



## **CHAPTER II**

### **MATERIALS AND METHODS**

#### **2.1 Materials**

**2.1.1 Chemicals and instruments used in this study are indicated in the appendix**

#### **2.1.1 Human DNA and blood samples**

DNA and blood samples used in this study were kindly provided by Assoc. Prof. Dr. Thanusak Tatu, the Hematology and Health Technology Research Center, Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University.

#### **2.2 Methods**

##### **2.2.1 Study of molecular background of silent $\alpha$ -thalassemia**

##### **2.2.1.1 phenol/chloroform DNA preparation**

The genomic DNA was isolated from peripheral blood leukocyte using standard phenol/chloroform technique as described (Sambrook Joseph 2001). EDTA-blood samples were centrifuged at 3,000 rpm for 10 min. After plasma layer was discarded,

200  $\mu$ l of buffy coat containing leukocyte was carefully aspirated and transferred to new microcentrifuge tube, followed by the addition of 1,000  $\mu$ l of 0.5% Non-idet P-40. The tube was then mixed vigorously, centrifuged at 5,000 rpm for 5 min and supernatant discarded. The entire washing step was repeated for 3 times or until clear supernatant was obtained and white WBC pellet was observed at the bottom of the microcentrifuge tube. The lysis step commenced with the addition of 500  $\mu$ l of lysis buffer, 25  $\mu$ l of 10% SDS and 20  $\mu$ l of 5 mg/ml proteinase K to the WBC pellet, followed by overnight-incubation at 37°C. After complete cell lysis was observed, the phenol/chloroform DNA extraction started with the addition, to the cell lysis mixture, of 100  $\mu$ l of 5X ANE buffer, 300  $\mu$ l of saturated phenol and 300  $\mu$ l of chloroform. The mixture was then vigorously shaken, spun for 5 min at 5,000 rpm and double layers of solution was obtained. The lower layer containing organic solution was then carefully aspirated and discarded, leaving the colorless upper layer inside the tube. Then 2 subsequent phenol/chloroform extractions were carried out to ensure complete DNA enrichment as well as removal of cellular proteins. After the second phenol/chloroform extraction, 1,000  $\mu$ l of chloroform was added to the solution, the tube vigorous shaken, spun at 5,000 rpm for 5 min and supernatant carefully aspirated into a new clean microcentrifuge tube. The DNA was precipitated, using the salting out principle, by adding 50  $\mu$ l of 3M sodium acetate, 1,000  $\mu$ l of deeply cold absolute ethanol followed by gentle end-over-end mixing and white DNA thread formed. However, but no DNA thread was seen, the solution must be kept in -20°C for 30 min or overnight to enhance DNA precipitation. The DNA was enriched by centrifugation at 10,000 rpm for 10 min, the supernatant completely discarded and DNA

pellet washed with 1,000  $\mu\text{l}$  of 70% ethanol to remove excess phenol/chloroform. After washing with 70% ethanol, the DNA pellet was air-dried at room temperature in the safety cabinet for overnight. The dried DNA pellet was dissolved with 50  $\mu\text{l}$  of TE buffer and its quantity was determined by measuring the absorbance at 260 nm. The DNA concentration was finally calculated using the following equation :

$$\text{DNA } (\mu\text{g/ml}) = \text{OD}_{260}/\text{ml} \times 50 \mu\text{g/ml of genomic DNA}$$

(Note: 1  $\text{OD}_{260}/\text{ml} = 50 \mu\text{g/ml}$  of genomic DNA)

#### **2.2.1.2 Chelex™ direct DNA extraction**

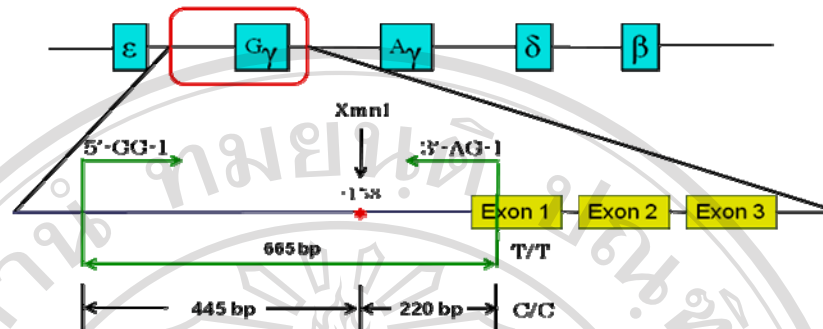
Chelex™ 100 is a chelating resin capable of binding  $\text{Mg}^{2+}$ , the metal ion essential for DNase action. The removal of  $\text{Mg}^{2+}$  then decreases the activity of this enzyme, preventing the genomic DNA from being degraded (Walsh, *et al* 1991).

The DNA was isolated from peripheral blood leukocyte. Practically, 200  $\mu\text{l}$  of whole EDTA blood or 40  $\mu\text{l}$  of buffycoat was washed with 1,000  $\mu\text{l}$  of 0.5% (v/v) Triton X-100 or lysis buffer to enrich white WBC pellet at the bottom of the tube which was subsequently washed once with 1-ml distilled water. The clean WBC pellet was then resuspended in 110- $\mu\text{l}$  distilled water, 1-2 drops of Chelex™-100 suspension added and incubated at 56°c for 2 hours or overnight. This incubation aimed to activate the Chelex™ resin to allow its maximum chelating activity. After 56°C-incubation, the tube was boiled in boiling water for 10 min, then mixed vigorously, centrifuged at 6,000 rpm for

1 min, supernatant, with DNA, transferred into new micro-centrifuge tube and stored at -20°C until use.

### 2.2.1.3 Detection of *Xmn*I- $\gamma$ polymorphism

A substitution of T for C at position -158 relative to the  $\gamma$ -globin gene cap site creates a cutting site for the restriction enzyme *Xmn*I (Gilman and Huisman 1985). The *Xmn*I- $\gamma$  site was detected by restriction analysis of specifically amplified PCR product of the  $\gamma$ -promoter region (Sampietro, *et al* 1992). The PCR was performed in a total volume of 25  $\mu$ l containing 50 ng of genomic DNA, 100  $\mu$ M of dNTPs, 1.25 units of Taq DNA polymerase (Fermentas), 0.4  $\mu$ M of primers [5'-GG-1 : 5'-AAC TGT TGC TTT ATA GGA TTT TTC A-3', 3'-AG-1 : 5'- GTC TGG ACT AGG AGC TTA TTG AT - 3'], as shown in Figure 2.1, in 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 2.5 mM MgCl<sub>2</sub>. A total of 35 cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primer annealing at 58°C for 2 min and primer extension at 72°C for 1 min. The initial denaturation time was extended to 5 min, while the final extension time was prolonged to 5 min. The 665-bp amplified products was produced and subsequently digested with the *Xmn*I restriction enzyme (NEW ENGLAND BioLabs) under the condition suggested by the manufacturer. The success digestion then yielded two digested fragments of 445 bp and 220 bp in the presence of *Xmn*I- $\gamma$  site.

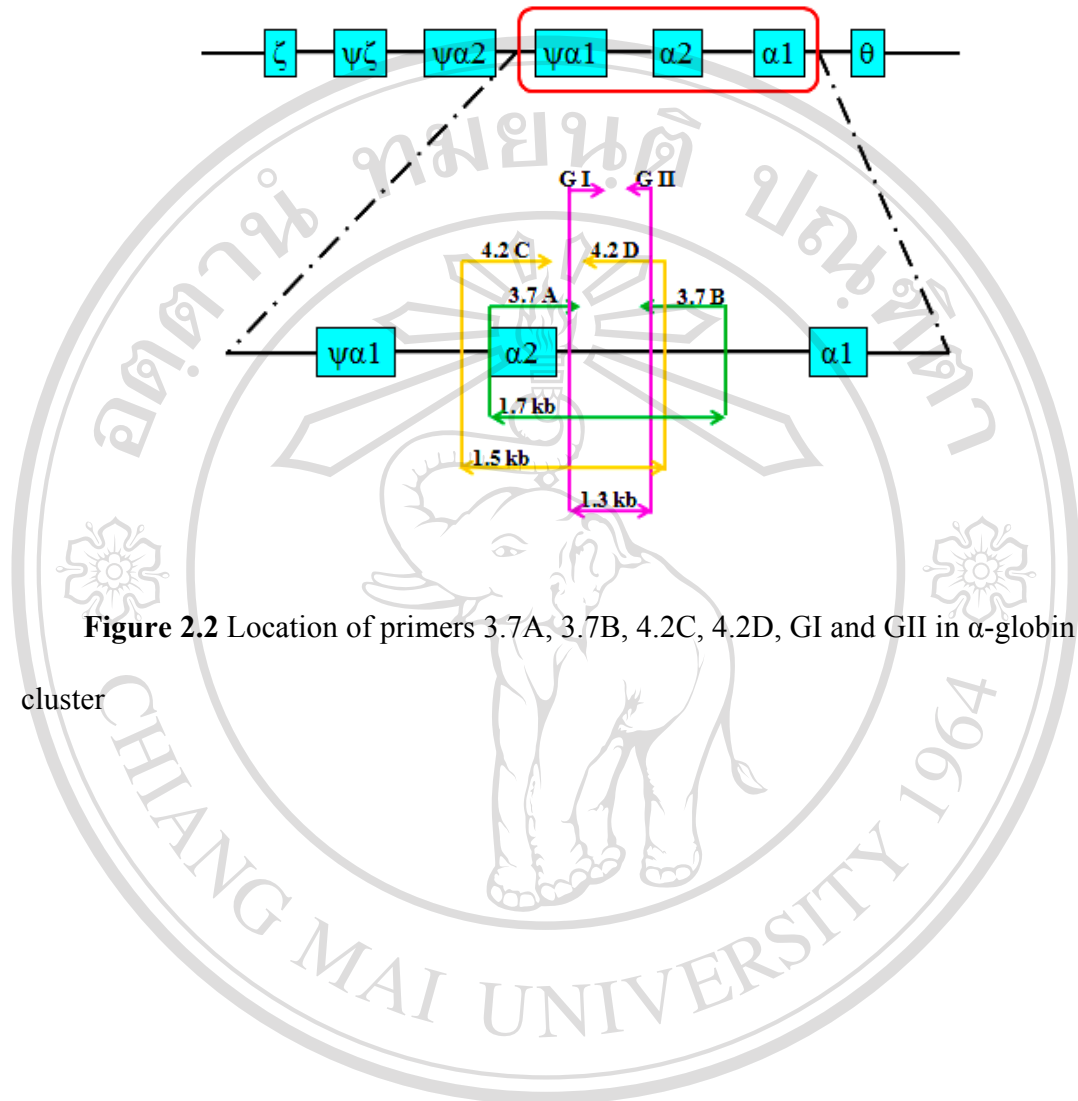


**Figure 2.1** Schematic picture of normal  $\gamma$ -globin gene cluster and  $XmnI$ - $G\gamma$  site. The binding position of 5'-GG-1 and 3'-AG-1 primers and  $XmnI$  cutting site are shown.

#### 2.2.1.4 Detection of $\alpha$ -thalassemia 2 (3.7-kb and 4.2-kb deletions)

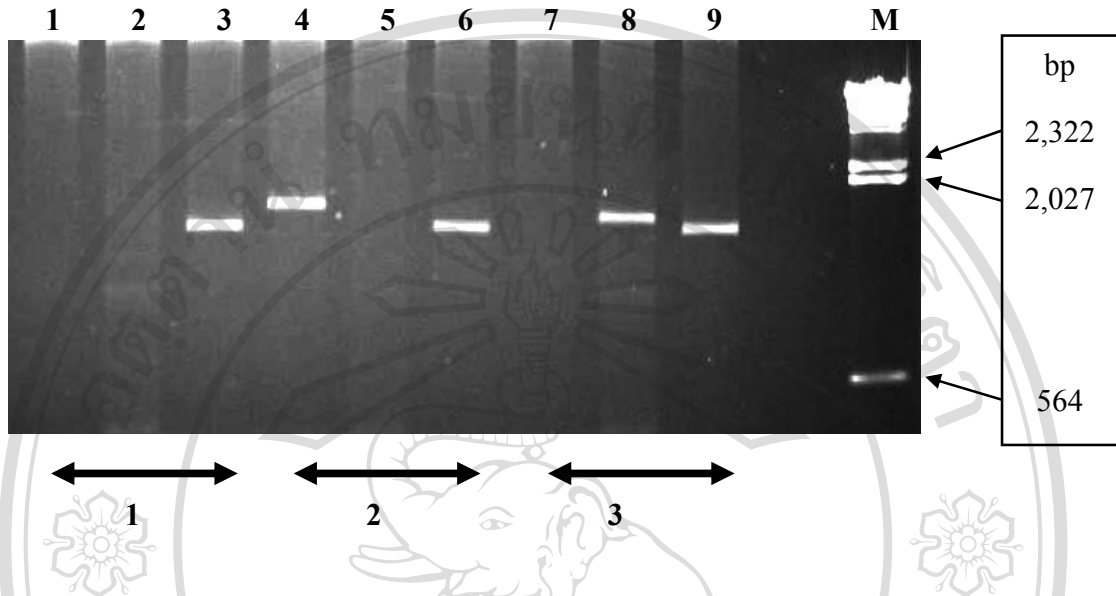
Genotyping of  $\alpha$ -thalassemia 2 with 3.7 kb and 4.2 kb deletion was carried out by PCR technique described elsewhere (Fucharoen, *et al* 2002). Technically, PCR was performed in three separate reactions; i.e. PCR for 3.7 kb deletion, PCR for 4.2 kb deletion and PCR for internal control, using the same PCR ingredients and PCR cycles. Primers used for identification of 3.7-kb deletion comprised **3.7A** : 5'-CCC AGA GCC AGG TTT GTT TAT CTG-3' and **3.7B** : 5'-GAG GCC CAA GGG GCA AGA AGC AT-3'. Primers for detecting 4.2-kb deletion were **4.2C** : 5'-GCT AGA GCA TTG GTG GTC ATG CC-3' and **4.2D** : 5'-TTC TGA CTC TGC CCA CAG CCT GA-3'. Primers for internal control consisted of **G1** : 5'-GCT GAC CTC CAA ATA CCG T-3' and **GII** : 5'-CTC TGC CTC CTT GGT TAA AA-3'. Location of each primer was shown in Figure 2.2. The PCR reaction for each primer pair was performed in a total volume of

25  $\mu$ l containing 200 ng of genomic DNA, 200  $\mu$ M of dNTPs, 1.5  $\mu$ M of each primer (3.7A + 3.7B, 4.2C + 4.2D, GI + GII), 1.5 units of DreamTaq™ DNA polymerase (Fermentas) in 75 mM Tris-HCl (pH 8.8), 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1% DMSO and 2.5 mM  $\text{MgCl}_2$ . A total of 22 cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1.30 min, primers annealing at 60°C for 1.30 min and primers extension at 68°C for 2 min. The initial denaturation time was extended to 5 min while the final extension time was prolonged to 7 min. A 1.7-kb amplified products were produced if 3.7-kb deletion was present, whereas 1.5-kb PCR products were generated in those having the 4.2-kb deletion. The 1.3-kb fragment generated from the primers GI + GII must be seen in all subjects to ensure the success of PCR reaction. The example of gel picture in this instance is shown in Figure 2.3.



**Figure 2.2** Location of primers 3.7A, 3.7B, 4.2C, 4.2D, G I and G II in  $\alpha$ -globin gene cluster

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**Figure 2.3** Agarose gel picture of amplified products for identifying the 3.7-kb and 4.2-kb deletions causing  $\alpha$ -thalassemia 2. Lanes 1, 2, 3 belong to sample No. 1, lanes 4, 5, 6 belong to sample No. 2 and lanes 7, 8, 9 belong to sample No. 3. Lanes 1, 4, 7 are products of primers 3.7A and 3.7B (1.7kb). Lanes 2, 5, 8 are products of primers 4.2C and 4.2D (1.5kb). Lanes 3, 6, 9 are products of primers GI and GII (1.3 kb). M represent Lamda HindIII fragment size standard. Note: sample No.1 was negative for 3.7 –kb and 4.2 –kb deletions. Samples No.2 and No. 3 were heterozygotes for 3.7 –kb deletion and 4.2 –kb deletion, respectively.

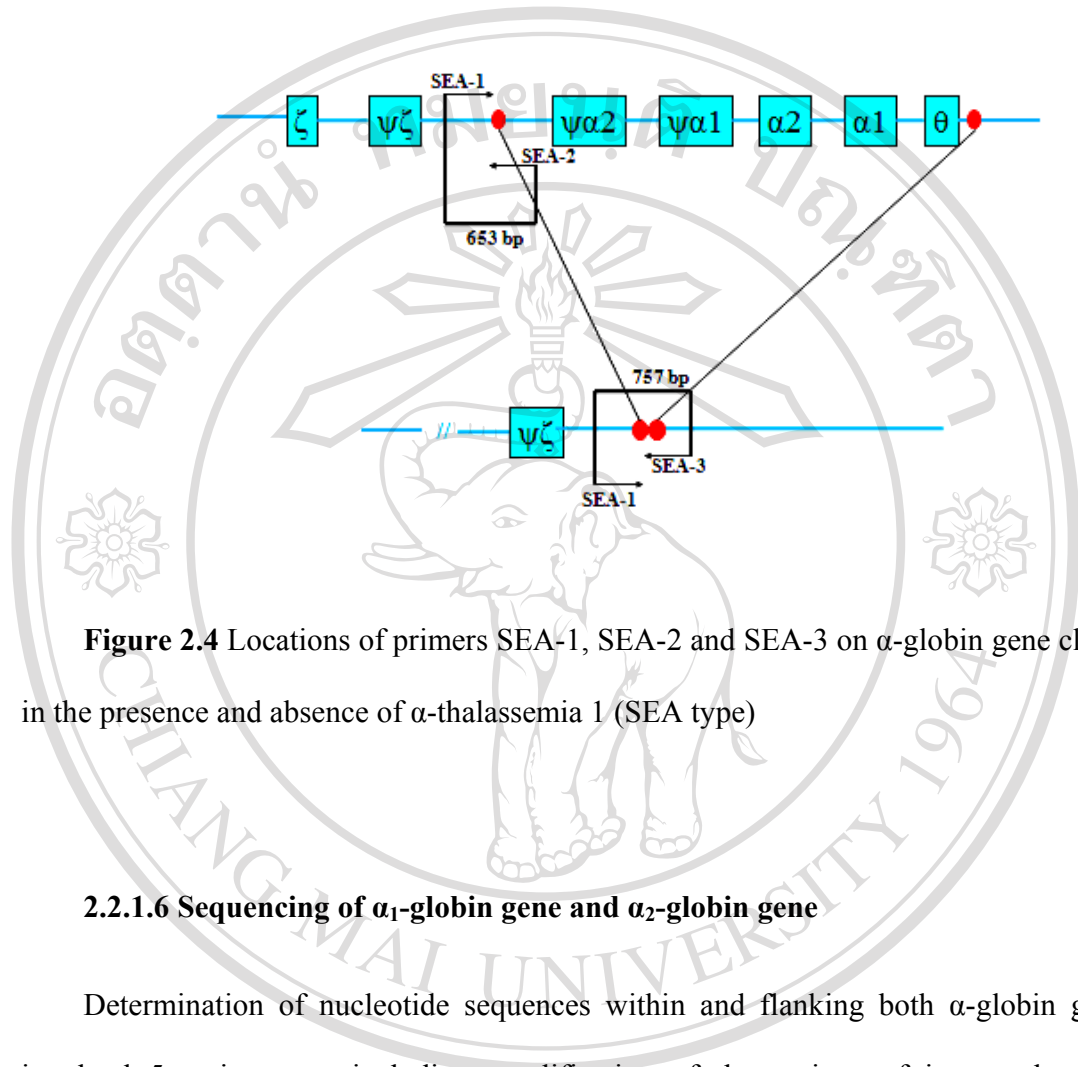
#### 2.2.1.5 Detection of $\alpha$ -thalassemia 1 (SEA type)

The so-called “Gap-PCR” utilizing a set of three primers (common primer “SEA-1” : 5’-TGA CTC CAA TAA ATG GAT GAG GA-3’; wild type specific “SEA-2” : 5’-GCC TGC GCC GGG GAA CGT AAC CA-3’ and SEA breakpoint specific “SEA-3” :



5'-CGC CAA AGA TGG CTA CTC GGA GA-3') was employed to identify  $\alpha$ -globin gene deletion causing  $\alpha$ -thalassemia 1 (SEA type). All three primers were newly designed using PCR primer designing software together with the reference nucleotide sequences of  $\alpha$ -globin gene cluster obtained from GenBank database (access code: AE006462). The location of each primer on the  $\alpha$ -globin gene cluster is shown in Figure 2.4. Validity of these three primers were successfully determined using known subject who is one of the parents of HbH disease.

The PCR was performed in a total volume of 25  $\mu$ l containing 500 ng of genomic DNA, 100  $\mu$ M of dNTPs, 2.5 units of i-Taq DNA polomerase (Intron), 0.1  $\mu$ M of SEA-1 and 0.07  $\mu$ M of each primers SEA-2 and SEA-3 in 10  $\mu$ M Tris-HCl (pH 8.3), 50 mM KCl, 2% DMSO and 2.5 mM MgCl<sub>2</sub>. A total of 40 cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primers annealing at 60°C for 1 min and primers extension at 72°C for 1 min. In the initial denaturation was extended to 5 min while the final extension was prolonged to 7 min. The 757-bp amplified product was produced in the presence of the  $\alpha$ -thalassemia 1 of SEA deletion, whereas the 653- bp fragment was obtained in those negative for SEA deletion.



**Figure 2.4** Locations of primers SEA-1, SEA-2 and SEA-3 on  $\alpha$ -globin gene cluster in the presence and absence of  $\alpha$ -thalassemia 1 (SEA type)

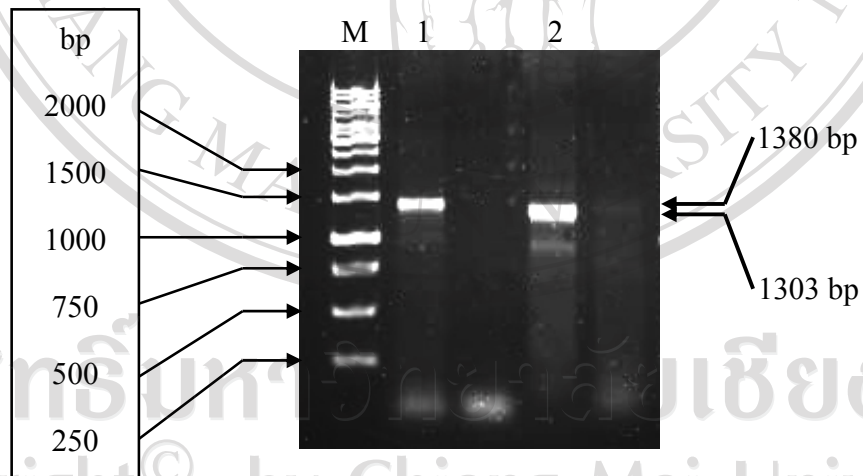
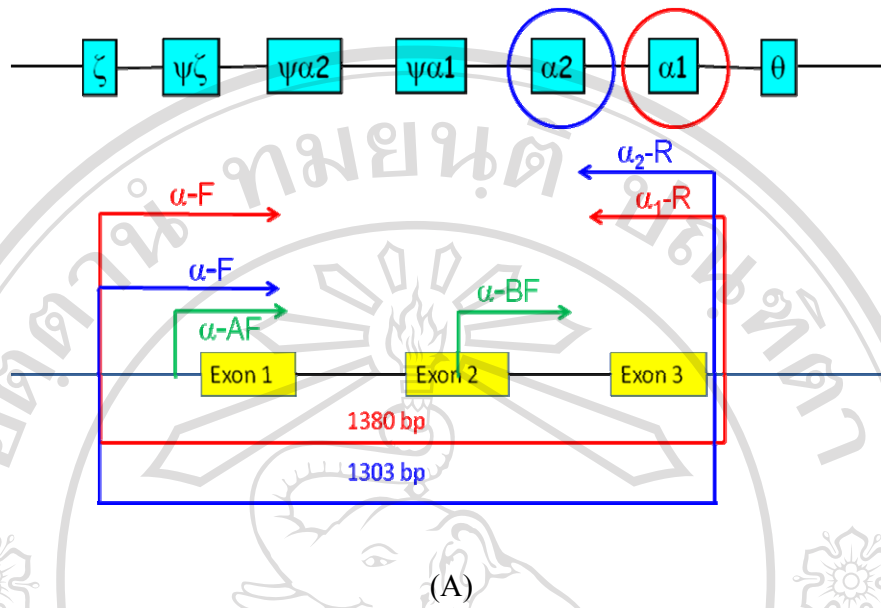
#### 2.2.1.6 Sequencing of $\alpha_1$ -globin gene and $\alpha_2$ -globin gene

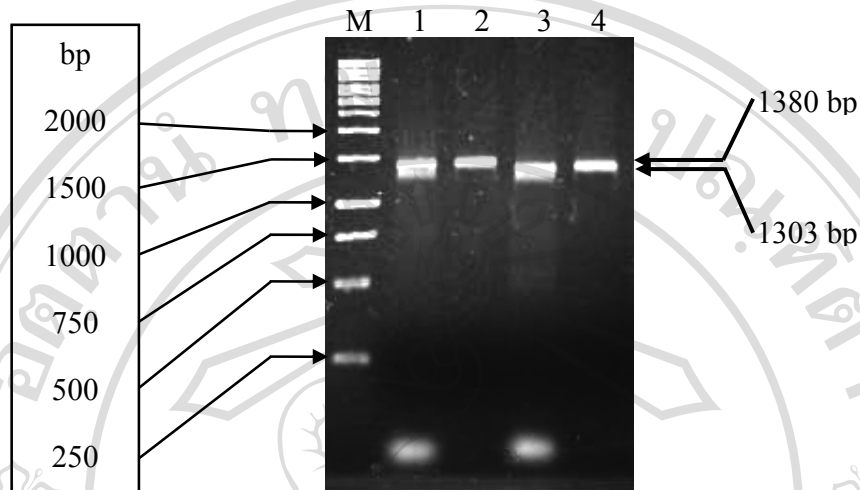
Determination of nucleotide sequences within and flanking both  $\alpha$ -globin genes involved 5 major steps including amplification of the regions of interest by PCR technique, cleaning up of PCR products, carrying out sequencing reaction, purification of extension products and automated sequence analysis (Sutcharitchan, *et al* 2005).

**Amplification of regions of interest** : Three oligonucleotide primers ( $\alpha$ -F : 5'-CGC GCA TTC CTC TCC GCC C-3',  $\alpha_1$ -R : 5'-ATG CCT GGC ACG TTT GCT GAG GGA A-3' and  $\alpha_2$ -R : 5'-AGA CCA GGA AGG GCC GGT G-3') were used for amplification, as shown in Figure 2.5 (A). Primer pair  $\alpha$ -F +  $\alpha_1$ -R were for  $\alpha_1$ -globin

gene while primers  $\alpha$ -F +  $\alpha_2$ -R were for  $\alpha_2$ -globin gene. Binding locations of each primer on  $\alpha$ -globin gene cluster are shown on Figure 2.5. Amplification was performed in a total volume of 50  $\mu$ l containing 200 ng of genomic DNA in 5 mM Tris-HCl (pH 8.8), 25 mM KCl, 2.5 mM  $MgSO_4$ , 100  $\mu$ M of dNTPs, 10% DMSO, 1.25 units of Pfu DNA polymerase (Fermentas) and 0.2  $\mu$ M of each primers in the set. A total of 37 cycles was carried out with each cycle comprising DNA denaturation at 98°C for 1 min, primer annealing at 58°C for 1 min and primers extension at 72°C for 1.30 min. While, initial denaturation was extended to 15 min and final extension was prolonged to 5 min.

PCR product were separated in 1.5% agarose gel electrophoresis and made visible with ethidium bromide and UV light from the Del Documentation System (BIO-RAD). The size of the DNA fragments were 1380 bp for  $\alpha_1$ -globin gene and 1303 bp for  $\alpha_2$ -globin gene as shown in Figure 2.5 (B).





(C)

**Figure 2.5** (A) Locations of PCR primers ( $\alpha$ -F,  $\alpha_1$ -R and  $\alpha_2$ -R) and sequencing primers ( $\alpha$ -AF and  $\alpha$ -BF) on  $\alpha$ -globin gene cluster. (B) Agarose gel pictures of PCR products of primers  $\alpha$ -F+ $\alpha_1$ -R (Lanes 1),  $\alpha$ -F+ $\alpha_2$ -R (Lane 2) with fragment size of 1,380 bp and 1,303 bp, respectively. (C) Agarose gel pictures of PCR products of primers  $\alpha$ -F+ $\alpha_1$ -R,  $\alpha$ -F+ $\alpha_2$ -R before (Lanes 1, 3) and after purification (Lanes 2, 4). M represented 1-kb DNA size standard.

**Cleaning-up of PCR product :** PCR products were cleaned up using the NucleoSpin<sup>®</sup>Extract II kit [MACHEREY-NAGEL GmbH & Co.KG (Germany)], both from agarose gel excisions and directly from the PCR tube. By using this protocol, contaminations such as salts and soluble macromolecular components are removed by a simple washing step leaving the DNA bound to silica membrane. The adsorbed DNA was then enriched by eluting out of the silica membrane by distilled water. The entire protocol was suggested by the manufacturer. Practically, take a clean scalpel to excise the DNA fragment from an agarose gel. The gel slice containing the fragment of interest was excised carefully to minimize the gel volume, weight determined and transferred into the new clean tube. 200 µl of Buffer NT was added to each 100 mg of agarose gel. The mixture was then incubated at 50°C for 5-10 min with intermittent vortexing until the gel slices are dissolved. Thereafter, a NucleoSpin<sup>®</sup>Extract II Column was placed into a 2-ml NucleoSpin Collecting Tube, sample loaded, centrifuged for 1 min at 11,000 g and flow-through discarded. Subsequently, the NucleoSpin<sup>®</sup>Extract II Column was placed back into the collecting tube, 600 µl of buffer NT3 added and centrifuged for 1 min at 11,000 g, flow-through discarded and placed back into the collecting tube. The centrifugation was repeated for 2 min at 11,000 g to remove the Buffer NT3 completely. Finally, the NucleoSpin<sup>®</sup>Extract II Column was placed into a clean 1.5-ml microcentrifuge tube, followed by addition of 15-50 µl of elution Buffer NE. The column soaked with Buffer NE was left at room temperature for 1 min to assure complete elution of DNA, i.e. to increase DNA yield. The column was finally centrifuged for

1 min at 11,000 g and the flow-through containing DNA fragments collected. The cleaned DNA fragments were then stored at  $-20^{\circ}\text{C}$  until use, as shown in Figure 2.5 (C).

**Sequencing reaction :** The sequencing reaction was carried out under the Sanger's Dideoxy Chain Termination principle using the commercial nucleotide sequencing kit. Two primers including  $\alpha$ -AF (5'-CCC CAA GCA TAA ACC CTG GC-3') and  $\alpha$ -BF (5'-GGC TCT GCC CAG GTT AAG GG-3') were used to sequence both  $\alpha$ -globin genes (Figure 2.5 (A) for their binding locations). The reaction was performed in a total volume of 20  $\mu\text{l}$  containing 8  $\mu\text{l}$  of ABI Prism<sup>®</sup> BigDye Terminator Ready Reaction Mix, 3.2  $\mu\text{M}$  of the  $\alpha$ -AF and  $\alpha$ -BF primers, 5 $\times$ Q-solution and 10-100 ng of cleaned PCR products. Deionized distilled water was added to make up the total volume to 20  $\mu\text{l}$ . The thermal cycles comprised initial DNA denaturation at  $95^{\circ}\text{C}$  for 2 min, followed by 25 cycles of denaturation at  $98^{\circ}\text{C}$  for 30 seconds, primer annealing at  $50^{\circ}\text{C}$  for 30 seconds and primer extension at  $60^{\circ}\text{C}$  for 4 min.

**Purification of extension products :** After sequencing reaction, the extension products were generated. The extension products were then purified by precipitating by salting out principle using ethanol and sodium acetate. Practically, the entire content of each extension product was transferred into a microcentrifuge tube containing 14.5  $\mu\text{l}$  of deionized distilled water, 3  $\mu\text{l}$  of 3 M sodium acetate, pH 4.6 and 62.5  $\mu\text{l}$  of absolute ethanol, mixed thoroughly and left at room temperature for 15 min, spun at 10,000 rpm in a microcentrifuge for 20 min and supernatant discarded. The pellet was then washed

with 250  $\mu$ l of 70% ethanol, spun at 10,000 rpm for 10 min, dried by a 1 min heating on the heat block at 90°C and finally stored at -20°C until use.

**Automated nucleotide sequencing :** The precipitated extension products were resuspended in 20  $\mu$ l of HI-DI™ Formamide (ABI Prism®), vigorously mixed and spun down. The suspension was then heated at 95°C for 2 min, immediately chilled on ice bath. Separation of the extension products was accomplished using the ABI PRISM®3130 Genetic Analyzer (Applied Biosystems) which is based on the capillary electrophoresis principle. The raw data from capillary electrophoresis collected by ABI PRISM Sequencing Analysis software V5.3.1 (PE Applied Biosystems) and analyzed by Chromas Lite 2.01 (Technelysium Pty Ltd.). Sequencing data was manually compared with the reference sequence of the  $\alpha$ -globin genes (GeneBank : HUMHBA4).

### 2.2.2 Development of multiplex allele-specific PCR

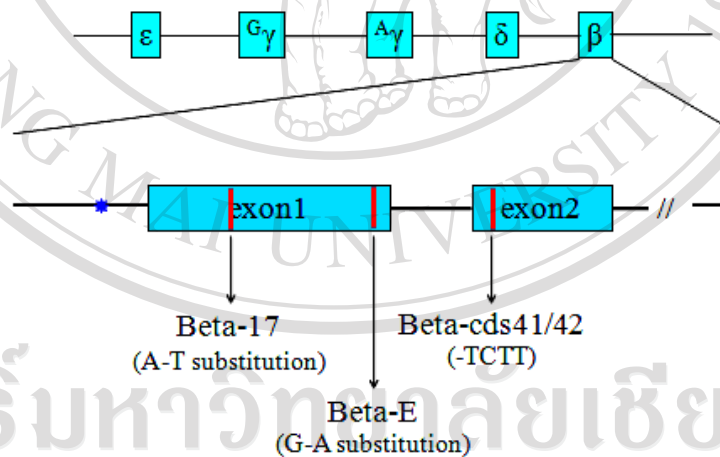
The ultimate aim of this part of thesis was to develop multiplex PCR protocol capable of simultaneous detection of severe types of thalassemia and hemoglobinopathies commonly occur across Thailand including, HbE ( $\beta^E$ ), A-T substitution at codon 17 ( $\beta^{17}$ ) of  $\beta$ -globin gene, TCTT deletion at codons 41/42 ( $\beta^{41/42}$ ) of  $\beta$ -globin gene and  $\alpha$ -thalassemia 1 (SEA type) (--<sup>SEA</sup>) (Figures 2.6, 2.4). Since this protocol was initiate in this study, then oligonucleotide primers used in the PCR reaction as well as all the conditions had to be optimized. The practical details are described in the forthcoming paragraphs.



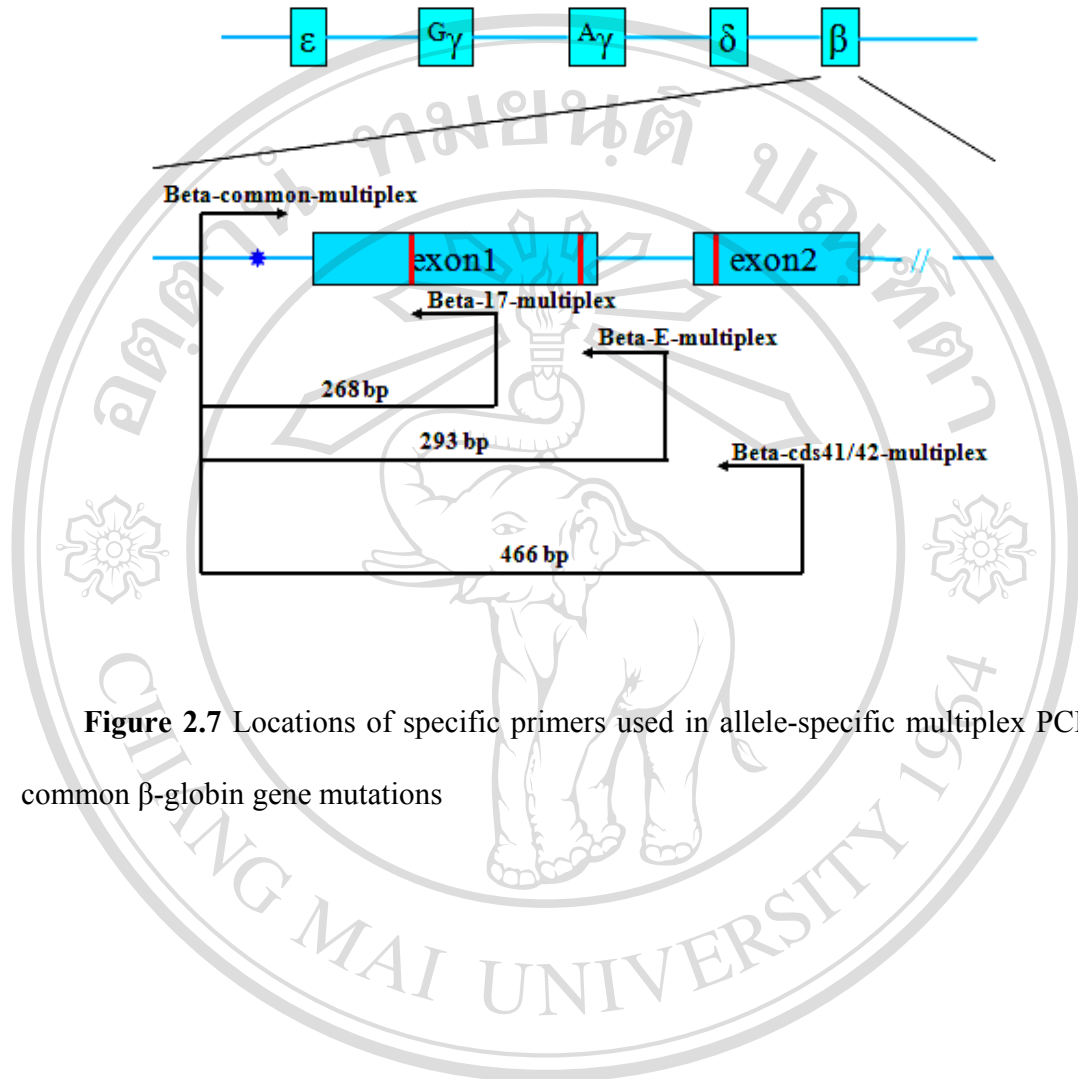
### 2.2.2.1 Design of PCR primers

The new set of PCR primers were designed by using the PCR primer designing software using the nucleotide sequences of  $\alpha$ -globin and  $\beta$ -globin genes obtained from GenBank database (<http://www.ncbi.nlm.nih.gov/>) with access code “HUMHBB” for  $\beta$ -globin gene and “AE006462” for  $\alpha$ -globin gene. Once the nucleotide sequences were obtained, the locations of these 4 common mutations ( $\beta^E$ ,  $\beta^{17}$ ,  $\beta^{41/42}$ , --<sup>SEA</sup>) were mapped using the information from the Globin Gene Server (<http://globin.cse.psu.edu/>). After the positions of the 4 common mutations were localized on the  $\alpha$ - and  $\beta$ -globin genes, several primer pairs were created by the primer design software as stated above. The major consideration in designing PCR primers for this newly developed protocol was the specificity of the test, size overlapping of amplified products. Since the new PCR protocol was the allele-specific multiplex PCR for detecting mutations on  $\alpha$ - and  $\beta$ -globin genes, the size of amplified products must not overlapped and be at least 20-bp different and, for  $\beta$ -globin gene, the 3'-end specifically complementary to the mutation points. Moreover, the third or fourth nucleotides from 3'-end of mutant-primers of  $\beta$ -globin mutations were altered to enhance specificity of the test (Carl w. Dieffenbach 1995). In addition, other primer properties were also strictly taken into account including GC content,  $\Delta G$  of primer priming,  $T_m$ , hair pin loop formation and false priming rate as well as length of each primer. All of these primers must have GC content between 40-60%,  $\Delta G$  of -0.25 to 0.25 kcal/mol, as least hair pin loop formation and false priming rate as possible and less than 25 bp in length. Finally, 4 primers for  $\beta$ -globin gene mutations were generated including one common forward primer (Beta-common-multiplex), one

$\beta^E$ -specific reverse primer (Beta-E-multiplex), one  $\beta^{17}$ -specific reverse primer (Beta-17-multiplex) and one  $\beta^{41/42}$  specific reverse primer (Beta-cds41/42-multiplex). For  $\alpha$ -thalassemia 1 (SEA type) ( $--^{SEA}$ ), 3 primers were created comprising common forward primer (SEA-1-multiplex), wild-type reverse primer (SEA-2-multiplex) and SEA-specific reverse primer (SEA-3-multiplex). The amplified products of primers SEA-1-multiplex and SEA-2-multiplex were also used as an internal control. The detail of the newly designed primers including sequences, length, location in GenBank database were shown in Table 2.1 and annealing site of primers for  $\alpha$ -thalassemia 1 (SEA type) and  $\beta$ -globin gene mutations in Figures 2.4 and 2.7, respectively.



**Figure 2.6** Schematic locations of  $\beta$ -globin gene common mutations in Thailand and cause severe  $\beta$ -thalassemia



**Figure 2.7** Locations of specific primers used in allele-specific multiplex PCR for common  $\beta$ -globin gene mutations

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**Table 2.1**  $\beta$ -globin gene primers and  $\alpha$ -globin gene primers newly designed for use in the developed allele-specific multiplex PCR described in this thesis

Names	Sequence (5'-3')	Length (bp)	Location
Beta-common-multiplex	AAG AGC CAA GGA CAG GTA CGG CTG T	25	61993-62017 <sup>a</sup>
Beta-17-multiplex	CCA ACT TCA TCC ACG TTC ACG TA	23	62238-62260 <sup>a</sup>
Beta-E-multiplex	CGT ACC AAC CTG CCC AGG GCC AT	23	62265-62287 <sup>a</sup>
Beta-cds41/42-multiplex	AGA TCC CCA AAG GAC TCA ACC T	22	62437-62462 <sup>a</sup>
SEA-1-multiplex	TGA CTC CAA TAA ATG GAT GAG GA	23	155228-155250 <sup>b</sup>
SEA-2-multiplex	GCC TGC GCC GGG GAA CGT AAC CA	23	155552-155571 <sup>b</sup>
SEA-3-multiplex	CGC CAA AGA TGG CTA CTC GGA GA	23	175268-175290 <sup>b</sup>

<sup>a</sup> HUMHBB, <sup>b</sup> AE006462

### 2.2.2.2 Optimization of developed multiplex allele-specific PCR

The multiplex allele-specific PCR developed in this thesis is based on the use of mixture of all 7 primers designed for simultaneous genotyping, in a single PCR reaction, of  $\alpha$ -thalassemia 1 (SEA type) and 3 common  $\beta$ -thalassemia mutations commonly found in Thai. Then, optimization of the technique was necessary; i.e. to obtain maximal yields and clear differentiation of amplified products on agarose gel electrophoresis. The condition to be optimized included 1). Efficiency of newly designed  $\beta$ -globin gene primers, 2). Efficiency of newly designed  $\alpha$ -thalassemia (SEA type) primers, 3). Efficiency of multiplex allele-specific PCR, 4). Quantity of primers, 5). Quantity of DMSO, 6). Quantity of  $MgCl_2$ , 7). Quantity of dNTPs and 8). Quantity of genomic DNA.

**Test of efficiency of  $\beta$ -globin gene primers :** The PCR was performed in a total volume of 25  $\mu$ l containing 5  $\mu$ l of genomic DNA (Chelex™ extracted), 100  $\mu$ M of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 4.0 ng (0.600  $\mu$ M) of Beta-common-multiplex and 2.0 ng (0.300  $\mu$ M) of the rest of primers were tested, in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.5 mM  $MgCl_2$  and 4.0 % DMSO. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. Success of the experiment was indicated by the presence of specific amplified fragments, regardless of non-specific products.

**Test of efficiency of  $\alpha$ -thalassemia (SEA type) primers:** The PCR was performed in a total volume of 25  $\mu$ l containing 5  $\mu$ l of genomic DNA (Chelex™ extracted), 100  $\mu$ M of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 4.0 ng (0.600  $\mu$ M) of SEA-1- multiplex and 2.0 ng (0.300  $\mu$ M) of the rest of primers were tested, in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.5 mM MgCl<sub>2</sub> and 4.0 % DMSO. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. Success of the experiment was also indicated by the presence of specific amplified fragments, regardless of non-specific products.

**Test of efficiency of multiplex allele-specific PCR:** The PCR was performed in a total volume of 25  $\mu$ l containing 5  $\mu$ l of genomic DNA (Chelex™ extracted), 100  $\mu$ M of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 4.0 ng (0.600  $\mu$ M) of Beta-common-multiplex and SEA-1-multiplex, 2.0 ng (0.300  $\mu$ M) of rest primers, as shown in table 1, in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.5 mM MgCl<sub>2</sub> and 4.0 % DMSO. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. Success of the experiment was also indicated by the presence of specific amplified fragments, regardless of non-specific products.

**Optimization of primer quantities:** The PCR was performed in a total volume of 25  $\mu\text{l}$  containing 5  $\mu\text{l}$  of genomic DNA (Chelex<sup>TM</sup> extracted), 100  $\mu\text{M}$  of dNTPs, 0.5 units i-Taq DNA polymerase (Intron) in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.5 mM  $\text{MgCl}_2$  and 4.0 % DMSO. 2.0 ng (0.300  $\mu\text{M}$ ) and 1.0 ng (0.150  $\mu\text{M}$ ), respectively, of the both primer “Beta-common-multiplex” and “SEA-1-multiplex”, and 1.0 ng (0.150  $\mu\text{M}$ ) 0.5 ng (0.075  $\mu\text{M}$ ), respectively, of the rest of primers were tested. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The amount of primers used in the reaction yielding the best amplified products was selected and used in subsequent experiment.

**Optimization of concentration of DMSO:** The PCR was performed in a total volume of 25  $\mu\text{l}$  was containing 5  $\mu\text{l}$  of genomic DNA (Chelex<sup>TM</sup> extracted), 100  $\mu\text{M}$  of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng (0.150  $\mu\text{M}$ ) of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.5 ng (0.075  $\mu\text{M}$ ) of the rest of primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.5 mM  $\text{MgCl}_2$ . Five concentrations of DMSO (1.0%, 2.0%, 4.0%, 6.0% and 8.0 %) were tested. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The concentration yielding the best amplified products was then chosen.

**Optimization of concentration of MgCl<sub>2</sub>:** The PCR was performed in a total volume of 25 µl containing 5 µl of genomic DNA (Chelex™ extracted), 100 µM of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng (0.150 µM) of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.5 ng (0.075 µM) of the rest of primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl and 2.0 % DMSO. Five concentrations of MgCl<sub>2</sub> (1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM and 3.5 mM) were evaluated. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The concentration yielding the best amplified products was then chosen.

**Titration of dNTPs:** The PCR was performed in a total volume of 25 µl containing 5 µl of genomic DNA (Chelex™ extracted), 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng (0.150 µM) of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.5 ng (0.075 µM) of the rest of primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.0 % DMSO and 2.0 MgCl<sub>2</sub>. The concentration of dNTPs to be tested were 60 µM, 80 µM, 100 µM, 120 µM and 140 µM. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The concentration yielding the best amplified products was then selected.



**Titration of DNA:** The PCR was performed in a total volume of 25  $\mu\text{l}$  was performed containing 140  $\mu\text{M}$  of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng (0.150  $\mu\text{M}$ ) of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.5 ng (0.075  $\mu\text{M}$ ) of the rest of primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.0 % DMSO and 2.0  $\text{MgCl}_2$ . The amount of Chelex<sup>TM</sup>-extracted genomic DNA to be tested were 3  $\mu\text{l}$ , 4  $\mu\text{l}$ , 5  $\mu\text{l}$ , 6  $\mu\text{l}$  and 7  $\mu\text{l}$ . A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The amount of genomic DNA yielding the best amplified products was then selected.

**Titration of SEA-2-multiplex primer:** Owing to the signal of amplified products of SEA-1-multiplex and SEA-3-multiplex were less than those of SEA-1-multiplex and SEA-2-multiplex, the amount of SEA-2-multiplex was then titrated again in order to obtain higher amount of products generated from the primers SEA-1-multiplex and SEA-3-multiplex. The PCR was performed in a total volume of 25  $\mu\text{l}$  containing 5  $\mu\text{l}$  of genomic DNA (Chelex<sup>TM</sup> extracted), 140  $\mu\text{M}$  of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.5 ng of the rest of primers except SEA-2-multiplex primer in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.0 % DMSO and 2.0  $\text{MgCl}_2$ . The concentration of SEA-2-multiplex primers tested was in the series of 0.031 (0.004  $\mu\text{M}$ ), 0.063 (0.008  $\mu\text{M}$ ) 0.125 (0.176  $\mu\text{M}$ ), 0.25 (0.035  $\mu\text{M}$ ) and 0.5 ng (0.070  $\mu\text{M}$ ). A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min,

primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min.

**Validation of final optimized condition:** After all the conditions were optimized, overall procedures of the newly developed “multiplex allele-specific PCR” were repeated to confirm efficiency of the technique. The PCR was performed a total volume of 25  $\mu$ l containing 5  $\mu$ l of genomic DNA (Chelex™ extracted), 140  $\mu$ M of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 1.0 ng (0.150  $\mu$ M) of “Beta-common-multiplex” and “SEA-1-multiplex” primers, 0.25 ng (0.035  $\mu$ M) “SEA-2-multiplex” primers and 0.5 ng (0.075  $\mu$ M) of the rest of primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.0 % DMSO and 2.0 MgCl<sub>2</sub>. A total of 42 thermal cycles was carried out in MJ PCT-200 Thermal Cycler with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min and primers extension at 72°C for 1 min. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min.

However, in repeating the newly developed “multiplex allele-specific PCR” on different thermal cycler (MyCycler, Bio-Rad), the previously optimized condition was slightly modified to obtain clear amplified fragments. The modification was lowering the amounts to  $\beta$ -globin gene specific primers to 25% of the original optimized ones. Thus, the final working amount of  $\beta$ -globin gene specific primers were 0.25 ng (0.038  $\mu$ M) for Beta-common-multiplex and 0.125 ng (0.018  $\mu$ M) for the rest. In addition, the thermal cycles were also slightly modified. Thus, the new thermal cycles was composed of 37 cycles and started with denaturation at 95°C for 1 min, primer annealing at 62°C for

30 sec and primer extension at 72°C for 30 sec. The durations of initial denaturation and final extension remained the same.

### **2.2.2.3 Determination of double heterozygous state using the developed multiplex allele-specific PCR**

After all the conditions of the newly developed multiplex allele-specific PCR were optimized and validated, it was employed to detect the double heterozygous form that probably exist in those samples labelled BH and EH in the Table 1.1. The PCR was performed a total volume of 25 µl containing 5 µl of genomic DNA (Chelex™ extracted), 140 µM of dNTPs, 0.5 units i-Taq DNA polymerase (Intron), 0.25 ng (0.038 µM) for “Beta-common-multiplex” primer and 0.125 ng (0.018 µM) for the rest of beta primers, 1.0 ng (0.150 µM) of “SEA-1-multiplex” primers, 0.25 ng (0.035 µM) “SEA-2-multiplex” primers and 0.5 ng (0.075 µM) of “SEA-3-multiplex” primers in 10 mM Tris-HCl (pH 8.3), 50 mM HCl, 2.0 % DMSO and 2.0 MgCl<sub>2</sub>. A total of 37 thermal cycles was carried out in thermal cycler (MyCycler, Bio-Rad) with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 62°C for 30 sec and primers extension at 72°C for 30 sec. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min. The PCR products was separated in the agarose gel electrophoresis and visualized under the Gel Documentation system (Bio-rad).