DNA, 200 μM of each dNTP, 0.02 units *Taq* DNA polymerase (QIGEN GmbH, Hiden, Germany), 0.375 μM of each primer (P_1 : 5' - GCG ATC TGG GCT CTG TGT TCT –3', P_2: 5' – GTT CCC TGA GCC CCG ACA CG –3', P_3 5'-GCC TTG AAC TCC TGG ACT TAA-3') in 10 μM Tris pH 8.6, 50 mM KCl, 0.00125 % xylene cyanol and 1.5mM $MgCl_2$. A total of 40 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 30 sec, primer annealing at 58°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 5 min while the final extension was prolonged to 7 min. For SEA deletion type, a 314-bp fragment was found in those negative for SEA deletion, while 314-bp and 188-bp amplified fragments were seen in the heterozygous state and only 188-bp fragment were produced in the homozygotes (Figure 2.2).

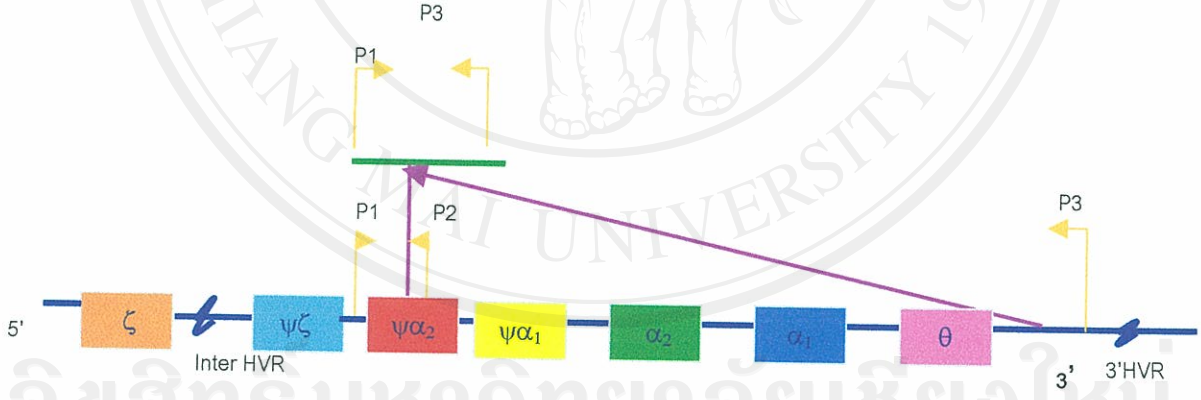

Figure 2.1 Schematic representation of location of primers for detection of α-thalassemia –1 (SEA) type.

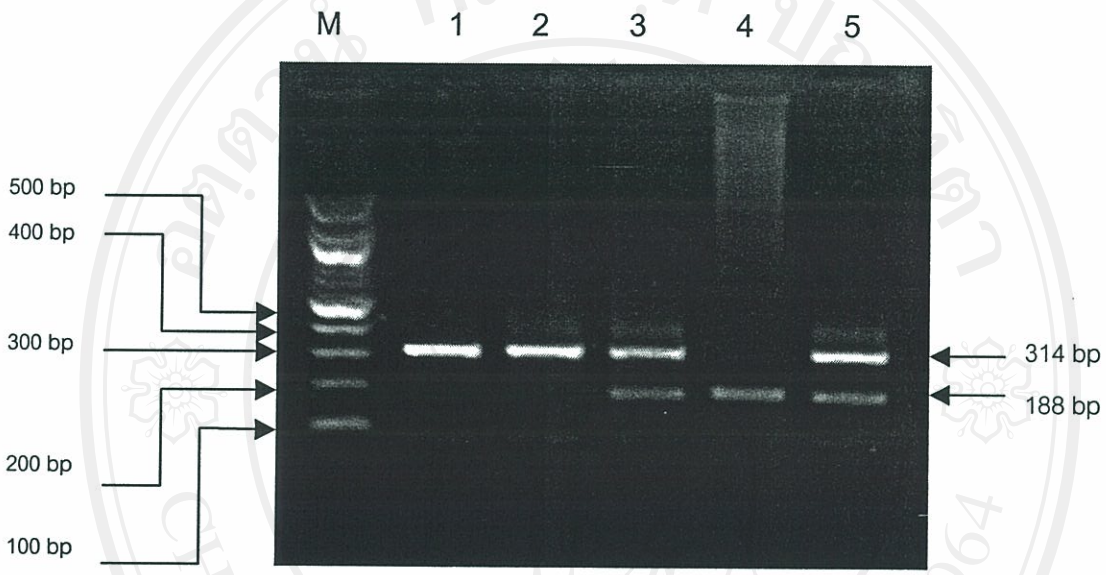

Figure 2.2 Agarose gel electrophoresis for detection of α-thalassemia –1 (SEA) type. Lane M is pME-80J3 *Eco*1741 and *Pvu*I digest DNA size marker. Lane 1 and 2 represent cases without α-thalassemia–1 (SEA) deletion where only the 314-bp fragment is present. Lane 3 and 5 represent those heterozygous for α-thalassemia–1 (SEA) deletion; 188–bp and 314-bp fragments are present. Lane 4 represents the case homozygous for α-thalassemia-1 (SEA) or Bart's hydrops fetalis in which only 188-bp amplified products is seen.

2.3 Amplification Refractory Mutation System (ARMS)-PCR for detecting β-thalassemia mutations

ARMS-PCR is also known as allele-specific PCR (AS-PCR) or PCR amplification of specific allele (PASA). It was first described by Newton *et al*, 1989 (Newton, *et al* 1989) to detect point mutations and small deletions/insertions in genomic DNA. Old and his colleagues were the first to use this technique in the characterization of β-thalassemia mutation (Old, *et al* 1990). Since then, the technique has become widely employed to detect β-thalassemia mutations around the world. At present, there are two types of ARMS-PCR generally carried out in the molecular laboratories which are **single ARMS-PCR and multiplex ARMS-PCR.**

Single ARMS-PCR

The standard ARMS was generally performed in two reactions (two tubes) using 3 oligonucleotide primers (common, normal and mutant primers). In the first tube, two primers including common and normal primers were utilized to produce wild type amplified products. In the second tube, however, the common primer was utilized with the mutant primer to generate the amplified products encompassing the mutated point (Figure 2.3). If the sample was negative for the mutation to be determined, only amplified products generated from common and normal primers were observed. However, if the subject was homozygous for the mutation, only the products from common and mutant primers were generated. In addition, the amplified products were produced in both tubes in those heterozygous for the mutation investigated. In addition to β-globin specific primers, α-globin specific primers (P1 and P2) were also added in the reaction as an internal control.

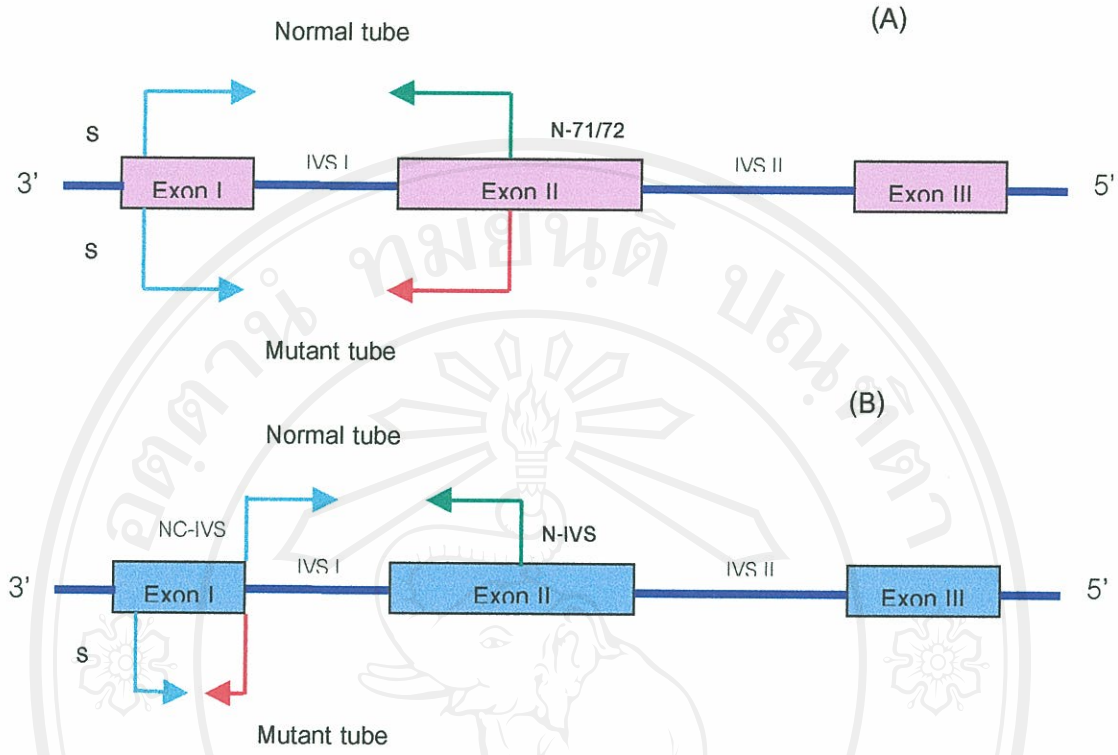

Figure 2.3 Model of primers localization of the single ARMS-PCR. (A) Single ARMS-PCR Cds 71/72, (B) single ARMS-PCR IVS I-nt 1 G-T.

The multiplex ARMS-PCR

In multiplex ARMS-PCR, more than one mutations can simultaneously detected in a single reaction. The reaction mixtures were still divided into 2 tubes i.e. for detecting wild and mutant types separately resembling to that performed in the single ARMS-PCR. However, in addition to the α-globin specific internal control primers (P1 and P2), more than one β-globin specific primers were added in each tube to detect the mutations of interest. In general, each multiplex ARMS-PCR master mix contained 3 or 4 β-globin specific primers (one common primer and two or three primers specific to different β-thalassemia mutations) in addition to a pair of internal control primers mentioned above (Figure 2.4). The main theme of the multiplex ARMS-PCR was that the amplified products generated from each β-thalassemia mutation specific primer must have size-

differences so that they could easily be revealed on a simple agarose gel electrophoresis routinely carried out in most molecular laboratories.

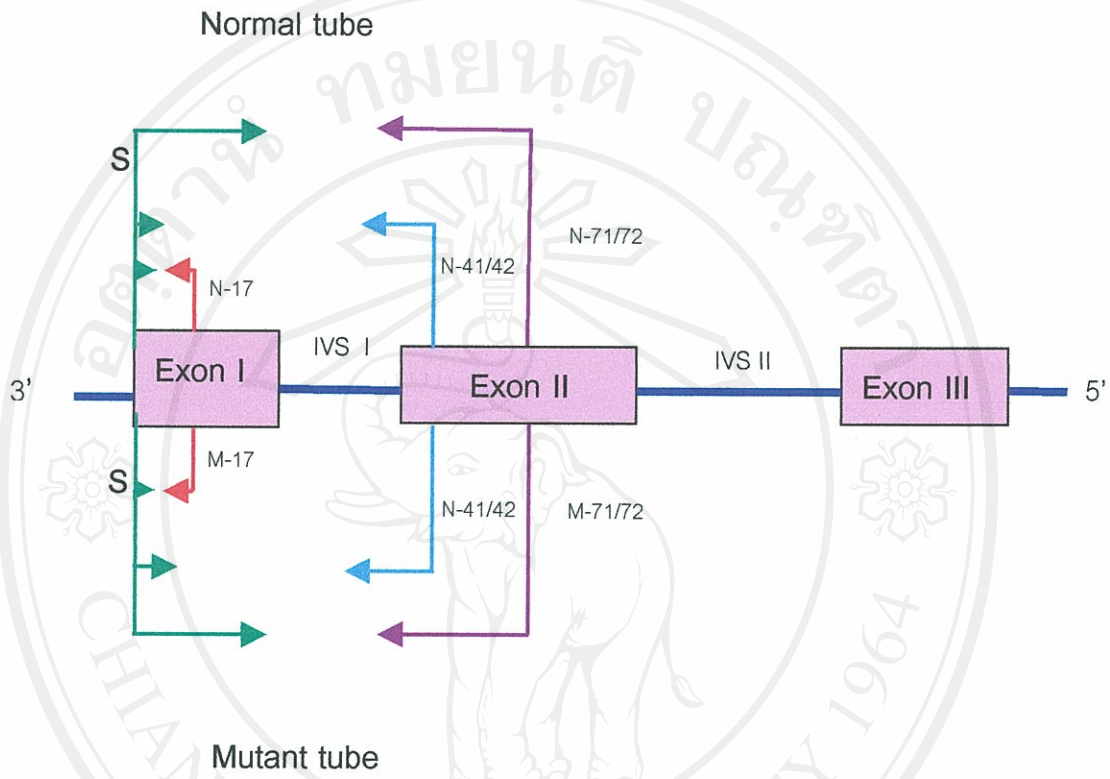

Figure 2.4 Model of primer location of multiplex ARMS-PCR

General rules for ARMS-PCR primer design

The ARMS-PCR primers was designed so that they were able to discriminate between templates which differ at a specific single nucleotide residue. The primers were designed so that the 3' terminal base was able to distinguish the different alleles; i.e. normal and mutant alleles. The ARMS-PCR primers are usually about 30 bases long with ~50 % GC content, should not have self complementary sequences of more than or equal to 4 bp and should not have more than or equal to 4 bp at its 3' end complementary to other primers (Lo 1998). The followings are the sequences of oligonucleotide primers used in ARMS-PCR to detect β-thalassemia mutation commonly found in Thailand and also used through out this thesis.

ARMS primers sequence for characterization of β-thalassemia mutations (Weatherall and Clegg 2001) (www.PrimerBiosoft.com)

1. Common primers (sense primers)

NC-IVS 5'-CCC CTT CCT ATG ACA TGA ACT TAA -3'

S 5'-ACC TCA CCC TGT GGA GCC AC- 3'

2. Codons 41/42 (-TTCT) (anti-sense primers)

N-41/42 ; 5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA-3'

M-41/42 ; 5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3'

3. Codons 17 (A-T) (anti-sense primers)

N-17 ; 5'-CTC ACC ACC AAC TTC ATC CAC GTT CAC ATT- 3'

M-17 ; 5'-CTC ACC ACC AAC TTC AGC CAC GTT CAG ATA-3'

4. Codons 71/72 (+A) (anti-sense primers)

N-71/72 ; 5'-GAG GTT GTC CAG GTG AGC CAG GCC ATC AAT- 3'

M-71/72; 5'-AGG TTG TCC AGG TGA GCC AGG CCA TCA ATT- 3'

5. IVS I nt1(G-T) (anti-sense primers)

N-IVS; 5'-GAT GAA GTT GGT GGT GAG GCC CTG GGT AGG- 3'

M-IVS ; 5'-TTA AAC CTG TCT TGT AAC CTT GAT ACC GAA- 3'

6. Codon 26 (G-A) or Hb E (anti-sense primers)

Hb E-N; 5'-TAA CCT TGA TAC CAA CCT GCC CAG GGC GTC -3'

Hb E-M ; 5'-TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT- 3'

7. Internal control primer

P_1 ; 5'-GCG ATC TGG GCT CTG TGT TCT-3' (sense primers)

P_2 ;5'-GTT CCC TGA GCC CCG ACA CG-3' (anti-sense primers)

2.4.1 Single ARMS-PCR reaction mix

The PCR was performed in two tubes as previously mentioned. In each tube, the reaction was carried out in a total volume of 25 μl containing 150 ng genomic DNA, 200 μM of each dNTPs, 0.6 units *Taq* DNA polymerase (QIAGEN GmbH, Hiden, Gemany), 100 ng of β-globin specific common primer (S: 5'-ACC TCA CCC TGT GGA GCC AC-3'), 200 ng of P_1 primer (5'-GCG ATC TGG GCT CTG TGT TCT–3') and 200 ng of P_2-primer (5'– GTT CCC TGA GCC CCG ACA CG–3') in 10 mM Tris pH 8.8, 50 mM KCL and 1.5 mM $MgCl_2$. The wild type specific primers were used in the normal tube and the mutant specific primers were used in the mutant tube. The optimal quantities of these β-globin specific primers were determined in the study of this thesis. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95^0C for 2 min, primer annealing at 65^0C for 1 min and primer extension at 72^0C for 2 min. The initial denaturation was extended to 5 min. The last extension was also extended to 7 min.

2.4.2 Multiplex ARMS-PCR reaction mix

Like the single ARMS-PCR, the multiplex ARMS-PCR was also performed in two tubes; i.e. normal and mutant tubes. In both tubes, a total volume of 25 μl was performed containing 250 ng genomic DNA, 300 μM of each dNTPs, 0.8 units *Taq* DNA polymerase (QIAGEN GmbH, Hiden, Gemany), 400 ng of β-globin specific common primer (S: 5'-ACC TCA CCC TGT GGA GCC AC- 3'), 200 ng P_1 primer (5'-GCG ATC TGG GCT CTG TGT TCT –3') , 200 ng P_2 primer (5'– GTT CCC TGA GCC CCG ACA CG –3') in 10 mM Tris pH 8.8, 50 mM KCL and 1.5 mM $MgCl_2$. In normal tube, 2 or 3 primers specific to different wild type sequences were utilized. 2 or 3 mutant specific primers were added into the mutant tube. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95^0C for 2 min, primer annealing at 65^0C for 1 min and primer extension at 72^0C for 2 min ; the initial denaturation was extended to 5 min. The last extension was also extended to 7 min.

2.5 Optimization of ARMS-PCR

As ARMS-PCR was firstly introduced in to this laboratory, optimization of the system was required. The optimization of ARMS-PCR technique carried out in this thesis started with the single ARMS-PCR in which the annealing temperature was optimized first, followed by the search for the optimal amount of the oligonucleotide primers used, then the optimized system for single ARMS-PCR was used for further optimization of the multiplex ARMS-PCR technique.

2.5.1 Optimization of annealing temperature for single ARMS-PCR

Annealing temperature are the most important parameter in the PCR reaction including the ARMS-PCR. As suggested by Old in 1990, the length of ARMS-PCR primer of 30 bp with a 65^0C-annealing temperature could prevent non-specific binding, thus, improving the specificity of the ARMS-PCR primer (Old, *et al* 1990). In this study, the optimal annealing temperature was determined by performing the single ARMS-PCR using the standard PCR protocol (Sambrook and Russell 2001). The PCR mix was the same as that previously described in 2.4.1. The titration of annealing temperature from 59^0- 69^0C was accomplished by using the Eppendorf Thermal Cycle with the thermal gradient option.

2.5.2 Titration of primers for single ARMS-PCR

The optimal amount of primers used in the reaction is also important to the success of the ARMS-PCR. In this study, the search for optimal quantities of the primers was carried out by using the PCR system mentioned in 2.4.1 with the optimized annealing temperature of 65^0C. The titration was undertaken for all primers within the concentrations range of 0.05 - 0.2 μM.

2.5.3 Optimization of multiplex ARMS-PCR

As more than one mutations were simultaneously determined in the Multiplex ARMS-PCR, the amount of critical ingredients utilized in the reaction must be optimized to ensure the sufficiency of amplified products. In the present study, the optimization was carried for $MgCl_2$, dNTP and common primer concentrations. Four combinations of β-specific primers were used in the multiplex ARMS-PCR including; 1) codon 17 (A-T) and codons 41/42 (-TTCT), 2) codon 17 (A-T) and HbE, 3) condons 41/42 (-TTCT) and HbE, 4) condon 17 (A-T) and codons 41/42 (-TTCT) and codons 71/72 (+A).

2.5.3.1 Optimization of dNTP and $MgCl_2$ concentrations

The multiplex ARMS-PCR was carried out as described in 2.4.2 using the combination of wild type primers of condon 17 (A-T) and codons 41/42 (-TTCT) and codons 71/72 (+A) with the dNTP concentration of 200, 300, 400 μM and 2.0 mM $MgCl_2$. The yield of amplified products at each dNTP concentration was examined. The optimal concentration of dNTP for efficient multiplex ARMS-PCR was evaluated from the signal of the PCR products on agarose gel electrophoresis.

For the standardization of $MgCl_2$ concentration, the multiplex ARMS-PCR was carried out using the condition described in 2.4.2 using the combination of wild type primers of condon 17 (A-T) and codons 41/42 (-TTCT) and codons 71/72 (+A) with the fixed amount of dNTP at the optimized amount of dNTP of 300 μM. The concentrations of $MgCl_2$ to be tested were 1.5, 1.8, 2.1, 2.3 and 2.5 mM. The optimal concentration of $MgCl_2$ for efficient multiplex ARMS-PCR was evaluated from the signal of the PCR products on 2.0% agarose gel electrophoresis.

2.5.3.2 Optimization of the amount of β-specific common primer

In the multiplex ARMS-PCR, only one common primer was designed to pair with all the wild type and mutant specific primers. Thus, optimization of the amount of this common primer should be essential for good and efficient task. By using the multiplex ARMS-PCR condition mentioned in 2.4.2 using the combination of wild type primers of

condon 17 (A-T) and codons 41/42 (-TTCT) and codons 71/72 (+A) with optimized concentration of dNTP and $MgCl_2$ of 300 μM and 2.1 mM, respectively, the titration of common primer at the concentration of 0.2, 0.3, 0.4 and 0.5 μM was performed. The optimal concentration of common primer for efficient multiplex ARMS-PCR was evaluated from the signal of the PCR products on 2.0% agarose gel electrophoresis.

2.6 Evaluation of the optimal numbers of white blood cells for ARMS-PCR

Ten blood samples collected from hematological normal individuals was diluted with NSS at dilution from 1:2 to 1: 64 to produce a series of WBC numbers. DNA was then extracted from diluted blood by using the Chelex® method described in 2.3.1. The optimized single ARMS-PCR using the wild type primer of codons 41/42 (-TTCT) and optimized multiplex ARMS-PCR using the combination of wild type primers of condon 17 (A-T) and codons 41/42 (-TTCT) and codons 71/72 (+A) were undertaken using the DNA extracted from these diluted blood samples. The optimal amount of WBC for efficient ARMS-PCR was evaluated from the signal of the PCR products on 2.0% agarose gel electrophoresis.

2.7 Assessment of application potential of ARMS-PCR technique in the β-thalassemia heterozygote screenning

Four groups of pregnant women were recruited in this study according to the OFT results, HbA_2 levels and α-thalassemia 1 (SEA) genotype as described in 2.1.2. The optimized single ARMS-PCR and the optimized multiplex ARMS-PCR for all mutations were performed in all blood samples. The results generated from both ARMS-PCR techniques were analyzed to evaluate if they were consistent with the OFT results, HbA_2 levels and α-thalassemia 1 (SEA) genotype.

2.8 Assessment of application potential of ARMS-PCR technique in HbE screenning

A group of fifty pregnant women were recruited in this study according to the OFT results, HbE levels as described in 2.1.2. The optimized single ARMS-PCR was performed in all blood samples. The results generated from the ARMS-PCR techniques were analyzed to evaluate if they were consistent with the HbE levels generated from HPLC.

2.9 Assessment of application potential of ARMS-PCR for prenatal diagnosis (PND)

In this study, the high risk couples were initially screened for β-thalassemia mutations by both ARMS-PCR techniques. To identify β-thalassemia mutations of the *in utero* fetuses, the fetal samples were collected obstetrically to obtain cord blood or chorionic villi samples (CVS) (Tongsong, *et al* 2000). Genomic DNA from the cord blood was extracted by using Chelex® method and genomic DNA from CVS was extracted by using QIAamp DNA Minikit. The detection of β-thalassemia mutations of the fetal samples was accomplished using both single and multiplex ARMS-PCR. For cord blood samples, ARMS-PCR results were assessed whether they were consistent with those generated from HPLC or not. If not, nucleotide sequencing of the β-globin gene was performed. In case of CVS, the ARMS-PCR results were confirmed by nucleotide sequencing technique.

2.10 Nucleotide sequencing of β-globin gene exons

2.10.1 Amplification of the β-globin gene exons

The three exons of the β-globin gene were amplified using different primer pairs. Amplification of exons 1 and 2 were accomplished in a 0.2–ml-thin-wall tube. The PCR was performed in a total volume of 50 μl containing 5 μl genomic DNA, 200 μM of each dNTPs, 0.02 unit of Ampli*Taq* Gold® DNA polymerase (Applied Biosystems, Foster City,

CA, USA) and 0.4 μM of primer mix S-Exon 1-2 (5'-CCA ACT CCT AAG CCA GTG CC-3'), A-Exon 1-2 (5'-GGG AAA CAG ACG AAT GAT TGC A-3') in 10 mM Tris pH 8.8, 50 mM KCL and 2.5 mM $MgCl_2$. A total of 40 thermal cycles was carried out with each cycle comprising DNA denaturation at 94^{0}C, primer annealing at 60^{0}C for 1 min and primer extension at 72^{0}C for 2 min; the initial denaturation was extended to 95^{0}C for 15 min while the final extension was prolonged to 10 min (Sanguansermsri, *et al* 2000).

For the amplification of exon 3, PCR was performed in a total volume of 50 μl containing 5 μl genomic DNA, 200 μM of each dNTPs, 0.02 unit of Ampli*Taq* Gold® DNA polymerase (Ampplied Biosystems, Foster City, CA, USA), 0.5 μM of primer mix S-Exon 3 (5'-TCC CTA ATC TCT TTC TTT CAG G-3'),and A-Exon 3 (5'-GCT AGT TCA AAC CTT GGG AAA A-3') in 10 mM Tris pH 8.8, 50 mM KCl and 1.5 mM $MgCl_2$. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94^{0}C for 45 seconds, primer annealing at 55^{0}C for 1 min and primer extension at 72^{0}C for 1.30 min; the initial denaturation was extended to 95^{0}C for 15 min while the final extension was prolonged to 7 min.

PCR products were separated in 1.5 % agarose gel electrophoresis and made visible with ethidium bromide and medium-wavelength UV light from a transilluminator (Figure 2.5). The size of the DNA fragments were 760 and 659 bp, respectively .

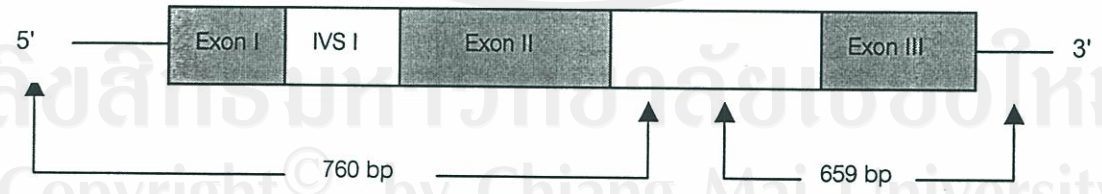

Figure 2.5 Model of primer locations of direct nucleotide sequencing of β-globin gene (*not to correct scale)

2.10.2 Nucleotide sequencing

2.10.2.1 Purification of amplicons

The amplicons were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hiden, Germany). The kit was designed to separated soluble PCR products ranging in size from 100 bp to 10 kb, nucleotides, DNA polymerases and salts. Practically, the amplified products to be purified was poured into a spin column provided in the kit. The column was then spun at 14,000 rpm to remove all the ingredients left over from the amplification reaction, leaving only PCR products attached to the resin inside the column. The attached amplified fragments were then eluted out of the column using eluting buffer after 14,000 rpm centrifugation. The purified DNA was then stored at -20^{0}C until use.

2.10.2.2 Cycle sequencing reaction

The extension of sequencing reactions were performed in 0.2-ml PCR tubes. The reaction mixture contained 8 μl of ABI Prism® BigDye Terminator Ready Reaction Mix (Applied Biosystems), 3.2 μM of sequencing primer (S-Exon 1-2 or S- Exon 3) and 5-20 ng of purified DNA. Deionized distilled water was added to make up 20 μl. The thermal cycles comprised DNA denaturation at 96^{0}C for 10 seconds, primer annealing at 50^{0}C from 5 seconds and primer extension at 60^{0}C for 4 min in total of 25 cycle.

2.10.2.3 Precipitation of the sequencing extension products

The extension products were precipitated using a mixture of ethanol and sodium acetate. The entire content of each extension products was transferred into a tube containing 2.0 μl of 3 M sodium acetate (NaOAc), pH 4.6 and 50 μl of 95 % ethanol (EtOH), mixed thoroughly and left at room temperature for 15 min, spun at 14,000 rpm in a microcentrifuge for 30 min and supernatant discarded. The pellet was then washed with 250 μl of 70 EtOH, spun at 14,000 rpm for 25 min and dried by a 1-min heating on

the heat block at 90^{0}C. The precipitated extension products was stored at –20^0 C until separation by electrophoresis.

2.10.2.4 Automated DNA sequencing

The precipitated extension products were suspended in 12-25 µl template suppression reagent (TSR), mixed and spun down. The suspension was then heated at 95^{0}C for 2 min, immediately chilled on ice for 10 min, mixed and spun down again. The separation of the extension products was accomplished using the ABI Prism 310 Genetic Analyzer (Applied Biosystems) which is based on the capillary electrophoresis (CE) principle. The raw data from capillary electrophoresis on the machine was collected and analyzed by Macintosh-based software (Sequencing Analysis Software Version 2.1.1). Data was manually compared with the reference sequence of the β-globin gene (GeneBank U01317).