CHAPTER III
RESEARCH METHODOLOGY

1. Subjects and specimens

1.1 Processing of blood samples from pregnant women

Plasma specimens were collected from 5 ml peripheral blood of couples at risk of having severe thalassemia in the fetuses when they attended at the thalassemia project, Faculty of Associated Medical Sciences for routine DNA testing.

Maternal plasma was separated from EDTA blood by centrifugation at 3,000g for 10 min within a day of collection. Plasma was removed carefully to ensure that the buffy coat was undisturbed. The plasma sample was undergone a second centrifugation at 3000 rpm for 10 min, and the re-centrifuged plasma was collected and stored at $-20\ ^{0}C$ until analysis.

Sample size determination for comparison of two independent groups was calculated using the following formula:

$$n\ /group = \frac{2\sigma^2(Z_{\alpha/2} + Z_{\beta})^2}{(\mu_1-\mu_2)^2}$$

* : Pooled variance in group1,2 (σ^2) = 4.32

Alpha error level or confidence level 95% ($Z_{\alpha/2}$) = 1.96

Beta error level or statistical power 80% (Z_{β}) = 0.84

Different in mean between two groups ($\mu_1-\mu_2$) = 1.11

$$n\ /group = \frac{2x\ 5.12x(1.96 + 0.84)^2}{(1.11)^2}$$

$$= 55$$

* Base on the level of GAPDH gene in 37 maternal plasma DNA samples.

1.1.1 Maternal plasma DNA preparation

Plasma DNA was extracted with a QIAmp DNA Blood Mini Kit (Qiagen) using the "blood and body fluid protocol" provided by manufacturer. A total of 400 μL plasma sample was used for DNA extraction per column. The DNA was eluted into a final volume of 50 μl and 5 μL of the extracted DNA was used for PCR.

1.1.2 Real Time PCR

Amount of plasma DNA was determined using a *Taq*Man assay for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is present in all genome [32]. The TaqMan PCR analysis was carried in 25 μl reaction volumes containing plasma DNA template, 10 μM of each amplification primers (Forward primer 5'-CCCCACACACATGCACTTACC-3', reverse primer 5'-CCTAGTCCCA GGGCTTTGATT-3') and dual-labeled *Taq*Man probe (5'-(FAM)AAAGAGCTAG GAAGGACAGGCAACTTGGC(TAMRA)-3') in QuantiTect Probe PCR Master Mix kit (Qiagen). The PCR was carried out under the following conditions: incubate 95 ^{0}C for 10 minutes, to activate the *Taq* polymerase, followed by 40 cycles of 20 seconds at 94 ^{0}C and 1 minutes at 60 ^{0}C on a Rotor-Gene 3000 real-time DNA analysis system (Corbett Research, Sydney, Australia).

1.1.3 Detection of α-thalassemia genes

1.1.3.1 α-Thalassemia 1 (SEA type)

Identification of the α-thalassemia 1 (SEA type) was performed using the multiplex PCR methodology described previously [150, 151]. The PCR was carried out in 25 μl reaction containing DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1 M betaine, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, specific primers (A7, A9 and A1B) and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR amplification was performed with an initial heat activation step of 3 minutes at 94 °C followed by 40 cycles of 94 °C 1 minute, 55 °C 1 minute, 72 °C 1 minute with an additional final extension at 72 °C for 10 minutes using the GeneAmp PCR 9600 system (Perkin-Elmer Cetus Co., USA). The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

1.1.3.2 α-Thalassemia 2 ($-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb deletions)

Identification of the α^{+}-thalassemia ($-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb) using the multiplex PCR methodology was performed as described previously [152]. The PCR was carried out in 50 µl reaction containing DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 5% DMSO, 3.0 mM $MgCl_2$, 200 µM dNTPs, specific primers (A, B, C, D, αG26 and αG27) and 1 unit of *Taq* DNA polymerase (BIOTOOLS B&M Labs, S.A., Spain). PCR amplification was performed with an initial heat activation step of 3 minutes at 94 °C followed by 10 cycles of 94 °C 30 seconds, 60 °C 30 seconds, 68 °C 2 minutes, followed by 20 cycles of 94 °C 30 seconds, 60 °C 30 seconds, 68 °C 2 minutes (plus 20 seconds every cycle) using a DNA Thermal Cycle 480 (Perkin-Elmer Wellesley, MA, USA). The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

1.1.3.3 Hb Constant Spring (α^{ConSp}) and Hb Pakse' (α^{PS})

Identification of α^{ConSp} and α^{PS} genes using the multiplex PCR methodology was performed as described previously [153-155]. The PCR was carried out in 50 µl reaction containing DNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 4.8% DMSO, 1 M betaine, 3.0 mM $MgCl_2$, 200 µM each of dNTPs, specific primers (αG2, C3, αG17 and αG18) and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR amplification was performed with an initial heat activation step of 3 minutes at 94 °C followed by 30 cycles of 94 °C 1 minute, 65 °C 1.5 minutes, 72 °C 1 minute with an additional final extension at 72 °C for 10 minutes using the GeneAmp PCR 9600 system (Perkin-Elmer Cetus Co., USA). The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

1.2 Processing of blood samples from the subjects with abnormal Hb

EDTA blood of the subjects with uncharacterized abnormal Hb and their family members sent from various hospitals to the Faculty of Associated Medical Sciences were collected and used in this study.

1.2.1 Hematological and Hb analysis

Hematological data including Hb, Hct, MCV, MCH, MCHC and erythrocyte count were collected on an automated blood cell counter. Hb were analyzed using automated cation exchange high performance liquid chromatography (HPLC) hemoglobin analyzer (VariantTM; Bio-Rad Laboratories, Hercules, Calif., USA), LPLC and Capillary Zone Electrophoresis (SEBIA), according to the manufacturing protocols.

1.2.2 DNA preparation

Genomic DNA was prepared as follows: mix 500 µl of 3% dextran in normal saline solution with 500 µl of whole blood and stand for 30 min at room temperature. Transfer the upper layer of solution into a new tube, centrifuge at 8,000 rpm for 5 min at room temperature and discard supernatant fluid. Add 300 µl of 75 mM NaCl / 25mM EDTA buffer (pH 8.0) into the pellet and mix by vortex then add 15 µl of 20% SDS and 15 µl of pronase E (10 mg/ml). The solution is incubated for 6-12 hours at 37 ^{0}C. Add 85 µl of 6M NaCl solution and mix for 5 min. Centrifuge at 10,000 rpm for 5 min at room temperature. Transfer the upper layer to a new tube containing 800 µl of cold ethanol. Mix and keep the tube at – 80 ^{0}C for 15 min. Centrifuge at 12,000 rpm for 10 min at 4 ^{0}C. DNA pellet was collected and washed with 200 µl of cold 70 % ethanol dried up and dissolved in 100 µl sterile distilled water [156].

1.2.3 Screening for known Hb variant

Screening for known Hb variants were examined using allele specific PCR developed previously.

1.2.3.1 Routine PCR analysis of common variants.

(1) Multiplex ASPCR for detection of Hb S, Hb D-punjab and Hb Tak [157]

As shown in Figure 5, primers G37, G40 and G33 were used for detection of β^{S}, $\beta^{D\text{-}Punjaab}$, β^{Tak} mutations, respectively. Primers S1, G7 and H5 were common primers for Hb S, Hb D-punjab and Hb Tak, respectively. With this system primers G7 and H5 could be used as internal control of amplification. The PCR reaction mixture (50 µl) contains template DNA, specific amount of each primer

(i.e. 7.5 pmol of H5, S1 and G37, 4.5 pmol of G33, 75 pmol of G7 and 60 pmol of G40), 200 μmole/l dNTPs and 1 unit Taq DNA polymerase (Promega, Madison, WI, USA.) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA). A total of 30 cycles after initial heating at 94 ^{0}C for 3 min was performed under the following PCR conditions: 94 ^{0}C for 30 sec, 60 ^{0}C for 30 sec and 72 ^{0}C for 1 min. The amplified DNA product was analyzed by electrophoresis on a 2% agarose gel and visualized under UV-light after ethhidium bromide staining (Figure 5).

(2) ASPCR for detection of Hb Korle-Bu [2]

The specific primers, G39 and S3, were used for detection of $\beta^{Korle\text{-}Bu}$ and two addition primers, γ4 and γ5, were used as internal control of amplification. The PCR reaction mixture (50 μl) contains template DNA, 15 pmole of specific primers and 7.5 pmol of common primers, 200 μM dNTPs and 1 unit Taq DNA polymerase (Promega Co., USA) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with an initial heat activation step of 3 minutes at 94 ^{0}C followed by 30 cycles of 94 ^{0}C for 1 minute and 65 ^{0}C for 1 minute 30 seconds. Then, the amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethhidium bromide staining (Figure 6).

(3) ASPCR for detection of Hb Q-Thailand [92]

The specific primers, αG20 and B, were used for detection of α^{QT}, primers C and D, were used for detection of α_2-globin gene and two addition primers, γ4 and γ5 were used as internal control of amplification. The PCR reaction mixture (50 μl) contains template DNA, 60 pmol of each C and D primers, 15 pmol of specific primers αG20 and B, 30 pmol of each internal control primer, 200 μM dNTPs and 2.5 unit Taq DNA polymerase (Promega Co., USA) in 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM KCl, 1 mM EDTA, o.1% triton X-100, 5% glycerol (v/v), 5% dimethyl sulphoxide (DMSO) and 3 mM MgCl2. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with

an initial heat activation step of 3 minutes at 94 ^{0}C followed by 30 cycles of 94 ^{0}C for 1 minute and 65 ^{0}C for 1 minute 30 seconds. Then, the amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethhidium bromide staining (Figure 7).

(4) Multiplex PCR for identification of Hb Queens and Hb Siam [96]

The multiplex PCR reaction mixure (50 µl) contains genomic DNA, 15 pmole of primers C1, αG 19 and αG35, 30 pmole of primer B, 200 µmole/l dNTPs and 1 unit Taq DNA polymerase (Promega, Madison, WI, USA.) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA). A total of 35 cycles after initial heating at 94 ^{0}C for 3 min was performed under the following PCR conditions: 94 ^{0}C for 30 sec, 60 ^{0}C for 30 sec and 72 ^{0}C for 1 min. The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

(5) ASPCR for detection of Hb Hope [158]

The specific primers, G41 and H5, were used for detection of β^{Hope} and two addition primers, γ4 and γ5, were used as internal control of amplification. The PCR reaction mixture (50 µl) contains template DNA, 15 pmole of each primer, 200 µM dNTPs and 1 unit Taq DNA polymerase (Promega Co., USA) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with an initial heat activation step of 3 minutes at 94 ^{0}C followed by 30 cycles of 94 ^{0}C for 1 minute and 65 ^{0}C for 1 minute 30 seconds. Then, the amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethhidium bromide staining (Figure 8).

(6) Multiplex ASPCR for detection of Hb Pyrgos and Hb J-Bangkok [159]

Primer G36 and G32 used for detection of $\beta^{J\text{-}Bangkok}$ and β^{Pyrgos}, respectively. Primer S3 was common primer. With this system primer γ4 and γ5 could be used as internal control of amplification. The multiplex PCR reaction

mixure (50 μl) contains genomic DNA, 15 pmole of primers G36, G32 and S3, 30 pmol of primers γ4 and γ5, 200 μmole/l dNTPs and 1 unit Taq DNA polymerase (Promega, Madison, WI, USA.) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA). A total of 30 cycles after initial heating at 94 ^{0}C for 3 minute was performed under the following PCR conditions: 94 ^{0}C for 1 minute and 65 ^{0}C for 1 minute 30 seconds. The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

(7) ASPCR for detection of Hb Beijing [160]

The specific primers, αG23 and C3, were used for detection of $\alpha^{Beijing}$ and two addition primers, C1 and C3, were used as internal control of amplification. The PCR reaction mixture (50 μl) contains template DNA, 45 pmole of C3, 15 pmol of C1, αG23 primers, 200 μM dNTPs, 1 M betain, 5% DMSO and 1 unit Taq DNA polymerase (Promega Co., USA) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with an initial heat activation step of 3 minutes at 94 ^{0}C followed by 10 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds and 68 ^{0}C for 2 minute and 20 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds and 68 ^{0}C for 2 minutes (plus 20 seconds every cycle). The amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethidium bromide staining.

1.2.3.2 Analysis for unknown hemoglobin variants.

Entire α- and β-globin genes was amplified using specific primers as shown in Table 4.

(1) α-Globin gene amplification

Selective amplification of α1- and α2-globin genes were obtained by PCR with two separate sets of primers. Primers (C1+B) and (C1+C3) are α1- and α2-globin genes specific, repectively. Each PCR reaction (50μl) contained DNA template, 200 μM dNTPs, 0.75 M betain, 5% DMSO and 1 unit Taq DNA polymerase (Promega Co., USA) in 12 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM

MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with an initial heat activation step of 3 minutes at 94 ^{0}C followed by 10 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds and 68 ^{0}C for 2 minute and 20 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds and 68 ^{0}C for 2 minutes (plus 20 seconds every cycle). Then, the amplified DNA product was analyzed by electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethhidium bromide staining.

(2) β-Globin gene amplification

The PCR reaction for amplification of β-globin gene as performed using 2 sets of primer pairs. Primers (S1+S3) were use for amplification of exon 1 whereas primers (YK15+F13) were used for amplification of exon 2 and exon 3. Each PCR reaction mixture (50 μl) contains template DNA, 30 pmole of each primer, 200 μM dNTPs and 1 unit Taq DNA polymerase (Promega Co., USA) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin. Amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin Elmer Cetus Co., USA) with an initial heat activation step of 3 minutes at 94 ^{0}C followed by 30 cycles of 94 ^{0}C for 1 minute and 65 ^{0}C for 1 minute 30 seconds. Then, the amplified DNA product was analyzed by Electrophoresis on a 1.5% agarose gel and visualized under UV-light after ethhidium bromide staining.

(3) DNA sequencing of globin gene by automated DNA sequencer

Direct DNA sequencing of the amplified fragment was performed on an ABI Prism cycle sequencing dye primer ready reaction kit according to the manufacturer's instructions (Perkin-Elmer Biosystems Corp., Norwalk, CT, USA) at the Bioservice Unit (BSU), National Science and Technology for Development Agency, Thailand.

2. Development of multiplex ASPCR for differential diagnosis of Hb Hope and Hb Phimai

A multiplex ASPCR for simultaneous detection of Hb Hope and Hb Phimai mutations was developed as shown in Figure 15. With this simultaneous detection system, G64 specific primer was used with a common primer S3 for β^{Phimai} mutation and G41 specific primer was used with a common primer H5 for β^{Hope} mutation, to produce specific fragments of 131 bp and 333 bp, respectively. In each reaction tube, γ4 and γ5 were additional primers used to produce the fragments of 578 bp for PCR control [161]. The multiplex PCR reaction mixure (50 μl) contains 50-200 ng genomic DNA, 15 pmol of G41 and H5 primers and 30 pmol of G64 and S3 primers, 200 mmol/l dNTPs and 1 unit Taq DNA polymerase (New England Biolabs, Inc.: U.S.) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatine and 3 mM $MgCl_2$. The amplification reaction was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA, USA). A total of 30 cycles after initial heating at 94oC for 3 min were performed under the following PCR conditions: 94 °C for 45 s, 65 °C for 1 min and 72 °C for 45 s. The amplified product was analyzed on 1.5% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

3. Haplotype analysis

3.1 α-Globin gene haplotype analysis

Six polymorphic sites within α-globin gene cluster were used to define haplotype. These includes the *Xba*I site in the upstream of the ζ2-globin gene, the *Bgl*I site in the upstream of inter-ζ HVR, the inter-ζ HVR (IZHVR) site between ζ2- and ψζ1-globin genes, the *Acc*I site in the down stream of the ψα2-globin gene, the *Rsa*I site in the upstream of the α2-globin gene and the *Pst*I site in the upstream of the α1-globin gene which were determined by the polymerase chain reaction followed by restriction enzyme digestions, as described previously [162]. The α–globin gene cluster with the position of six polymorphic sites was shown in Figure 16. Amplification primers used for each polymorphic sites were listed in Table 5. The digestion reaction of PCR product for each polymorphic site contained

PCR product	8 μl
10X restriction buffer	2 μl
BSA 10 μg/μl	0.2 μl
H2O	8.8 μl
Restriction enzyme	1.0 μl

Digestion was carried out for 2 hours at 37 °C before addition of 1 μl loading dye and analyzed on 1.5% agarose gel electrophoresis.

3.2 β-Globin gene haplotype analysis

Seven polymorphic restriction sites in the β-globin gene cluster shown in Figure 17. Including the *Hinc*II site 5' of the ε-globin gene, the *Hind*III sites in the $^{G}\gamma$ and $^{A}\gamma$-globin genes, the *Hinc*II site in the ψβ-globin gene in the its 3' region, the *Ava*II and *Bam*HI in the β-globin gene and its 3' were determined using PCR followed by restriction endonuclease digestion. β-Globin gene haplotypes were defined as described [163]. Amplification primers used for each polymorphic site were listed in the Table 6. The digestion reaction of PCR product for each polymorphic site contained

PCR product	8 μl
10X restriction buffer	2 μl
100 mM spermidine	0.2 μl
H2O	8.8 μl
Restriction enzyme	1.0 μl

Digestion was carried out for 2 hours at 37 °C before addition of 1 μl loading dye and analyzed on 1.5% agarose gel electrophoresis.

4. Oligonucleotide primers

Table 4 Sequences of oligonucleotide primers and their calculated melting temperatures

Primer	Sequence (5'→3')	Tm (^{0}C)
B	GAGGCCCAAGGGGCAAGAAGCAT	69
C	GCTAGAGCATTGGTGGTCATGCC	60.5
C1	TGGAGGGTGGAGACGTCCTGG	65
C3	CCATTGTTGGCACATTCCGGGACA	69
D	TTCTGACTCTGCCCACAGCCTGA	62.6
γ4	GGCCTAAAACCACAGAGT	49
γ5	CCAGAAGCGAGTGTGTGGAA	58
S1	TGTCATCACTTAGACCTCAC	46
S3	TCCCATAGACTCACCCTGAA	54
YK15	TCTCTCTGCCTATTGGTCTA	49
F13	AATGCACTGACCTCCCACAT	56
G7	GATACAATGTATCATGCCTCT	47
G32	CACGACAACCTCAAGGA	54
G33	CTGGCCCACAAGTATCACAC	54
G36	ACTCCTGATGCTGTTATGGA	60
G37	AGTAACGGCAGACTTCTCCA	62
G39	GTGCTCGGTGCCTTTATGA	53
G40	TGCACTGGTGGGGTGAATTG	64
G41	TATCAGAAAGTGGTGGCTGA	60
G28R	ACCACCAACTTCATCCACGC	64
G64	AAAGTGCTCGGTGCCTTTAG	58.3
H5	GCAGCCTCACCTTCTTTCATGG	70
αG2	GCTGACCTCCAAATACCGTC	64
αG17	AGATGGCGCCTTCCTCTCAGG	64
αG18	ACGGCTACCGAGGCTCCAGCA	72
αG19	CAACGTCAAGGCCGCCTGGC	70
αG35	TTCTCCCCGCAGGATGTTCCG	65.6
αG20	CAACGCCGTGGCGCACGTGC	72
αG23	TCAAGGCCGCCTGGGGTAAT	61.1

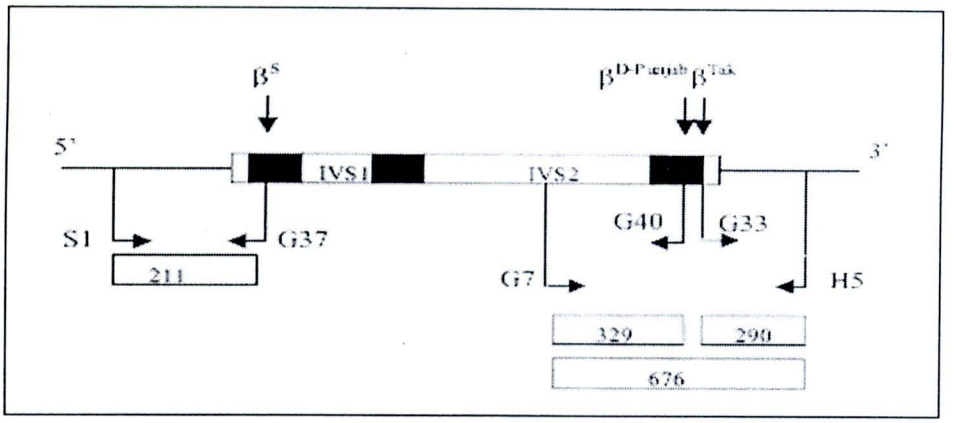
Table 5 Sequences of oligonucleotide primers used for analysis of α-globin gene haplotypes [164]

Primers	**Sequences (5'→3')**	**Polymorphic site**
αG26 (sense)	CTCAACAGATGCCAGCCAAACAGAC	*Xba*I-5'ζ2
αG27 (antisense)	GAACCATTACACCAAGTGAAGGACG	
αG29 (sense)	CCAGGACACAGATGGAGGCTAATGT	*Bgl*I / IZHVR
αG30 (antisense)	TTGGGGTTGGGTGCATGAGGC	
αG31 (sense)	AGGCATGGGCCGCCATTCCTGG	*Acc*I-3'ψα2
αG32 (antisense)	CTTGATCTGGGCTGAGCC	
αG24 (sense)	GCACCTCCCACCCTCCCCCTC	*Rsa*I-5'α2
αG25 (antisense)	GTCTCCACCCTCCACCCGCCACTC	
αG33 (sense)	GGTTGATGCTCCAGCCGGTTCCAGCTATTGC	*Pst*I-5'α1
αG34 (antisense)	CCAGAAGAGTGCCGGGCC	

Table 6 Sequences of oligonucleotide primers used for analysis of β-globin gene haplotypes [163]

Primers	**Sequences (5'→3')**	**Polymorphic site**
SF1 (sense)	GCCCATCACCAAGGCAAT	*Bam* HI-3'β
SF2 (antisense)	GCTCTACGGATGTGTGAGAT	
SF3 (sense)	GTTTGTGTGTGTGAGAGC	*Hind* III-Gγ
SF4 (antisense)	TCTTTAGGCATGCGTCAACA	
SF5 (sense)	TTAACGTCTTCAGCCTACAA	*Hind* III-Aγ
SF6 (antisense)	CAATCTGCACACTTGAGGGGC	
SF7 (sense)	GGCACATGGATCGAATTGAA	*Hinc* II-5'ε
SF8 (antisense)	ACCATGATGCCAGGCCTGAC	
SF9 (sense)	GGGAACAGAAGTTGAGAGATAG	*Hinc* II-ψβ
SF10 (antisense)	CTCTTTTCTTGCAGGATTGC	
SF11 (sense)	GCTGGATGAACAAACATTCC	*Hinc* II-3'ψβ
SF12 (antisense)	AAGGAGCACCCACTAGCTCA	
YK15 (sense)	TCTCTCTGCCTATTGGTCTA	*Ava* II-β
G8 (antisense)	GCTTGGACTCCAGAATAATCC	

A.



B.

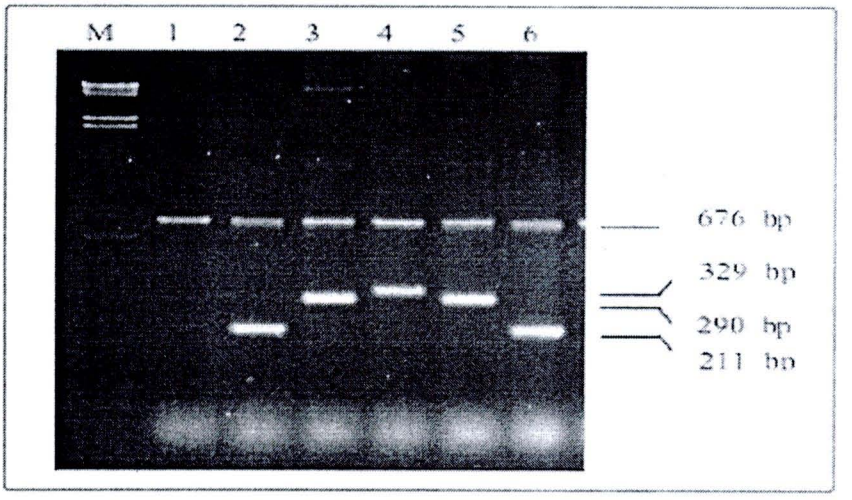
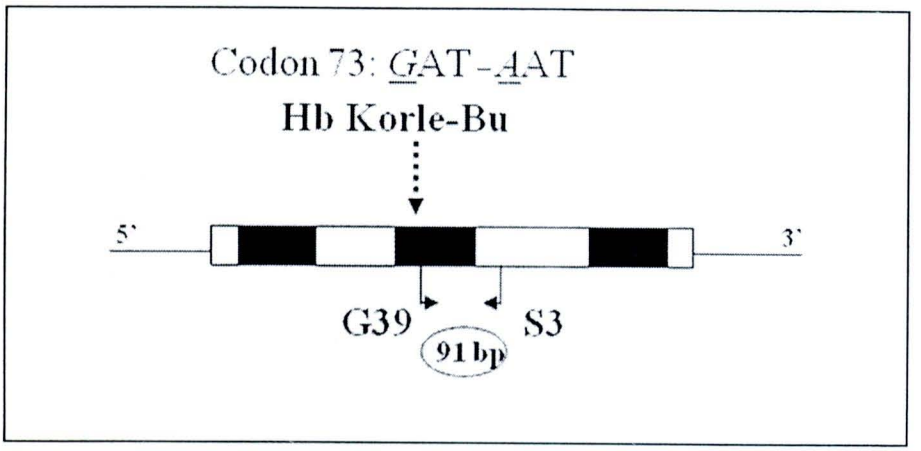


Figure 5 A simultaneous multiplex allele-specific PCR for differentiation of Hb S, Hb D-Punjab and Hb Tak

A: The locations and orientation of primers (S1&G37), (G7&G40) and (G33&H5) that produce fragments of 211 bp, 329 bp and 290 bp, specific for Hb S, Hb D-Punjab and Hb Tak are indicated. The 676 bp fragment generated from primers (G7&H5) is an internal control for the PCR amplification.

B: A representative agarose gel electrophoresis of the multiplex allele specific PCR analysis. Lane 1: normal control, lane 2&6: Hb S carriers, lane 3&5: Hb Tak carriers and lane 4: Hb D-Punjab carrier. M represents the λ/*Hind* III size markers.

A.



B.

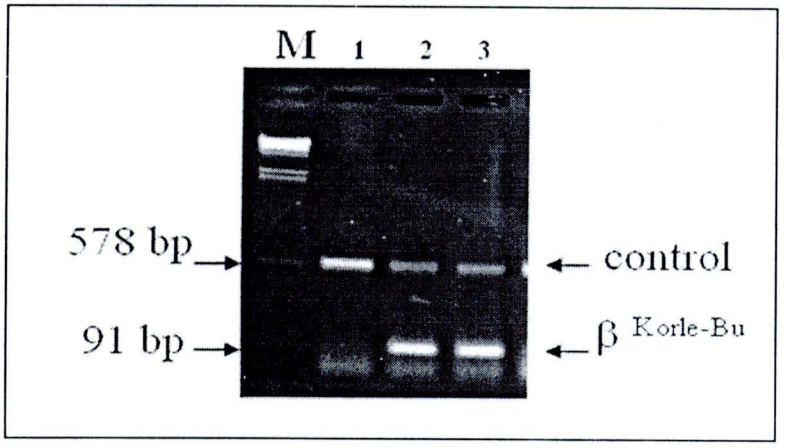
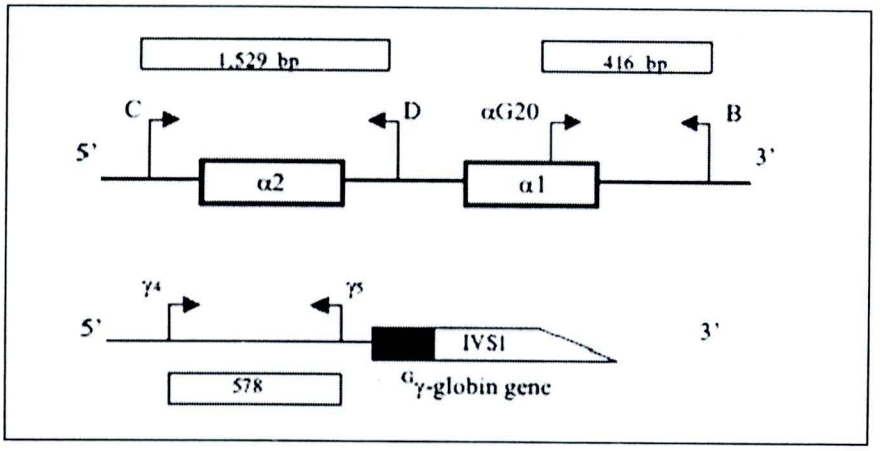


Figure 6 An allele specific PCR for detection of Hb Korle-Bu

A: The locations and orientations of primers G39&S3 on the β-globin gene and the size of amplified fragment is depicted. The 578 bp is an internal control fragment of the $^{G}\gamma$-globin promoter whereas the 91 bp indicates the presence of the $\beta^{Korle\text{-}Bu}$ gene.

B: A representative agarose gel electrophoresis of an allele specific PCR analysis. Lane 1: normal control, lane 2&3: Hb Korle-Bu carriers. M represents the λ/*Hind* III size markers.

A.



B.

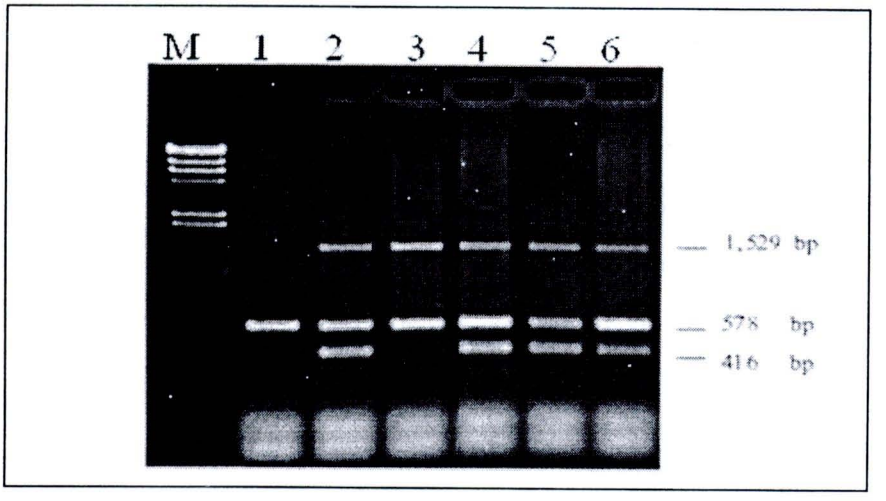
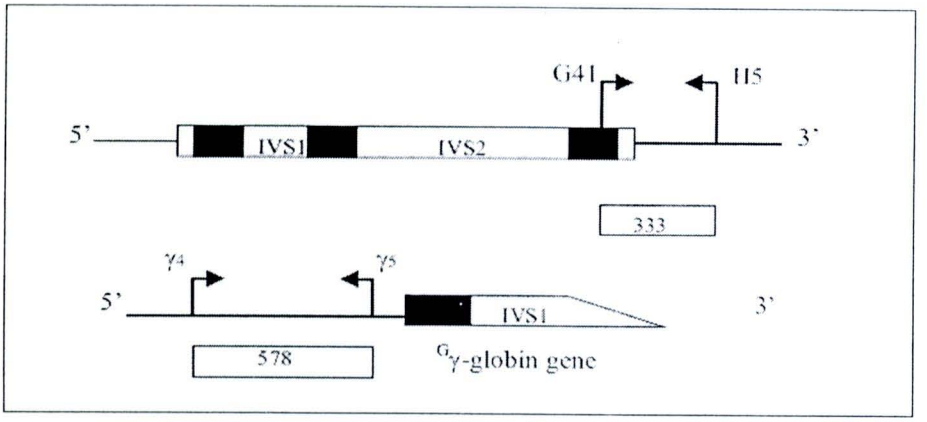


Figure 7 A simultaneous multiplex allele-specific PCR for differentiation of the $-\alpha^{4.2}$ and the $\alpha^{Q\text{-Thailand}}$ mutation in *cis*

A: The locations and orientation of primers (C&D) and (αG20&B) that produce fragments of 1,529 bp and 416 bp, specific for the $-\alpha^{4.2}$ and the $\alpha^{Q\text{-Thailand}}$, respectively. The 578 bp is an internal control fragment of the $^{G}\gamma$-globin promoter.

B: A representative agarose gel electrophoresis of the multiplex allele specific PCR analysis. Lane 1: normal control, lane 2&4-6: Hb Q-Thailand carriers, lane 3: $-\alpha^{4.2}$ carrier. M represents the λ/*Hind* III size markers.

A.



B.

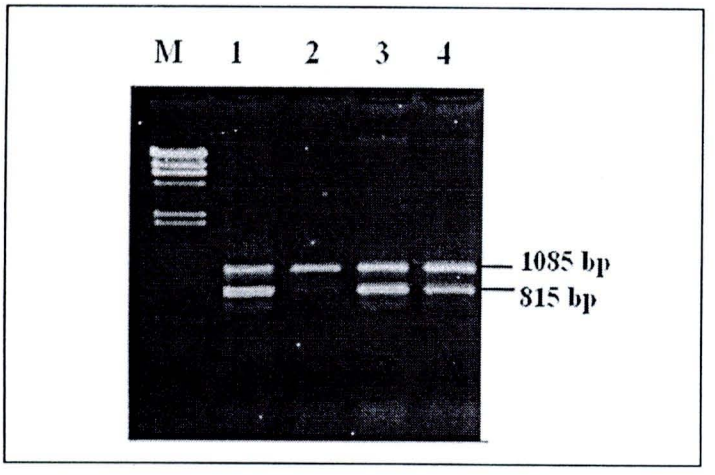
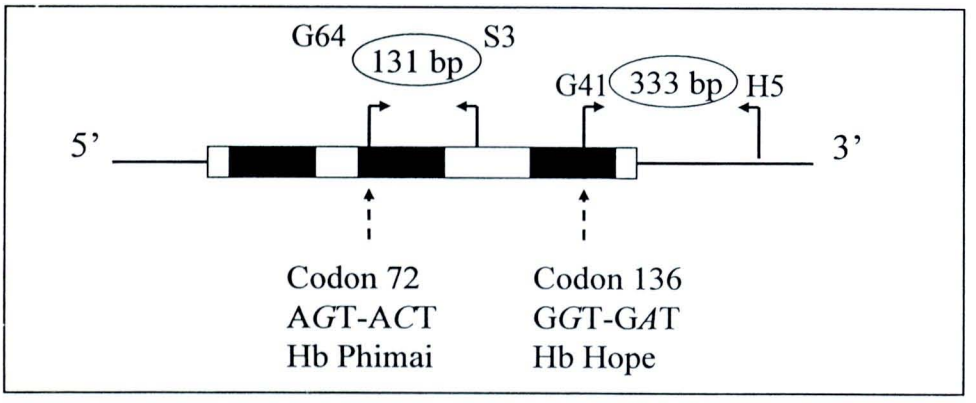


Figure 8 An allele specific PCR for detection of Hb Hope

A: The locations and orientations of primers G41&H5 on the β-globin gene and the size of amplified fragment is depicted. The 578 bp is an internal control fragment of the $^{G}\gamma$-globin promoter whereas the 333 bp indicates the presence of the β^{Hope} gene.

B: A representative agarose gel electrophoresis of an allele specific PCR analysis. Lane 1&3-4: Hb Hope carriers, lane 2: normal individual. M represents the λ/*Hind* III size markers.

A.



B.

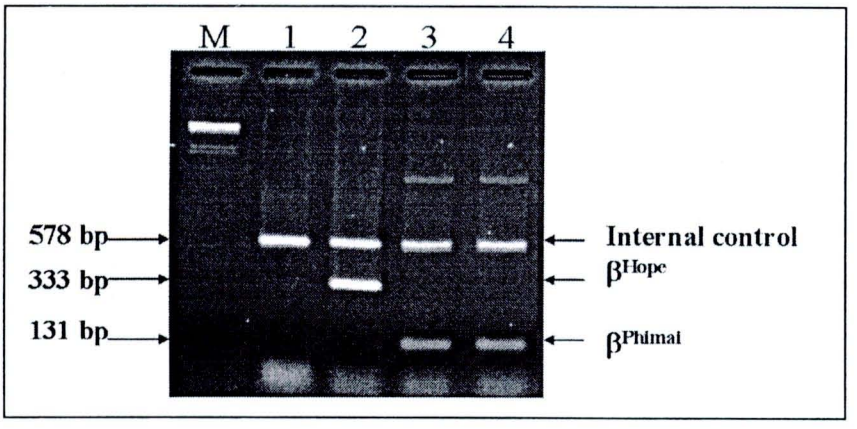


Figure 9 A simultaneous multiplex allele-specific PCR for differentiation of Hb Hope and Hb Phimai

A: The locations and orientation of primers (G41&H5) and (G64&S3) that produce fragments of 333 bp and 131 bp, specific for Hb Hope and Hb Phimai are indicated. The 578 bp is an internal control fragment of the $^{G}\gamma$-globin promoter.

B: A representative agarose gel electrophoresis of the multiplex allele specific PCR analysis. Lane 1: normal control, lane 2: Hb Hope carrier, lane 3&4: Hb Phimai carriers. M represents the λ/*Hind* III size markers.

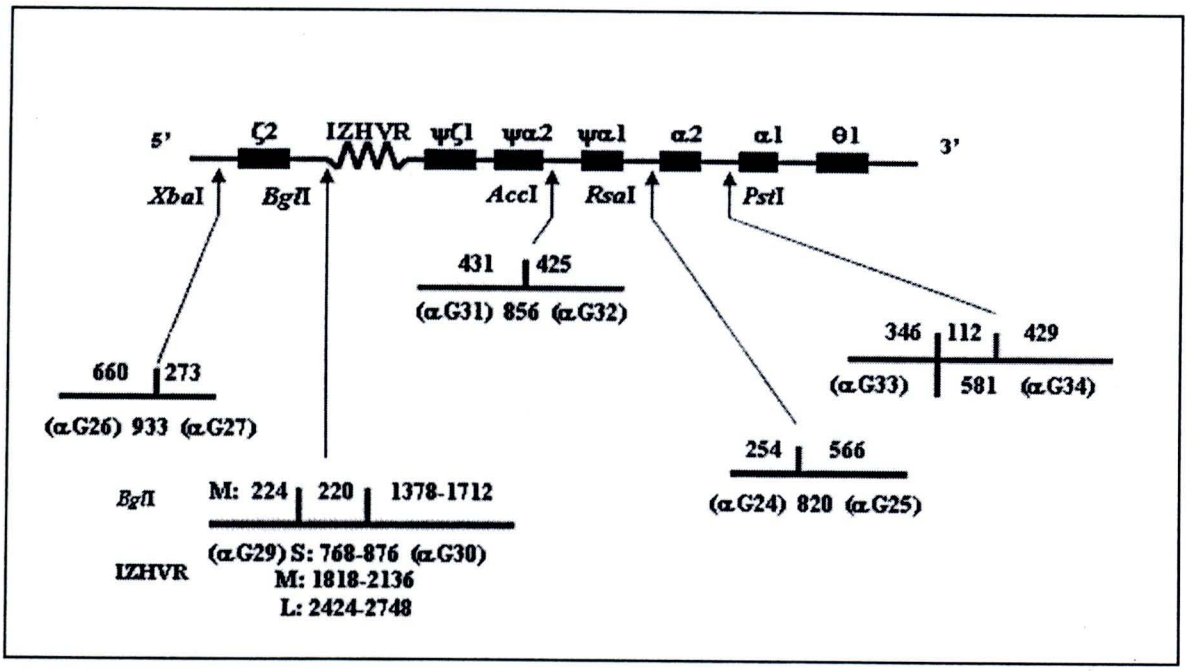


Figure 10 The α-globin gene cluster with the positions of six polymorphic sites used in this study. The regions amplified by PCR is indicated. Number along the lines are length in base pair of digested and undigested fragments. The name of each primer is shown in parentheses.

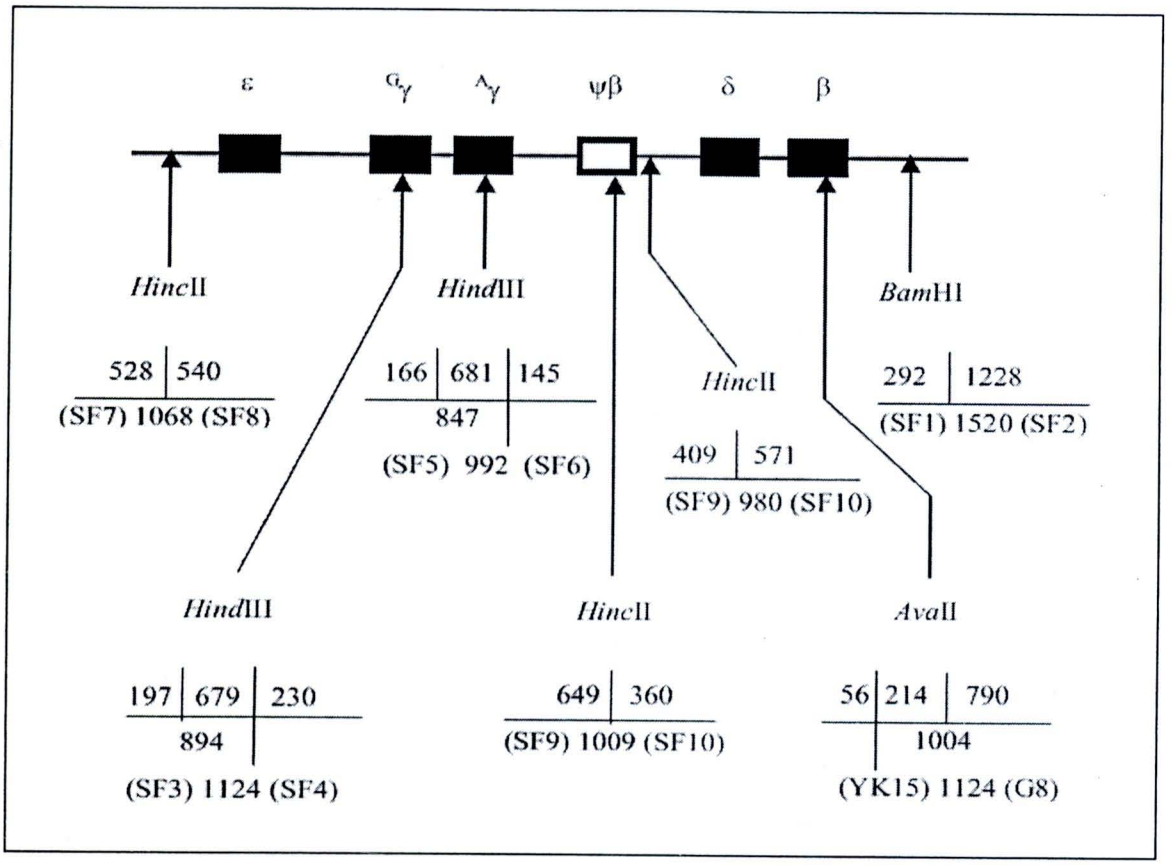


Figure 11 The β-globin gene cluster with the positions of seven polymorphic sites used in the construction of β-globin gene haplotype. The regions amplified by PCR are indicated. Number along the lines are length in base pair of digested and undigested fregments. The name of each primer is shown in parenthesis.