# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Materials

**2.1.1** Chemicals and instruments used in this thesis are indicated in the Appendix.

**2.1.2** Blood samples comprised both known and unknown blood samples. The known blood samples were the samples of which the globin gene mutations had already been characterized by the established molecular techniques. These samples were utilized in the PCR development steps. The unknown samples were the blood samples of which the globin gene mutations had not been characterized and used in the PCR evaluation steps. This study protocol was approved by the Research Ethic Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Approved notice number 118/2555).

### 2.2 Methods

This thesis was designed to include 2 principal investigations comprising 1) development of the in-house multiplex allele-specific PCR, 2) development of the in-house direct PCR from whole blood without DNA isolation.

**2.2.1 Development of the in-house multiplex allele-specific PCR**

The primary purpose of this thesis was to extent ability of the in-house multiplex allele-specific PCR previously set up in Dr. Thanusak's laboratory to be able to detect more common globin gene mutations simultaneously. The initial in-house multiplex allele-specific PCR set up in this laboratory was able to simultaneously indentify 4 globin gene mutations including the SEA-α thalassemia 1, $\beta^{41/42(-TTCT)}$, $\beta^{17(A-T)}$ and HbE alleles (Suwannasin 2009, Tatu, *et al* 2012). The additional globin gene mutations designed to add up the original PCR protocol included the α-thalassemia 2 (3.7-kb deletion), HbCS and $\beta^{-28(A-G)}$. This part of study consisted of 4 consecutive steps which were 1) extraction of genomic DNA, 2) determination of locations of allele-specific primers in the corresponding globin genes, 3) optimization of singleplex allele-specific PCR and 4). Optimization of the developed in-house mutiplex allele-specific PCR.

**2.2.1.1 Extraction of genomic DNA**

The genomic DNA used in this part of thesis was prepared from EDTA blood using the Chelex™ extraction method with some modifications (Polski, *et al* 1998, Walsh, *et al* 1991). A 100 μl EDTA blood sample was firstly washed in 1 ml 0.5% (v/v) Triton X-100 and then in 1 ml deionised water (DI) before adding 110 μl DI and 1-2 drops of Chelex™ suspension (5% (w/v) in DI). The mixture was then incubated overnight in a 56$^{o}$C-waterbath to activate the Chelex™ resin and then in a boiling water-bath for 10 minutes to release DNA from leukocytes. Finally, the tube was centrifuged at 15,000 g for 1 minute, supernatant harvested and kept at -20$^{o}$C until use.

**2.2.1.2 Determination of locations of allele-specific primers in the corresponding globin genes**

The oligonucleotide primers used in the study comprises those specific for 7 common globin gene mutations in Thailand; SEA-α thalassemia 1, α-thalassemia 2 (3.7-kb deletion), HbCS, HbE, $\beta^{17(A-T)}$, $\beta^{41/42\ (-TTCT)}$ and $\beta^{-28(A-G)}$ as well as those served as the internal control (Figure 2.1). Thus, overall 7 oligonucleotide primers were utilized including SEA1, SEA3, CS-2, Alpha3.7A, Alpha3.7B, αG-17 and C3 primers (Table 2.1). These oligonucleotide primers were taken from the published sources (Fucharoen, *et al* 2002, Suwannasin 2009, Tatu, *et al* 2012). However, before utilizing these oligonucleotide primers in the in-house multiplex allele-specific PCR, it was essential to re-assure the correct and exact binding sites of each primer on its corresponding globin gene. This was performed by aligning, either manual or computer-based, the nucleotide sequences of the primers with those of α-globin gene and β-globin gene clusters obtained from the GenBank database with access code "AE006462" for α-globin gene cluster and "HUMHBB" for β-globin gene cluster (http://www.ncbi.nlm.nih.gov/pubmed/). The computer-based alignment was performed using the "Primer Premier 5.0" software. The exact numerical locations of the globin gene mutations corresponding to the GenBank database were obtained from the Globin Gene Server (http://globin.cse.psu.edu/). The locations of each oligonucleotide primers are shown in Figure 2.2.

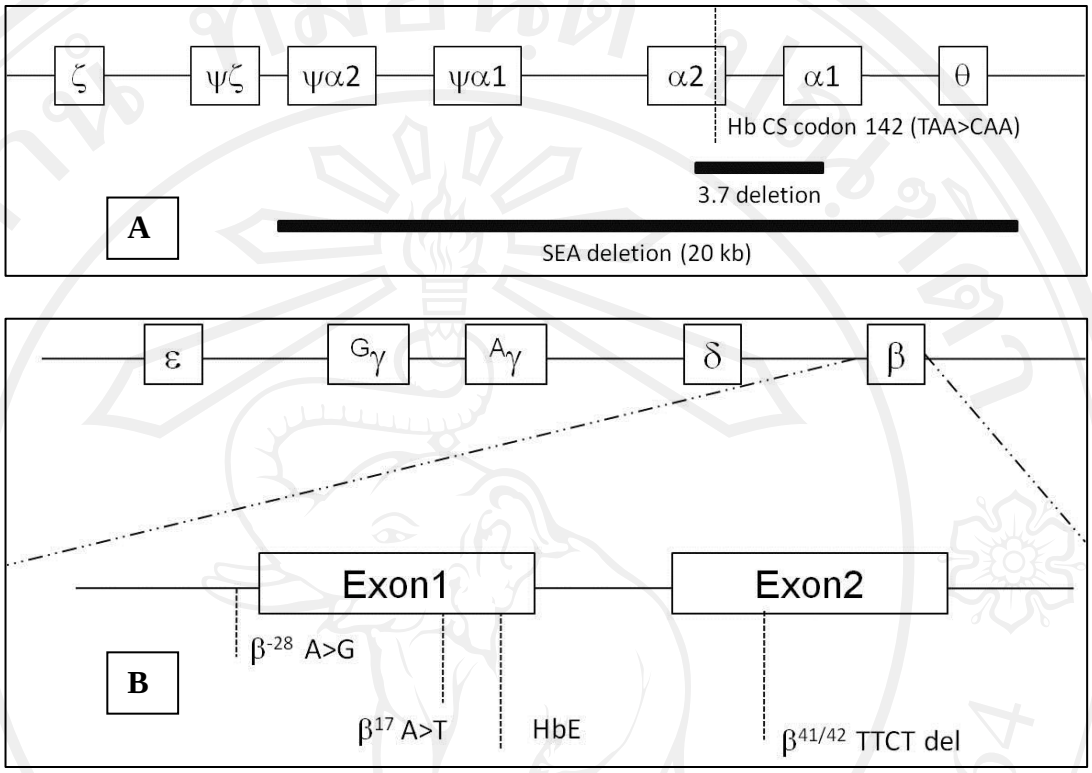


**Figure 2.1** Schematic locations of α-globin (A) and β-globin (B) genes common mutations in Thailand

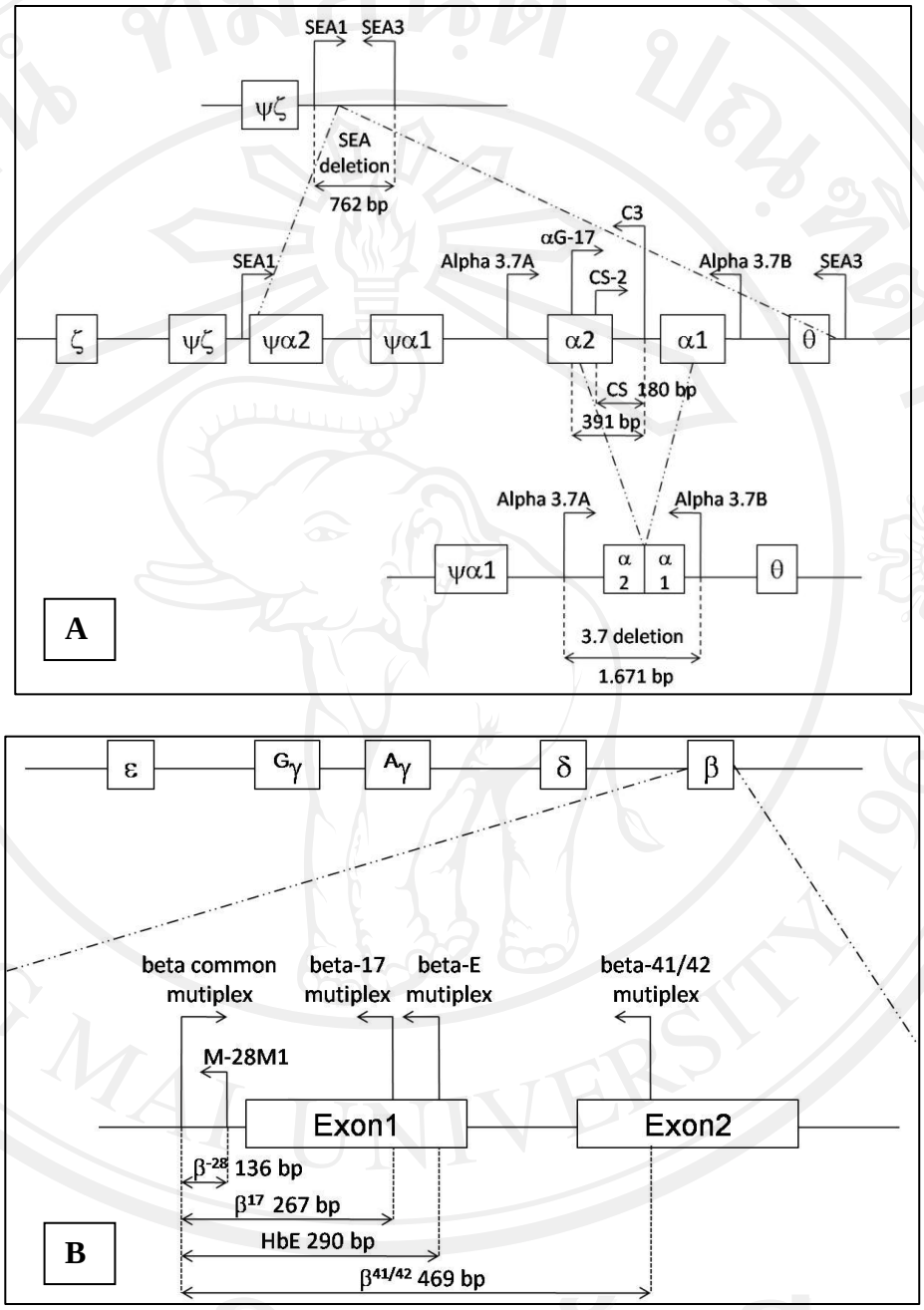


**Figure 2.2** Locations of oligonucleotide primers used in the multiplex allele-specific PCR for α-globin (A) and β-globin (B) genes mutations developed in this thesis

**Table 2.1** Names and sequences of oligonucleotide primers utilized in the developed PCR protocols

| **Primers** | **5'----------3'** | **Base pairs** |
|---|---|---|
| **SEA1** | TGACTCCAATAAATGGATGAGGA | 23 |
| **SEA2** | GCCTGCGCCGGGGAACGTAACCA | 23 |
| **SEA3** | CGCCAAAGATGGCTACTCGGAGA | 23 |
| **Alpha 3.7A** | CCCAGAGCCAGGTTTGTTTATCTG | 24 |
| **Alpha 3.7B** | GAGGCCCAAGGGGCAAGAAGCAT | 23 |
| **CS-2*** | GCTGACCTCCAAATACCCTCAA | 22 |
| **αG-17** | AGATGGCACCTTCCTCTCAGG | 21 |
| **C3** | CCATTGTTGGCACATTCCGG | 20 |
| **beta commom multiplex** | AAGAGCCAAGGACAGGTAAGGCTAT | 25 |
| **beta 17 multiplex** | CCAACTTCATCCACGTTCACGTA | 23 |
| **beta E multiplex** | CGTACCAACCTGCCCAGGGCCAT | 23 |
| **beta 41/42 multiplex** | AGATCCCCAAAGGACTCAACC T | 22 |
| **M-28M1*** | AGAAGCAAATGTAAGCAATACATGGCT CTGCCCTGCCATC | 40 |

Note: * = Underlined are the mismatch points.

**2.2.1.3 Optimization of the singleplex allele-specific PCR**

**A. Singleplex allele-specific PCR for internal control**

The α-globin cluster specific primers; αG-17 and C3 were utilized as the internal control primers. These primers were in fact created by other researcher (Fucharoen, *et al* 2003). The PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 μM αG-17 and 0.4 μM C3 primers, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 250 ng DNA template. The amplification reaction was carried out in 35 thermal cycles comprising denaturation at 95°C for 5 minutes in the first cycle and for 1 minute in subsequent 33 cycles, primer annealing at 60°C for 30 seconds and extension at 72°C for 1 minute 30 seconds in the 33 cycles and for 5 minutes during the last cycle. For PCR fragment analysis, 5 μl of PCR product was mixed with loading dye (Appendix C) and separated on a 2% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The αG-17 and C3 primers generated the 391-bp amplified products of the α-globin gene cluster (Figure 8(A)). The optimal condition was judged by presence of the 391-bp amplified product with or without non-specific one(s).

**B. Singleplex Gap-PCR for SEA-α thalassemia 1**

This singleplex Gap-PCR was a modification of that developed previously in Dr. Thanusak's laboratory (Suwannasin 2009, Tatu, *et al* 2012). This modifications consisted of optimization of annealing temperature, $MgCl_2$ concentration and amount of primers.

The 25 μl PCR contained the SEA1 and SEA3 primers (0.035 pmol/μl – 0.175 pmol/μl final concentration optimized in the "Checker board" fashion) (Table 2.2), 0.04 pmol/μl αG-17 and 0.4 pmol/μl C3 primers, 10 mM Tris pH 8.5, 50 mM KCl, 1, 1.5, 2, 2.5, 3.5 mM $MgCl_2$, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 250 ng DNA template. The amplification reaction was carried out in 35 thermal cycles comprising denaturation at 95$^{o}$C for 5 minutes in the first cycle and for 1 minute in subsequent 33 cycles, primer annealing 57-65 $^{o}$C for 30 seconds and extension at 72$^{o}$C for 30 seconds in the 33 cycles and for 7 minutes during the last cycle. For PCR fragment analysis, 5 μl of PCR product was mixed with loading dye (Appendix C) and separated on a 2% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc$^{TM}$ XR+ system (BioRad Laboratories, USA). The optimal condition was judged by presence of the 762-bp amplified product with or without non-specific one(s) in SEA α-thalassemia 1 and 391-bp amplified product of the internal control.

**C. Singleplex allele-specific PCR for Hb Constant Spring (HbCS)**

The singleplex allele-specific PCR for HbCS was that modified from the original protocol described elsewhere (Fucharoen, *et al* 2003). The modification included re-designing the CS-2 primer with the 3'-end mismatch in order to increase specificity and re-optimization of amount of αG-17 primer. (Table 4)

This PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$ , 0.4 μM each of the CS-2 and C3 primers, 0.016, 0.025, 0.034 and 0.04 μM αG-17 primer, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 250 ng DNA template. The amplification reaction was carried out in 35 thermal cycles comprising denaturation at 95$^{o}$C for 5 minutes in the first cycle and for 1 minute in subsequent 33 cycles, primer annealing at 60$^{o}$C for 30 seconds and extension at 72$^{o}$C for 1 minute 30 seconds in the 33 cycles and for 5 minutes during the last cycle. For PCR fragment analysis, 5 μl of PCR product was mixed with loading dye (Appendix C) and separated on the 2% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc$^{TM}$ XR+ system (BioRad Laboratories, USA). The optimal condition was determined by presence of the 180-bp amplified product with or without non-specific one(s) and the 391-bp internal control amplified product.

**D. Singleplex allele-specific PCR for α-thalassemia 2 (3.7-kb deletion)**

This amplification protocol was modified from the original version established elsewhere in order to increase yields (Fucharoen, *et al* 2002). The initial modifications ultimately comprised re-optimization of $MgCl_2$, betaine as PCR facilitator. Subsequently, the αG-17 and C3 primers served as internal control primers were added into the reaction and optimal numbers of thermal cycle determined.

In the initial modification, the PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 1, 2, 3 and 4 mM $MgCl_2$, 1.2 μM each of Alpha 3.7A and Alpha 3.7B primers, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.), 0.5, 1, 2, 3 M betaine and 250 ng DNA template. The amplification reaction was carried out in 22 thermal cycles comprising denaturation at 94°C for 6 minutes 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes in the first cycle. The subsequent 20 cycles were composed of denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes 20 seconds. The last cycle then comprised denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 7 minutes. Five μl PCR product was mixed with loading dye (Appendix C) and run on a 1.5% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The optimal condition was judged by presence of the 1,671-bp amplified product with or without non-specific one(s).

Subsequently, the PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, $MgCl_2$ (optimized quantity), 1.2 μM each of Alpha 3.7A and Alpha 3.7B primers, 0.025 μM αG-17 and 0.4 μM C3 primers, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.), betaine (optimized quantity) and 250 ng DNA template. The amplification reaction was carried out in 22, 32 thermal cycles comprising denaturation at 94°C for 6 minutes 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes in the first cycle. The subsequent 20, 30 cycles were composed of denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes 20 seconds. The last cycle then comprised denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 7 minutes. Five μl PCR product was mixed with loading dye (Appendix C) and run on a 1.5% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The optimal condition was judged by presence of the 1,671-bp amplified product in α-thalassemia 2 (3.7 kb deletion), 391-bp and 280-bp internal control products.

**Table 2.2** "Checker board" pattern used in optimization of oligonucleotide primers for α-thalassemia/hemoglobinopathies

| SEA1 / SEA3 | 0.25 µl (0.035 µM) | 0.5 µl (0.07 µM) | 0.75 µl (0.105 µM) | 1 µl (0.14 µM) | 1.25 µl (0.175 µM) |
|---|---|---|---|---|---|
| 0.25 µl (0.035 µM) | | | | | |
| 0.5 µl (0.07 µM) | | | | | |
| 0.75 µl (0.105 µM) | | | | | |
| 1 µl (0.14 µM) | | | | | |
| 1.25 µl (0.175 µM) | | | | | |

**2.2.1.4 Development and optimization of in-house multiplex allele-specific PCR**

After the singleplex allele-specific PCR protocols were optimized successfully, the multiplex allele-specific PCR were then developed. The development was initially performed by following the conditions set up in the singleplex allele-specific PCR. However, two in-house multiplex allele-specific PCR were developed in this thesis. These included the multiplex allele-specific PCR for both common α- and β-

thalassemia/hemoglobinopathies. The first protocol was proposed to detect SEA-α thalassemia 1, α-thalassemia 2 (3.7 kb deletion) and HbCS. The second protocol was aimed to indentify HbE, $\beta^{17(A-T)}$, $\beta^{41/42(-TTCT)}$ and $\beta^{-28(A-G)}$ simultaneously.

**A. In-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy**

Two steps of optimization were performed. In the first step, the SEA-specific primers were added into the PCR reaction optimized initially for the 3.7-kb deletion of the α-thalassemia 2. The second step was carried out after the first-step optimization gave satisfactory results. In the second step, a HbCS-specific oligonucletide primer was fortified.

In the first step, two optimization procedures were carried out regarding the amounts of SEA1 and SEA3 primers. The first procedure utilized the final concentration of these two primers optimized for the singleplex PCR. The second procedure utilized the final concentration of 0.4 pmol/μl. The PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 1 mM $MgCl_2$, 1.2 μM each of Alpha 3.7A and Alpha 3.7B primers, SEA1 and SEA3 primers (the final concentrations as optimized in the Section 2.2.1.3 A), 0.4 pmol/μl C3 primers, 0.025 pmol/μl αG-17, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.), 2 M betaine and 250 ng DNA template. The amplification reaction was carried out in 32 thermal cycles comprising denaturation at 94°C for 6 minutes 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes in the first cycle. The subsequent 30 cycles were composed of denaturation at 94°C for 1 minute 30 seconds,

primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes 20 seconds. The last cycle then comprised denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 7 minutes. Five μl PCR product was mixed with loading dye (Appendix C) and run on a 1.5% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The optimal condition was judged by presence of the 1,671-bp amplified product in α-thalassemia 2 (3.7 kb deletion), 762-bp amplified product in SEA α-thalassemia 1 and 391-bp internal control products.

For the second step of optimization, the reaction volume was increased to 50 μl as more ingradient was added. The optimization procedure involved titration of amount of $MgCl_2$ and numbers of thermal cycles. However, the amount of CS-2 primer to be used was the same as that previously optimized in the singleplex PCR (Section 2.2.1.3 B). The PCR was performed in a total volume of 50 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 1, 1.5, 2, 2.5, 3 and 3.5 mM $MgCl_2$, 1.2 μM each of Alpha 3.7A and Alpha 3.7B primers, SEA1 and SEA3 primers (the final concentrations as optimized in the Section 2.2.1.3 A), 0.4 μM each of CS-2 and C3 primers, 0.025 μM αG-17 primer, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.), 2 M betaine and 250 ng DNA template. The amplification reactions were carried out in 32, 31, 30, 29, 28 and 27 thermal cycles, each of which comprising denaturation at 94°C for 6 minutes 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at

68°C for 2 minutes in the first cycle. The subsequent 30, 29, 28, 27, 26, and 25 cycles were composed of denaturation at 94°C for 1 minutes 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 2 minutes 20 seconds. The last cycle then comprised denaturation at 94°C for 1 minute 30 seconds, primer annealing at 60°C for 1 minute 30 seconds and extension at 68°C for 7 minutes. Five μl PCR product was mixed with loading dye (Appendix C) and run on a 1.5% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The optimal condition was judged by presence of the 1,671-bp amplified product in α-thalassemia 2 (3.7 kb deletion), 762-bp amplified product in SEA α-thalassemia 1, 180-bp amplified product in HbCS and 391-bp internal control products.

**B. In-house multiplex allele-specific PCR for β-thalassemia and hemoglobinopathy**

This multiplex allele-specific PCR was a modification of that developed previously in Dr. Thanusak's laboratory (Suwannasin 2009). This modification consisted of the addition of internal control primers (αG-17 and C3) and M-28M1 primer into the original PCR mix. Then, the optimal conditions of β-globin mutation primers, $MgCl_2$ and the thermal cycles were determined.

The final concentration of M-28M1 primer was firstly titrated, followed respectively by that of "beta41/42 multiplex" and that of "beta 17 multiplex" primers.

After the concentrations of primer were optimized, the optimal final concentration of $MgCl_2$ was determined. Finally, the appropriate thermal cycles were also searched for.

The PCR was performed in a total volume of 25 μl containing 10 mM Tris pH 8.5, 50 mM KCl, 2, 2.5, 3, 3.5 and 4 mM $MgCl_2$, 0.4 μM each of αG-17 and C3 primers, 0.032 μM "beta common multiplex" primer, 0.018, 0.027, 0.036, 0.045 and 0.054 μM "beta 41/42 multiplex" primer, 0.018, 0.027, 0.036, 0.045 and 0.054 μM "beta17 multiplex" primer, 0.07, 0.14, 0.28, 0.56 μM M-28M1 primer and 0.018 μM "beta E multiplex" primers, 140 μM dNTPs, 2% DMSO, 0.1 units Taq-DNA polymerase (iTaq; iNtRON Biotechnology, Inc.), and 250 ng DNA template. The amplification reaction was carried out in 37, 32, 27, 26, 25, 24, 23 and 22 thermal cycles comprising denaturation at 95$^{o}$C for 5 minutes in the first cycle and for 1 minute in subsequent 35, 30, 25, 24, 23, 22, 21 and 20 cycles, primer annealing at 62$^{o}$C for 30 seconds and extension at 72$^{o}$C for 30 seconds in the 35, 30, 25, 24, 23, 22, 21, and 20 cycles and for 7 minutes during the last cycle. For PCR fragment analysis, 5 μl of PCR product was mixed with loading dye (Appendix C) and run on a 2% agarose gel in 0.5x TBE buffer (Appendix C) and then stained with ethidium bromide (Appendix C) for 10 minutes. Finally, the PCR product was visualized by Molecular Imager® Gel Doc$^{TM}$ XR+ system (BioRad Laboratories, USA). The success of this optimization was based on the presence of specific amplified products for $\beta^{41/42(-TTCT)}$ (469 bp), $\beta^{17(A-T)}$ (267 bp) , $\beta^{-28\ (A-G)}$ (156 bp) and HbE (290 bp) as well as those for an internal control (391 bp).

**2.2.1.5 Evaluation of efficiency of the developed in-house multiplex allele-specific PCR**

The developed in-house multiplex allele-specific PCR protocols for both α- and β-thalassemia/hemoglobinopathy were evaluated for their efficiencies in unknown samples. The results obtained by these developed PCR protocols were subsequently compared to those generated by the standard nucleotide sequencing.

**2.2.1.5.1 Identifications of globin gene mutation in unknown samples**

The venous blood samples were collected from 70 anonymous subjects using EDTA as an anticoagulant. Genomic DNA was prepared from these blood samples using the Chelex™ method described previously in Section 2.2.1.1. The identifications of mutations in the α- and β-globin genes were performed using the developed in-house multiplex allele-specific PCR optimized previously in Section 2.2.1.4. This thesis was ethically approved by The Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Approval notice number 118/2555).

**2.2.1.5.2 Confirmation of accuracy of detecting globin gene mutations in unknown samples**

To determine accuracy of the developed in-house multiplex allele-specific PCR in accurate identification of the globin gene mutation in the unknown samples, the nuclectide sequencing of the amplified products was performed. These involved 4 steps;

amplification of the interested regions, purification amplified products, extension reaction and automated nucleotide sequencing.

### 3.2 Amplification of the interested regions

For SEA-α thalassemia 1, α-thalassemia 2 (3.7-kb deletion) and HbCS, the developed in-house multiplex allele-specific PCR was employed employing all conditions that had been optimized. The specific amplified products of the SEA-α thalassemia 1 (762 bp), the α-thalassemia 2 (3.7-kb deletion) (1,671 bp) and the HbCS (180 bp) as shown in Figure 8 (A) were to be collected for subsequent steps.

For β-thalassemia, the whole β-globin gene was amplified following the protocol described elsewhere (Sirichotiyakul, *et al* 2003). Briefly, the PCR was performed in a total volume of 50 μl containing 5 μl DNA, 0.4 μM each of A primer (5'-CCA ACT CCT AAG CCA GTG CC-3') and B primer (5'-TGC AAT CAT TCG TCT GTT TCC C-3') (Figure 9), 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 140 μM dNTPs, 2% DMSO, 0.1 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.). The amplification reaction was carried out in 35 thermal cycles comprising denaturation at 95$^{o}$C for 5 minutes in the first cycle and for 1 minute in subsequent 33 cycles, primer annealing at 60$^{o}$C for 30 seconds and extension at 72$^{o}$C for 1 minute 30 seconds in the 33 cycles and for 7 minutes during the last cycle. The PCR products were separated in the agarose gel electrophoresis and visualized under the Molecular Imager® Gel Doc$^{TM}$ XR+ system (BioRad Laboratories, USA). The amplified product was the 780-bp fragments

spanning nucleotides 61980-62751 according to GenBank access code HUMHBB (Figure 2.3).

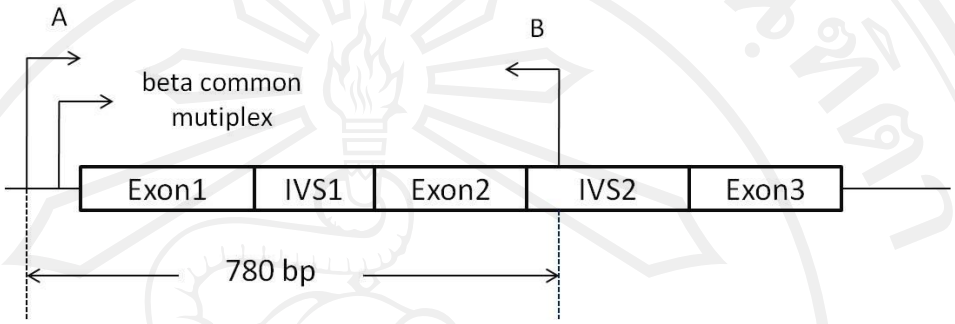


**Figure 2.3** Locations of PCR primers (A, B) and sequencing primer (beta common multiplex) used for β-globin gene sequencing

### 3.3 Purification of amplified products

The amplified products were cleaned up by using the NucleoSpin®Extract II (MACHEREY-NAGEL GmbH & Co.KG (Germany)). Briefly, the specific DNA band was excised from agarose gel and gel slices were solubilized in Buffer NTI (200 µl Buffer NTI per 100 mg agarose gel slices). The mixture was then incubated at 50°C for 5-10 minutes with intermittent vortexing at every 2-3 minutes until the gel slices were completely dissolved. Subsequently, the column was placed into a collection tube, dissolved gel loaded, centrifuged at 11,000g for 30 seconds and flow-through discarded. Thereafter, 700-µl Buffer NT3 was dropped into the column, centrifuged at 11,000g for 30 seconds, flow-through discarded and placed back into the collecting tube. The column was re-centrifuged at 11,000 g for 1 minute to remove Buffer NT3 completely. Finally, the column was placed into a new 1.5-mL microcentrifuge tube, 15-30 µl Buffer NE

added, incubated for 1 minute at room temperature, centrifuged at 11,000 g for 1 minute, flow-through collected and stored at -20°C until use.

### 3.4 Extension or sequencing reaction

The extension reactions for α- and β-globin gene specific sequences were not performed in this thesis. However, the extension reactions followed the standard dideoxy chain termination method of Sanger using the ABI Prism®BigDye terminator ready reaction mix. The forward PCR primers were utilized as the sequencing primers for SEA-α thalassemia 1, α-thalassemia 2 (3.7-kb deletion) and HbCS (Figure 2.2). In contrast, the "beta common multiplex" primer was used as the sequencing primer for the β-globin gene (Figure 9). In general, the extension reaction should be carried out under the so-called "the thermal cycling sequencing reaction" technique. In this reaction, the 20 ul amplification reaction was performed consisting 0.4 µM SEA1 for SEA α-thalassemia 1 or 1.2 µM Alpha 3.7A for α-thalassemia 2 (3.7 kb deletion) or 0.4 µM αG-17 for HbCS, 8 µl ABI Prism®BigDye terminator ready reaction mix, 2 µl 5XQ-solution and 10-100 ng of cleaned PCR products. The amplification reaction was carried out in 26 thermal cycles comprising denaturation at 95°C for 2 minutes in the first cycle and 98°C for 30 seconds in subsequent 25 cycles, primer annealing at 50°C for 30 seconds and extension at 60°C for 4 minutes.

### 3.5 Automated nucleotide sequencing

The automated nucleotide sequencing for α- and β-globin gene specific sequences also were not performed in this thesis. However, the nucleotide sequencing was based on separation of DNA fragments in the polyacrylamide gel electrophoresis (PAGE) which is

capable of separation 1-bp difference of the DNA. The fluorescent signals were detected by the detector installed in the automated sequencing machine, from which the nucleotide sequences was generated using the specific software.

In general practice, the nucleotide sequencing using the automated sequencing machine should be undertaken under the following procedure. The extension products were purified by ethanol/sodium acetate precipitation method. The extension product was transferred into a microcentrifuge tube containing 14.5 µl DI, 62.5 µl absolute ethanol and 3 µl of 3M sodium acetate added, mixed by vortex mixer and left at room temperature for 15 min. The mixtures were then centrifuged 10,000 rpm for 20 minutes and supernatant discarded. The pallet was washed with 250 µl of 70% ethanol, centrifuged at 10,000 rpm for 10 minutes and supernatant discarded. The pallet was dried in the heat box at 90$^{o}$C for 1 minute. The precipitated extension products were re-suspended in 25 µl of template suspension reagent (TSR; ABI Prism®BigDye™) before separating the extension fragments by the ABI Prism®Genetic analyzer (Applied Biosystems). The obtained nucleotide sequences were analyzed by the Chromaslite 2.01 software available free of charge from the internet (http://www.softpedia.com/get/Science-CAD/Chromas-Lite.shtml).

**2.2.2 Development of whole blood PCR protocols for identifying globin gene mutations**

Developement of the whole-blooded PCR involved 4 steps; 1) determining optimal thermal cycling pattern and suitable form of blood samples, 2) searching for the best PCR facilitators, 3) determining appropriate volume of blood samples used in the whole blood PCR, and 4) applying the developed technique in detecting the carriers of α- and β-thalassemia and hemoglobinopathies common in Thailand.

**2.2.2.1 Determining optimal thermal cycling pattern and suitable form of blood samples to be used in the PCR reaction**

Heating up the reaction leads to release of genomic DNA from the leukocytes (Jadaon, *et al* 2009, Mercier, *et al* 1990). Thus, extra repeating heat-cool steps was added prior to the typical PCR cycles set for PCR protocol for Gγ-promoter routinely employed in Dr. Thanusak's laboratory and the condition optimized. As 2 forms of whole blood samples may be encountered in the laboratory; e.g. blood lysate and fresh blood were tested. Three repeating extra heat-cool steps were initially tested for each type of blood samples used. In case of failure for three heat-cool steps, five and ten heat-cool steps were evaluated.

The 50-µl PCR reaction contained 0.4 µM 5'-GG-1 primer (5'-AAC TGT TGC TTT ATA GGA TTT TTC A -3'), 0.4 µM 3'-AG-1 primer (5'-GTC TGG ACT AGG AGC TTA TTG AT-3'), 11.7% (w/v) betaine, 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 0.05 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.)

and 2 μl whole EDTA blood. The reaction was heated for 3 minute at 94°C then cooled for 3 minute at 55°C and 3, 5 and 10 times repeating heat-cool steps were tested. Thereafter, an initial 5 minute denaturation at 95°C, followed by 33 cycles of 95°C denaturation for 1 minute, 60°C annealing for 30 seconds, and 72°C extension for 90 seconds. A final 5 minute extension at 72°C completed the reaction. 5 μl of each amplified product was analyzed by 1.5% agarose gel electrophoresis and visualized under the Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The expected amplicon size was 665 bp. The number of repeating extra heat-cool steps resulting in clear 665-bp amplicons was then chosen.

**2.2.2.2 Search for the best PCR facilitators**

Betaine and BSA were optimized in this study. The optimization was also based on the PCR reaction for Gγ-promoter routinely employed in Dr. Thanusak's laboratory. The facilitators were directly added into the reaction; either alone or in combination. The optimal amount of BSA between 0.2-1% (w/v), betaine between 5-15% (w/v) were determined by titration. The effect of betaine and BSA in combination was also evaluated. The 50-μl PCR reaction contained 0.4 μM 5'-GG-1 primer, 0.4 μM 3'-AG-1 primer, 0.2-1% (w/v) BSA or 5-15% (w/v) betaine, 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μM dNTPs, 0.05 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and 2 μl whole EDTA blood. The amplification took place under the thermal cycles optimized in the previous section. The amount of BSA or betaine resulting in clear 665-bp amplicons was then chosen.

**2.2.2.3 Determining appropriate volume of blood samples used in the whole blood PCR**

As heme has been shown to be inhibitor of the PCR reaction (Al-Soud and Radstrom 2000, Al-Soud and Radstrom 2001, Radstrom, *et al* 2004). Thus, optimal amount of whole blood to be utilized in the PCR reaction was the determined in the PCR protocol for Gγ-promoter set up in Dr. Thanusak's laboratory. The 50-µl PCR reaction contained 0.4 µM 5'-GG-1 primer, 0.4 µM 3'-AG-1 primer, the best PCR facilitator found in previous section, 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 0.05 units DNA polymerase (iTaq; iNtRON Biotechnology, Inc.). Whole EDTA blood (0.5, 1, 1.5 and 2 µl) was titrated in this PCR reaction. The amplification took place under the thermal cycles optimized in the previous section. The volume of whole EDTA blood resulting in clear 665-bp amplicons was then chosen.

**2.2.2.4 Applying the developed technique in detecting the carriers of α- and β-thalassemia and hemoglobinopathies common in Thailand**

To detect the globin gene mutations endemic in Thailand using the developed whole-blooded PCR, the optimized singleplex allele-specific PCR for each mutations were modified as shall be seen in the following parts.

**A. SEA-α thalassemia 1**

The PCR protocol employed was that described previously with primer quatities slightly modified (Suwannasin 2009, Tatu, *et al* 2012). The consecutive titration of SEA1 primer, followed by SEA2 and SEA3 primers was carried out. The total volume of PCR

reaction mixture was 25 µl containing 0.04, 0.07, 0.10 and 0.14 µM SEA1 primer, 0.028, 0.042, 0.056 and 0.07 µM SEA2 primer and 0.04, 0.07, 0.10 and 0.14 µM SEA3 primer (Table 4), 1x iProof GC buffer, 1.5 mM $MgCl_2$, 140 µM dNTPs, 2% DMSO, the best PCR facilitator found in section 2.2.2.2, 0.02 units iProof DNA polymerase (iProof; Bio-Rad, Hercules, CA) and fresh whole blood (the amount as optimized in Section 2.2.2.3). The reaction was heated for 3 minute at 94°C, then cooled for 3 minute at 55°C and this was repeated (number of repeated cycles as optimized in Section 2.2.2.1). Subseqeuently, the thermal cycles comprised 5-minute denaturation at 95°C, followed by 25 cycles of 95°C denaturation for 1 minute, 62°C annealing for 30 seconds and 72°C extension for 30 seconds. A final 5- minute extension at 72°C completed the reaction. 5 µl of each amplified product was analyzed by 2% agarose gel containing ethidium bromide (Appendix C) and were visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The expected amplicons were the 652-bp fragments generated from SEA1+SEA2 primers and the 762-bp products generated from SEA1+SEA3 primers.

**B. Hb Constant Spring**

The PCR protocol was slightly modified from that optimized in the singleplex protocol. The total volume of PCR reaction mixture was 25 µl containing 0.1, 0.2, 0.3 and 0.4 µM CS-2 primer, 0.025, 0.03, 0.04 and 0.05 µM αG-17 primer and 0.4 µM C3 primer (Table 4), 10 mM Tris pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, 2% DMSO, the best PCR facilitator found in section 2.2.2.2, 0.1 units DNA polymerase

(iTaq; iNtRON Biotechnology, Inc.) and fresh whole blood (the amount as optimized in Section 2.2.2.3). The reaction was heated for 3 minute at 94°C, then cooled for 3 minute at 55°C and this was repeated (number of repeated cycles as optimized in Section 2.2.2.1). Subseqeuently, the thermal cycles comprised 5 minute denaturation at 95°C, followed by 30 cycles of 95°C denaturation for 1 minute, 60°C annealing for 30 seconds, and 72°C extension for 1 minute. A final 5 minute extension at 72°C completed the reaction. 5 µl of each amplified product was analyzed by 2% agarose gel containing ethidium bromide (Appendix C) and were visualized by Molecular Imager® Gel Doc™ XR+ system (BioRad Laboratories, USA). The expected amplicons were the 180-bp fragments generated from CS2+C3 primers and the 391-bp products generated from αG-17+C3 primers.

**C. β-thalassemia ($\beta^{17}$, $\beta^{41/42}$, $\beta^{-28}$, HbE)**

The PCR protocol was that optimized for the multiplex in-house allele specific PCR for β-thalassemia described in Section 2.2.1.4. However, for the best result, the thermal cycles and the HbE specific primer ("beta E multiplex" primer) were titrated. The 25 µl-PCR reaction contained 0.032 µM "beta-common multiplex", 0.054 µM "beta41/42 multiplex", 0.054 µM "beta17 multiplex", 0.14 µM M-28M1, 0.025 µM αG-17 and 0.4 µM C3 and 0.0036, 0.0072, 0.0108, 0.0144 and 0.018 µM "beta E multiplex" primers in 10 mM Tris pH 8.5, 50 mM KCl, 4 mM $MgCl_2$, 140µM dNTPs, 2% DMSO, the best PCR facilitator found in section 2.2.2.2, 0.1 unit DNA polymerase (iTaq; iNtRON Biotechnology, Inc.) and fresh whole blood (the amount as optimized in Section 2.2.2.3).

The reaction was heated for 3 minute at 94°C, then cooled for 3 minute at 55°C and this was repeated (number of repeated cycles as optimized in Section 2.2.2.1). Subseqeuently, the thermal cycles comprised initial denaturation at 95$^{o}$C for 5 minutes and for 1 minute in subsequent 25 and 24 cycles, primer annealing at 62$^{o}$C for 30 seconds and extension at 72$^{o}$C for 30 seconds and final extension was prolonged to 7 minutes. 5 µl of each amplified product was analyzed by 2% agarose gel containing ethidium bromide (Appendix C) and were visualized by Molecular Imager® Gel Doc$^{TM}$ XR+ system (BioRad Laboratories, USA). The expected amplicons were the 391-bp products generated from αG-17 + C3 primers, the 267-bp products from beta-common multiplex + beta17 multiplex primers, the 469-bp products from beta-common multiplex + beta41/42 multiplex primers, the 156-bp products from beta-common multiplex + M-28M1 primers and the 290-bp fragments from beta-common multiplex + beta E multiplex primers.