CHAPTER II

MATERIALS AND METHODS

2.1 Subjects

Sixty patients were recruited from the Pediatric Thalassemia Clinic, Maharaj Nakorn Chiang Mai Hospital and the Department of Pediatrics, Faculty of Medicine, Chiang Mai University. By using the criterias described by Ho *et al.* in the chapter 1 (Ho, *et al.*, 1998) with slight modification the first criterias to look for was frequency of blood transfusion followed by present or absent of splenectomy and the age of onset. The patients were then classified into two groups; β-thalassemia intermedia (28 cases) and β-thalassemia major (32 cases) groups. Venous blood samples were collected from all subjects and kept in ethylenediamine tetraacetic acid (EDTA) as an anticoagulant.

2.2 Laboratory studies

2.2.1 **Hemoglobin** (Wintrobe, 1981)

Hemoglobin level was determined using the standard cyanmethemoglobin method. Twenty microlitre of EDTA blood was mixed with 5 ml of Drabkin solution and stand at RT for 10 minutes. The cyanmethemoglobin solution was then measured for optical density (OD) at 540 nm. The intensity of ODs was proportional to the hemoglobin level in samples. The concentration (in g/dl) of hemoglobin was obtained by comparing the ODs of the samples to that of the standard hemoglobin solution.

2.2.2 **Reticulocyte** (Jandl, 1987)

Messenger RNA (mRNA) in young erythrocytes can be stained with the supravital stains such as brillian cresyl blue or new methylene blue. The reticulocytes are the

stained erythrocytes seen under oil power field of light microscope as blue circles with the deep blue reticulum inside. Practically, three drops of EDTA blood were mixed with equal amount of 1% new methylene blue solution in 12x75 mm test tubes (Appendix-C). The mixture was allowed to stand for 15 min at room temperature and gently remixed before preparing smears on microscopic glass slides. To count the reticulocyte, a minimum 1000 red blood cells were examined under oil power field, in which the amont of reticulocytes was quantitated and expressed finally as percentage.

2.2.3 F cell quantitation by fluoromicroscopy (Thein, 1998)

F cells (FCs) on a thin dried blood smear can be visualized and quantified using fluorescence microscopy after immunostaining with monoclonal antibody to human γ-globin chain labelled with fluorochrome. About 1000 cells were normally counted in 10 consecutive fields using a 40x objective lens. The number of F cells relative to the total red blood cells in the examined field were determined.

2.2.3.1 Fixation and trypsinization

Thin blood smear were prepared on the scrupulously cleaned glass slides, usually using 1-2 μl whole blood and allowed to air-dry at least overnight. A circle of 5 mm diameter was made on a suitable area on the blood smear using a pensil or a diamond-head pen. The blood film was fixed in acetone: ethanol: methanol (6:2:2, v/v/v) for 20 min, air dried, rehydrated in PBS for 5 min, rinsed in DW and air-dried. Prior to incubation with antibody, trypsinization of dried blood smears is necessary to destroy the cross-linking between membrain proteins. The duration of trypsinization was critical. Over trypsinization could lead to lysis of red cells, while under trypsinization leads to reduced entry of antibody and diminished quality of stainning. To perform the trypsinization, the marked area of blood film was treated with 8 μl of trysin solution (Appendix-C) for exactly 15 min, washed in an excess amount of PBS for 5 min with

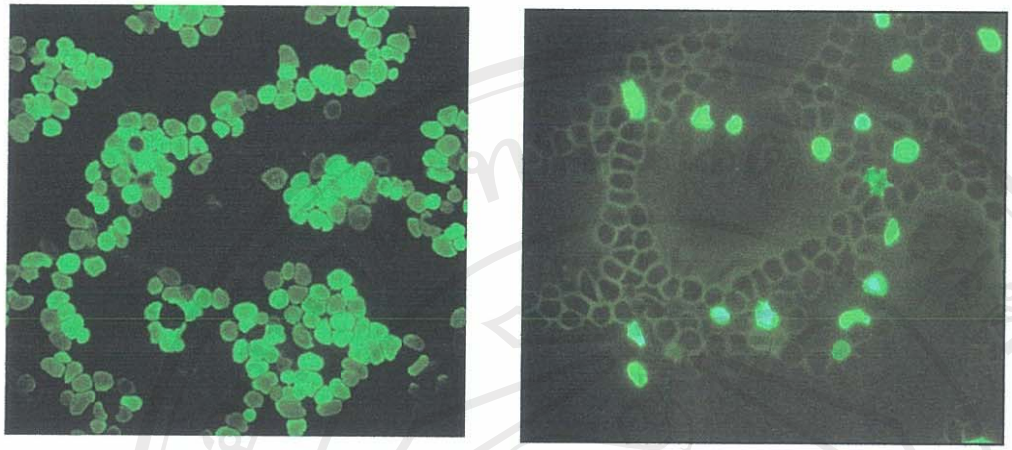
gentle agitation, rinsed in DW and air dried. Blood smears which are more than a few weeks old need more than 15 min of trypsinization.

2.2.3.2 Immunostaining of fixed blood smear

The trypsinized area on blood smears was incubated with 10 µl of fluorescien isothiocyanate (FITC)-conjugated monoclonal anti-human γ-globin chain (Appendix-C) at 37°C for 30 min in a moist chamber, washed in PBS with gentle agitation for 5 min and air dried. The stained smears were then washed in PBS for 5 min, rinsed in DW, air-dried and mounted in antifade (e.g. PBS:glycerol, 1:1).

2.2.3.3 FC analysis by fluorescence microscopy

The smear were observed under high power field (40x objective lens) using a fluorescence microscope (Olympus, BX60). F cells appeared as bright green circles while non-F cells (non-FCs) were seen as blank circles and the colour was identical to that of the background. FCs were examined per smear. On average, a single high power field (40X) with red cells in a single layer should contain ~ 250 cells. The FC numbers were expressed as a percentage of the total red cells scored (Figure 2.1).



a) b)

Figure 2.1 The appearance of FCs (a) and non-FCs (b) on blood smear after stained with FITC-conjugated mAb to human γ-globin chain. FCs are seen as bright green circles, while non-FCs appears blank circles.

2.2.4 Hb 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. With the help of sophisticated analytical software, hemoglobin fractions including HbF are automatically computed. This task was accomplished using the Bio-Rad VARIANT™ Hemoglobin Testing System (Bio-Rad Laboratories., 1994). Practically, 5 μl of EDTA blood was lysed in 1 ml of DW and placed in an autosampler inside the machine. The β-thal Short™ operating software was employed to control the separation profile of the machine. The hemoglobin separation lasts 6 min. The HbF was eluted first, followed by HbA 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 (Walsh, 1991) with some modifications (Sanguansermsri, *et al*., 1999). Chelex resin is a chelating agent that removes divalent cation including Mg^{++} which could act as cofactor for DNase enzyme. In practice, 40 µl of EDTA blood was mixed with 1 ml of 0.5% Triton X-100 in 1.5 ml tubes, vortexed and centrifuged at 14,000-rpm for 1 minute. The supernatant was removed, 1 ml of water added, centrifugated 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 minutes. 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 liberate the genomic DNA. The extracts were then stored at 4°C until use.

2.3.2 Mutagenically Separated PCR (MS-PCR) for detection of β-thalassemia mutations

The mutagenically separated polymerase chain reaction (MS-PCR) was originally described by Rust *et al*. (Rust, *et al*. 1993) as a general technique for the analysis of any point mutations. The principle of the technique involves the use of two allele-specific primers of different lengths that are separately complementary to a given DNA sequence except for a mismatch near the 3'-end of the primers. These additional and deliberately designed differences can markedly reduce cross-reaction in subsequent PCR cycles. A typical MS-PCR test contains three primers in the PCR mixture; the different products can be identified by their different sizes determined by the different primer sizes. Both normal and mutant alleles are amplified in the same reaction tube.

Subsequent gel electrophoresis shows at least one of the two allelic products at the same locus (Figure 2.2).

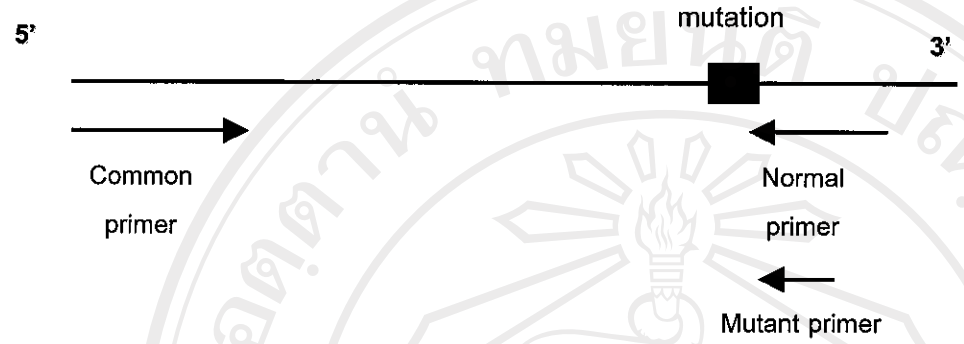


Figure 2.2 Schematic representation of the localization of primers used in the MS-PCR.

2.3.2.1 4 bp (-TTCT) deletion at codons 41/42 (codons 41/42; -TTCT)

The PCR was performed in a total volume of 25 µl containning 250 ng genomic DNA, 200 µM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 150 ng of primer N41 (N41: 5'-TTC CCA CCA TTA GGC TGC TGG TGG TCT ACC CTT GGA CCC AGA GGT TCT T-3'), 100 ng of primer C41 (C41: 5'-TCA TTC GTC TGT CCA TTC TAA AC-3') and 250 ng of primer M41 (M41: 5'-ACC CTT GGA CCC AGA GGT TGA G-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°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. For the codons 41/42, –TTCT mutation, a 351-bp fragment was found for the normal allele and a 324-bp fragment was observed in the mutant allele. Thus in those homozygous for this mutation, only 324-bp amplified fragment was seen, whereas two fragments (351 and 324 bp) were generated in

heterozygotes and only a 351-bp fragment was found in those negative for this particular mutation (Figure 3.1A).

2.3.2.2 A-T substitution at codon 17 (codon 17; A-T)

The PCR was performed in a total volume of 25 µl containning 250 ng genomic DNA, 200 µM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 150 ng of primer N17 (N17: 5'-ACC TGA CTC CTG AGG AGA AGA CTG CCG TTA CTG CCC TGT GGG ACA-3'), 100 ng of primer C17 (C17: 5'-GGC AGA GAG AGT CAG TGC CTA-3') and 100 ng of primer M17 (M17: 5'-TCT GCC GTT ACT GCC CTG TGG CAC-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°C for 1 min, primer annealing at 59°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. For the codon 17; (A-T), a 190-bp fragment was found for normal allele and a 170-bp fragment for homozygotes, while the 170-bp and 190-bp fragments were seen in those heterozygous for this condition (Figure 3.2A).

2.3.2.3 Adenine addition at codons 71/72 (Codons 71/72; +A)

The PCR was performed in a total volume of 25 µl containning 250 ng genomic DNA, 200 µM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 100 ng of primer N71 (N71: 5'-AGT GTC CGG TGC CTT TAG TG-3'), 100 ng of primer C71 (C71: 5'-CCT GAA GTT CTC AGG ATC-3') and 150 ng of primer M71 (M71: 5'-GTC AAG GCT CAT GGC AAG AAT CTG CTC GGT GCC TTT AAG-3') in 10 mM Tris pH 8.8, 50 mM KCl and 2.5 mM MgCl2. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min.

The codons 71/72 for the (+A) mutation, a 115 bp fragment was found for normal allele and fragments of 115 and 135 bp for the heterozygous state (Figure 3.3A).

2.3.2.4 A-G substitution at nucleotide -28 of β-globin promoter (NT-28; A-G)

The PCR was performed in a total volume of 25 μl containning 250 ng genomic DNA, 200 μM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 100 ng of primer N28 (N28: 5'-GAA GCA AAT GTA AGC AAT ACA TGG CTC TGC CCT GAC ATT-3'), 100 ng of primer C28 (C28: 5'-GCT TAC CAA GCT GTC ATT CC-3') and 100 ng of primer M28 (M28: 5'-AGA TGG CTC TGC CCT GAC ATC-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°C for 1 min, primer annealing at 59°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. For the NT-28, only 305-bp fragment was found in negative control and there were fragments of 305 and 287-bp in the heterozygous state. However, only the 287-bp amplified products was seen in those homozygous for this mutation (Figure 3.4A).

2.3.2.5 G-A substitution at codon 26 (Hb E)

The PCR was performed in a total volume of 25 μl containning 250 ng genomic DNA, 200 μM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 100 ng of primer NE (NE: 5'- CGT GGA TGA AGT TGG TGG AG -3'), 100 ng of primer CE (CE: 5'-GGC AGA GAG AGT CAG TGC CTA-3') and 150 ng of primer ME (ME: 5'-CTG CCC TGT GGG CAA GGT GAA CGT GGA TGA AGT TGG TGG AA-3') in 10 mM Tris pH 8.8, 50 mM KCl and 1.25 mM $MgCl_2$. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 1 min; the initial

denaturation was extended to 4 min while the final extension was prolonged to 5 min. For GAG-AAG mutation at codon 26, a 138 bp fragment was found in negative control and there were fragments of 160 and 138 bp in the heterozygous state, while homozygotes produced 160-bp amplified products (Figure 3.5A).

2.3.2.6 C-T substitution at nucleotide 654 within IVS 2 (IVS 2 nt 654; C-T)

The PCR was performed in a total volume of 25 μl containning 250 ng genomic DNA, 200 μM of each dNTP, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 100 ng of primer N654 (N654: 5'-CAT CAG TTA CAA TTT ATA TCC AGA AAT ATT TCT ACT A-3'), 100 ng of primer C654 (C654: 5'-CTT TCA GGG CAA TAA TGA TAC AAT G-3') and 100 ng of primer M654 (M654: 5'-GCA GAA ATA TTT ATA TGC AGA AAT ATT TCT ACT 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°C for 1 min, primer annealing at 59°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. For IVS2 nt 654 C-T mutation, a 134 bp fragment was found in normal allele and 134-bp and 115-bp in heterozygote, whereas only a 115-bp fragment was seen in those homozygous for this mutation (Figure 3.6A)

2.3.3 Determination of α-thalassemia-1 (SEA type) genotype

The Southeast Asian (SEA) deletional type of α-thalassemia-1 involves 19.304-kb fragment of α-globin gene cluster spanning from $\psi\alpha_2$-gene to the sequences 3' to the θ-globin gene. By using 3 primers (P1, P2, P3) that bind at the different sites inside and flanking the SEA deletion, one can easily determine the α-thalassemia-1 of this type by visualising the amplified products generated from each of primer pair (Figure 2.3).

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 (QIAGEN Gmbtt, Hiden,

Germany), 0.375 μM of each primers (P1: 5'-GCG ATC TGG GCT CTG TGT TCT-3', P2: 5'-GTT CCC TGA GCC CCG ACA CG-3', P3: 5'-GCC TTG AAC TCC TGG ACT TAA-3') in 10 mM Tris pH 8.6, 50 mM KCL, 0.00125% Xylene Cyanol and 1.5 mM $MgCl_2$. 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 340 bp fragment was found in negative control while 340-bp and 188-bp amplified fragments in the heterozygous state (Figure 2.4).

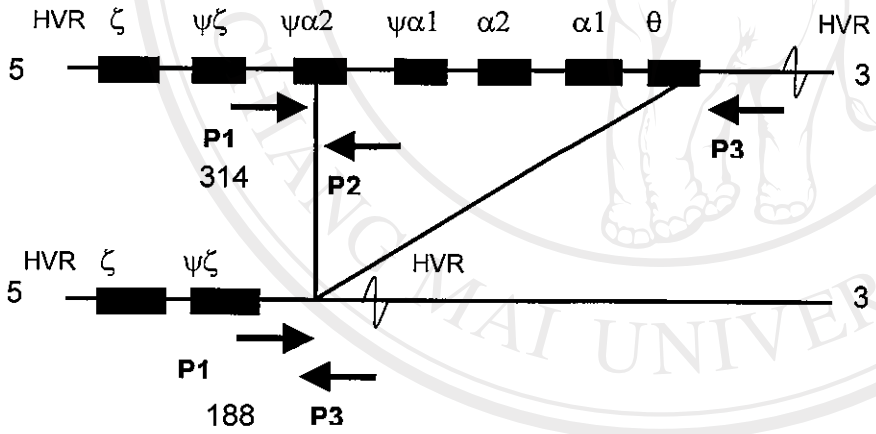


Figure 2.3 Schematic representation of the localization of 3-oligonucleotide primers (P1, P2, P3) used in Gap-PCR for α-thalassemia 1 (SEA type) identification as well as the amplified products created by the allele specific pairs of primer.

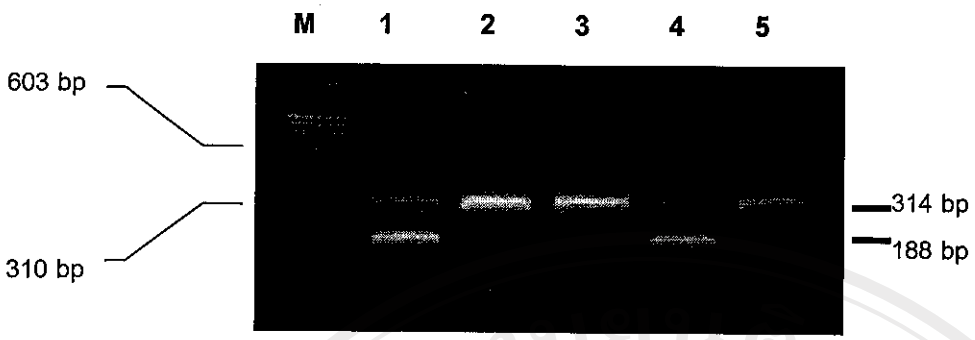


Figure 2.4 The result of Gap-PCR for α-thalassemia-1 (SEA-type). Lane M indicates the ϕX 174 Hae III digest DNA size marker. Lanes 2, 3, and 5 represents negative cases; 314-bp are present. Heterozygous state with the 314-bp and 188-bp fragments are seen in lanes 1 and 4.

2.3.4 Determination of the *Xmn*I-$^{G}\gamma$ site

A substitution of "T" for "C" at position −158 relative to the $^{G}\gamma$-globin gene cap site creates a cutting site for the restriction enzyme *Xmn*I (*Xmn*I-$^{G}\gamma$ site). The *Xmn*I-$^{G}\gamma$ site was detected by restriction analysis of sepecifically amplified PCR product of the $^{G}\gamma$-promoter region. The PCR was performed in a total volume of 25 μl containing 50 ng genomic DNA, 100 μM of each dNTPs, 0.5 units *Taq* DNA polymerase (QIAGEN Gmbtt, Hiden, Germany), 0.2 μM of each primer (5'-GG1: 5'-AAC TGT TGC TTT ATA GGA TTT TTC A-3' and 3'-AG-1: 5'-GTC TGG ACT AGG AGC TTA TTG AT-3') in 10 mM Tris pH 8.8, 50 mM KCL and 2.5 mM $MgCl_2$. A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 58°C for 2 min and primer extension at 72°C for 2 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min. The 665 bp PCR product was finally obtained (Figures 2.5).

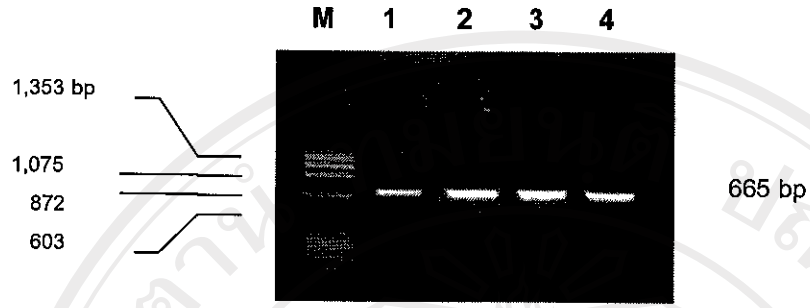


Figure 2.5 The result of *Xmn*I-$^{G}\gamma$ site. Lane M indicates the ϕX 174 Hae III digest DNA size marker. Lanes 1, 2, 3 and 4 represents 665-bp fragments.

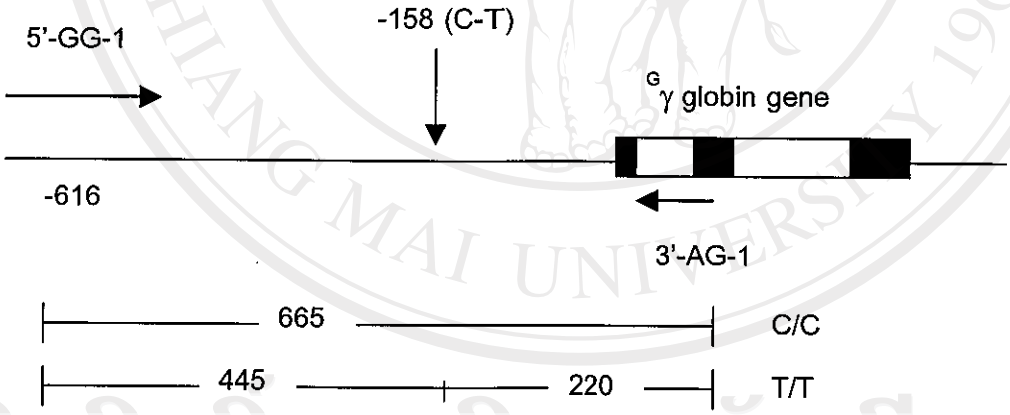


Figure 2.6 Schematic representation of 5' flaking region of the $^{G}\gamma$ - globin gene. Primers 5'-GG-1 and 3'-AG-1 were used to amplify, by PCR, the $^{G}\gamma$-promoter region for the detection of the C-T polymorphism at the position –158 to the cap site.

***Xmn*I restriction digestion**

The *Xmn*I restriction digestion was carried out in a 1.5-ml microcentrifuge tube. The 20 µl reaction contained 15 µl amplified DNA, 2 µl of 10X NEB buffer 2, 0.2 µl of 100XBSA (NEB) and 20 units of *Xmn*I restriction enzyme (NEB) and 1.8 µl DW. The mixture was then incubated at 37°C at least 9 hrs before the separation of the digested products in 2% agarose gel. Two digested fragments (445 and 220 bp) were generated in those homozygous for the presence of the *Xmn*I-$^{G}\gamma$ site (*Xmn*I-$^{G}\gamma$, +/+). Three bands (665, 445, 220 bp) were seen in heterozygote (*Xmn*I-$^{G}\gamma$, +/-), while only a 665-bp undigested band was visualised in those homozygous for the absence of the *Xmn*I-$^{G}\gamma$ site (*Xmn*I-$^{G}\gamma$, -/-) (Figures 2.7).

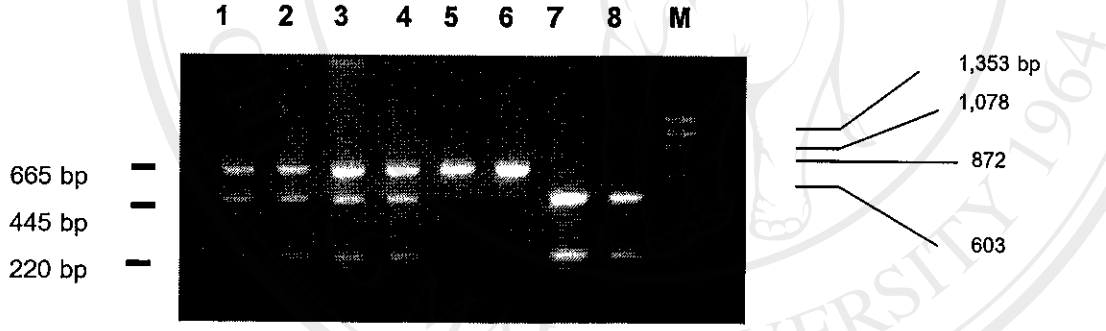


Figure 2.7 *Xmn*I digested fragments run on a 2% agarose gel. Lanes 7 and 8 represents individuals homozygous. Lanes 1 to 4 represent heterozygote state and normal state in lanes 5 and 6 for the *Xmn*I-$^{G}\gamma$ site. ϕX174 DNA (Hae III digest) was used as a size marker (Lane M) with sizes (in base pairs, bp) of the digested fragments shown.

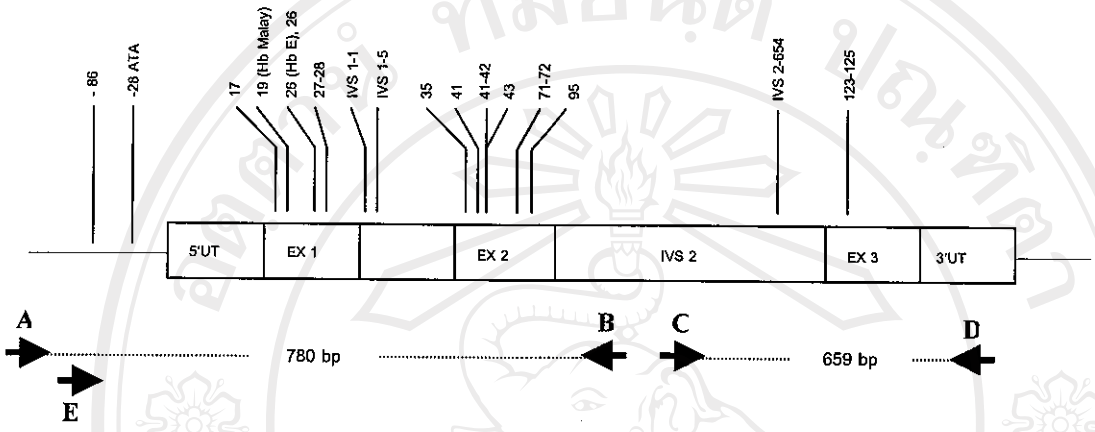
2.3.5 Nucleotide sequencing of β-globin gene exons

2.3.5.1 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, one unit of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA) and 0.5 μM of primer mix A (5'-CCA ACT CCT AAG CCA GTG CC-3'), B (5'-TGC AAT CAT TCG TCT GTT TCC C-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°C for 45 seconds, primer annealing at 60°C for 1 min and primer extension at 72°C for 2 min; the initial denaturation was extended to 95°C for 15 min while the final extension was prolonged to 10 min.

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, one unit of AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA), 0.5 μM of primer mix C: (5'-TCC CTA ATC TCT TTC TTT CAG G-3'), and D (5'-TTT TCC AAG GTT TGA ACT AGC-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°C for 45 seconds, primer annealing at 55°C for 1 min and primer extension at 72°C for 1.30 min; the initial denaturation was extended to 95°C for 15 min while the final extension was prolonged to 10 min.

PCR was performed in a GeneAmp® PCR System 9700 thermal cycle (Applied Biosystems, Foster City, CA). PCR products were separated in 3% agarose gel electrophoresis and made visible with ethidium bromide and medium-wavelength UV light from a transluminator (Figure 2.8). The size of the DNA fragments were 780 and 659-bp, respectively (Sirichotiyakul, *et al.*, 2003).



Primers sequences: A: 5' – CCA ACT CCT AAG CCA GTG CC – 3'

B: 5' – TGC AAT CAT TCG TCT GTT TCC C – 3'

C: 5' – TCC CTA ATC TCT TTC TTT CAG G – 3'

D: 5' – TTT TCC CAA GGT TTG AAC TAG C – 3'

E: 5' – AGA AGA GCC AAG GAC AGG TAC G – 3'

Figure 2.8 Diagram of the β-globin gene demonstrated 19 mutations that has been found in Thailand (modified from Sutcharitchan P, *et al.* Reverse dot-blot detection of Thai β-thalassaemia mutations. Br J Haematol 1995; 90: 811) and sequences of primers used for PCR and sequencing (Sirichotiyakul, *et al.*, 2003).

2.3.5.2 Nucleotide sequencing

a) Cycle sequencing reaction

As the template for sequencing reaction, the amplicons were purified using the QIA quick PCR purification kit (QIAGEN Gmbtt, Hiden, Germany). The kit was designed to separate single or double stranded PCR products ranging in size from 100 bp to 10 kb from primers, nucleotides, polymerases and salt. 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 rmp to remove all the ingradients 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 sterile DW after 14,000-rmp centrifugation. The purified DNA was then stored at -20°C until use.

The extension or sequencing reactions were preformed 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. Deionized distilled water was added to make up 20 µl. The reactions were heated to 94°C for 2.30 min, followed by 25 cycles 10 seconds at 96°C, 5 seconds at 50°C, and 4 min at 60°C in a GeneAmp® PCR System 9700 thermal cycle (Applied Biosystems, Foster City, CA).

b) 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 transfered 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 minutes, 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 14000 rpm for 25 min and dried by a 1-min heating on the heat block at 90°C. The precipitated extension products was stored at -20°C until separation by the electrophoresis.

c) 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°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 standard sequence of the β-globin gene (GeneBank U01317).

2.4 Statistical analysis

The SPSS v 9.0 for Windows was employed to calculate descriptive and inferential statistics including mean, standard deviation, Student's unpaired *t*-test and Z-score for the differences of proportion.