CHAPTER II

RESEARCH DESIGNS AND METHODS

2.1. Research designs.

Whole blood samples from Hb Chiang Mai carrier's and normal subject's were collected and kept at -20°C during the experimental period. Genomic DNA were extracted from the whole blood samples by using a method modified from Steger and co-workers (1994) and Chi-Chia and co-workers (1993) and were used as template for PCR. α_2- and α_1-globin genes were specifically amplified by using a modified PCR system from Molchanova and co-workers (1994) and McKeown and co-workers (1994). Each specific globin gene PCR product was then electrophoresed by using the vertical agarose gel as the gel matrix and then purified with a gel purification kit (Promega, Madison, USA) which finally divided into two aliquotes. For cycle sequencing, each PCR purification product was used as DNA template for Chain-termination cycle sequencing system (Perkin Elmer, Cetus, USA). The sequencing products were then purified and prepared prior to being injected into an ABI 310 "PRISM" DNA sequencer (Perkin Elmer, Cetus, USA) and the point mutation expected was determined from the sequencing data obtained. From the sequencing data, the point mutation in α_1- or α_2-globin genes was confirmed by using specific restriction enzyme digestion technique in which the purified α_1- and α_2-globin genes PCR products were used as DNA templates.

Each procedure is given in detail later in this chapter. In Figure 12, a schematic diagram presenting the conclusion of the whole research design is shown.

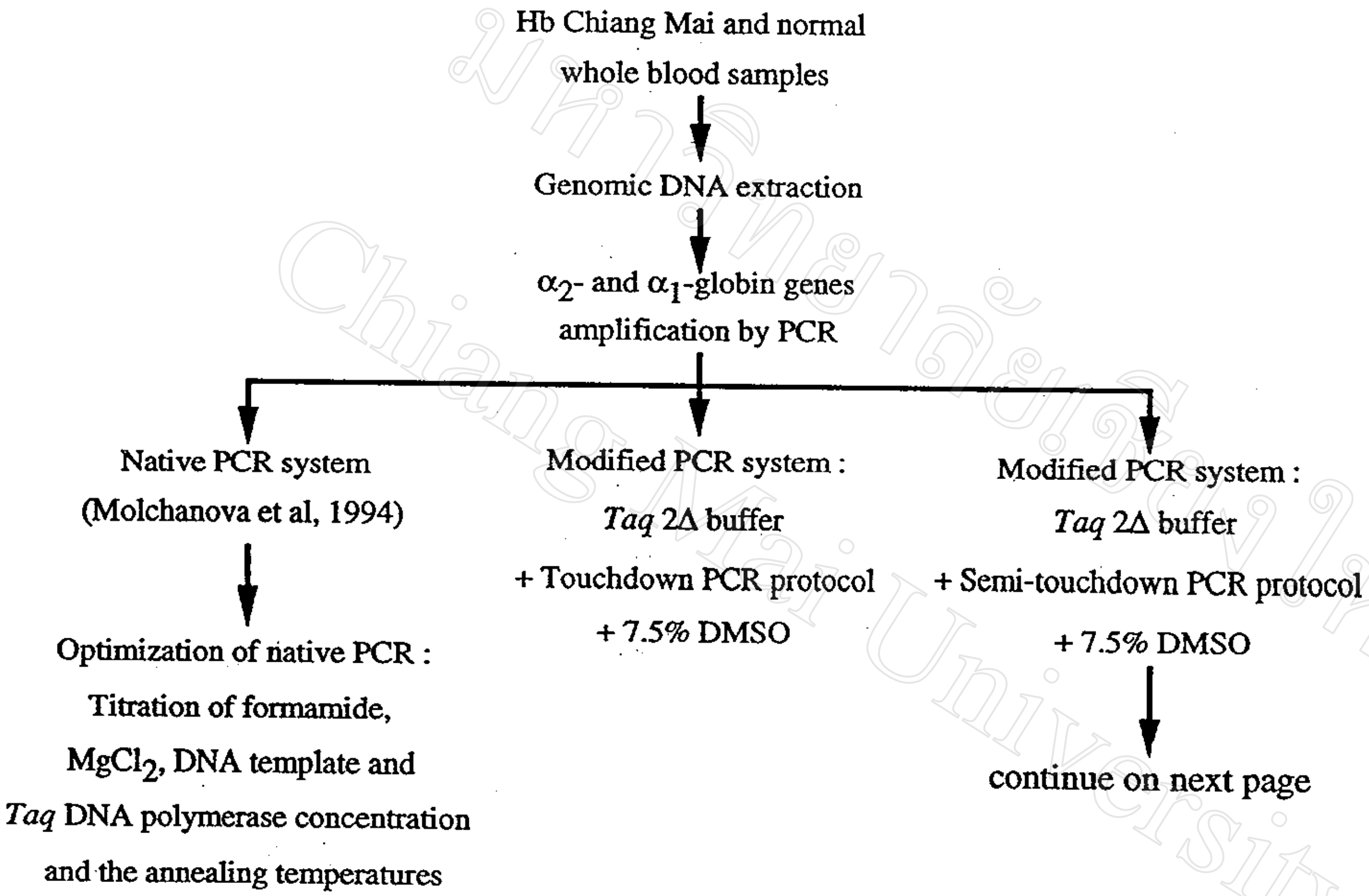

Figure 12. Diagrammatic representation of the research design in this study.

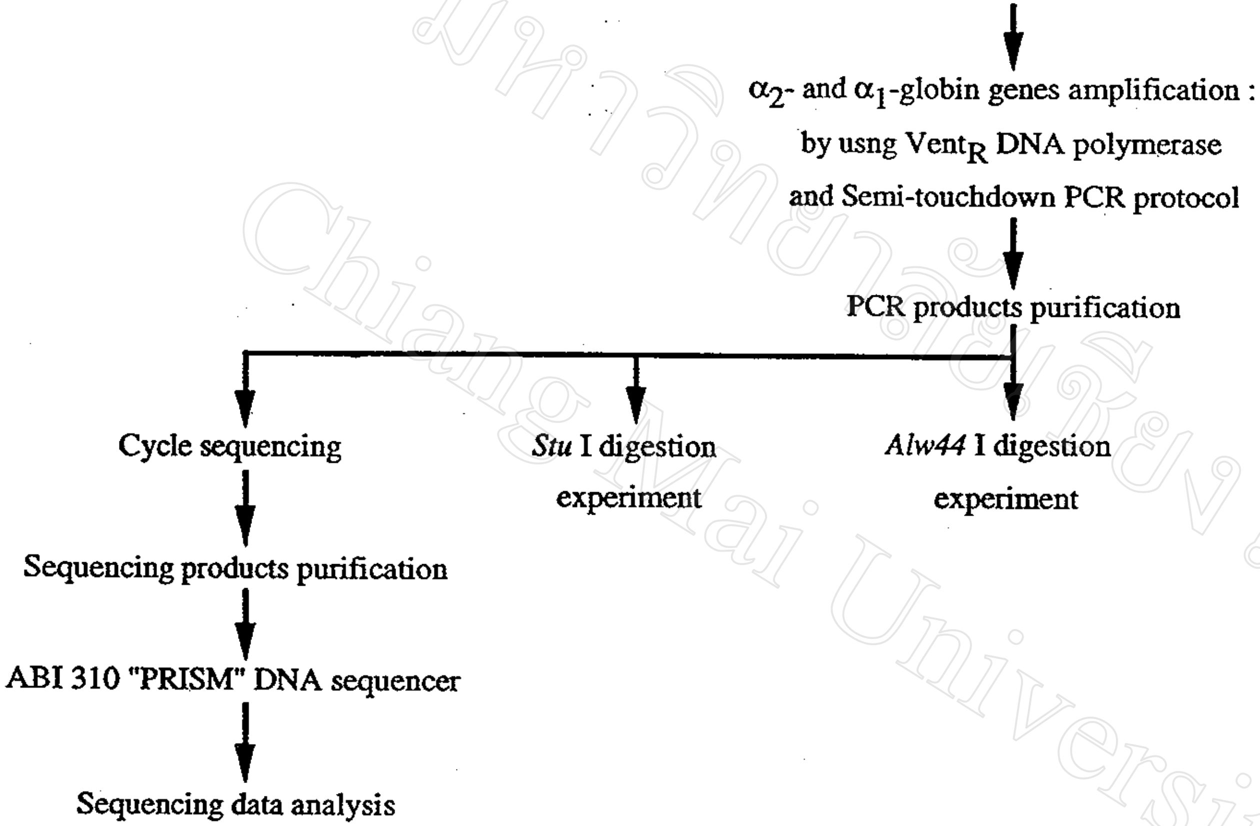

Figure 12.(continue) Diagrammatic representation of the research design in this study

2.2. Chemicals and materials.

Chemicals and materials used in the study are listed in the Appendix 1. Instruments and reagent preparations are also described in Appendix 2 and 3, respectively.

2.3. Study subjects.

Two subjects were used for study in this thesis. Hb Chiang Mai's carrier is the father of a β-thalassemia-major child (Sitthipreechacharn, 1994) who lives in Chiang Mai, Thailand. A normal sample (showing the Osmotic Fagility Test (OFT) value = 92) containing normal α-globin genes (αα/αα) was used as a negative control. Whole blood from these two subjects was collected from venous vessel by the officials of OPD 29th, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. Each 1 ml of the whole blood was aliquoted in 1.5 ml microfuge tubes containing 100 μl of 0.02 mg/ml EDTA in 0.9% NaCl and kept at -20°C during the experimental period.

2.4. Laboratory studies.

2.4.1. Genomic DNA isolation from whole blood.

Genomic DNA isolation was modified from Steger and co-workers (1994) and Chih-Chia and co-workers (1993) in order to used as little volume of the whole blood while maintaining the high purity and quantity of genomic DNA obtained. Genomic DNA were extracted from 50-200 μl of whole blood samples. They were washed once with 0.5% Triton x100/TE buffer pH 8.4 and 2-4 times with TE buffer pH 8.4. The red-colored supernatant containing haemoglobin and other proteins were discarded in each washing, and then centrifuged at 14,000 rpm for 1 min. The cell pellets was treated with proteinase-K at 45°C for 5 hr and then precipitated with 3 M NaOAc pH 5.6 and 95% EtOH. Finally, each was solubilized in 50 ml of TE buffer pH 8.4. The diagram of genomic DNA extraction is shown in Figure 13.

Nucleic acid and protein quantitation were performed by making a 1:200 dilution of stock genomic DNA solution with distilled-deionized water and measuring the optical density (OD) value with a Spectrophotometer at 260 and 280 nm, respectively. A simple equation to estimate the DNA quantity and purity was as follows ;

DNA (ng/µl) = OD_{260} x 50 x 200

when

OD_{260} = the OD measured at 260 nm

50 = DNA co-efficient

200 = dilution factor

Note :

1. Genomic DNA concentration should be 500 ng/µl or higher. This would facillitate the PCR optimization experiments specially in the pipetting step while the other components are already fixed in volume.

2. The higher the OD_{260}/OD_{280} value, the purer the nucleic acid from contaminated proteins. Normally, more than 1.2 is acceptable.

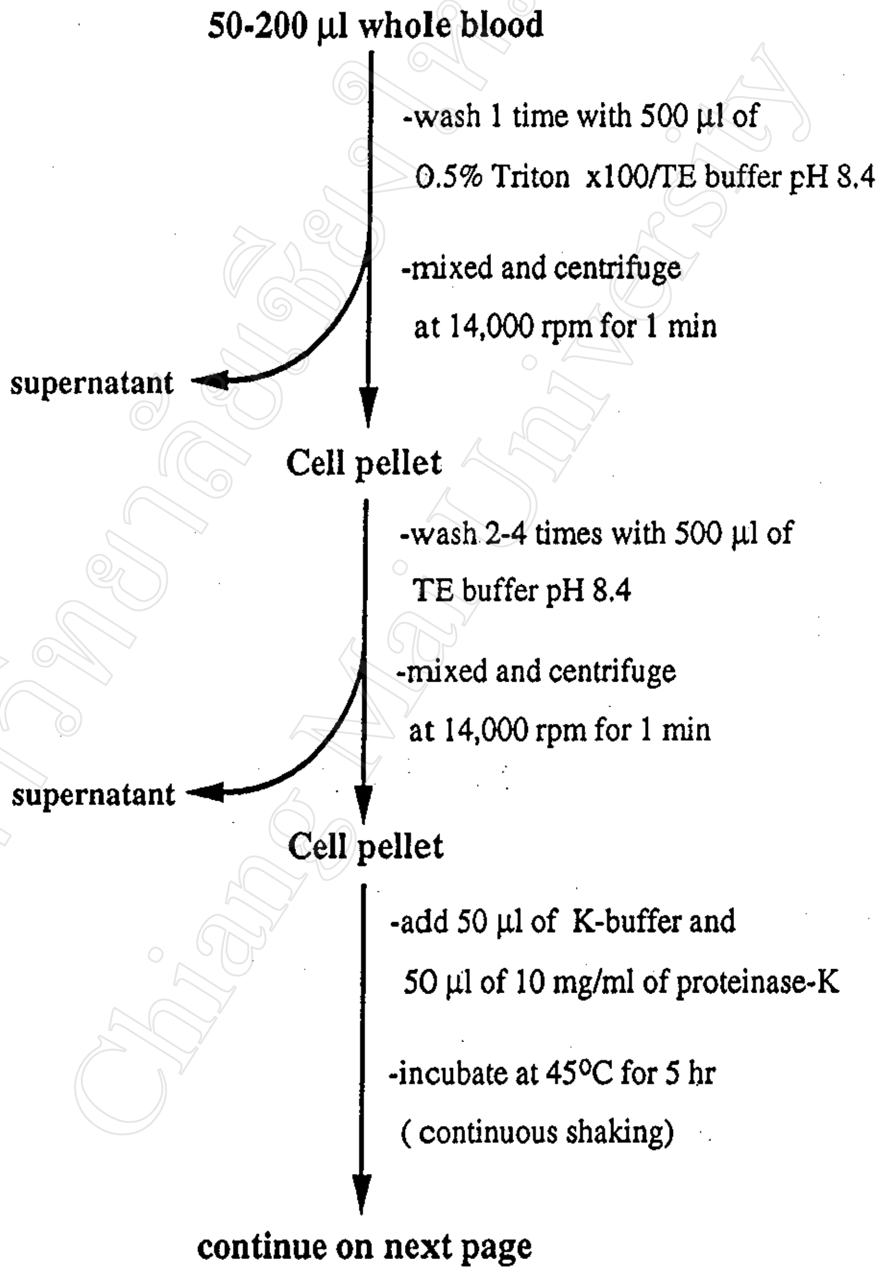

Figure 13. Diagrammatic representation of genomic DNA isolation from whole blood.

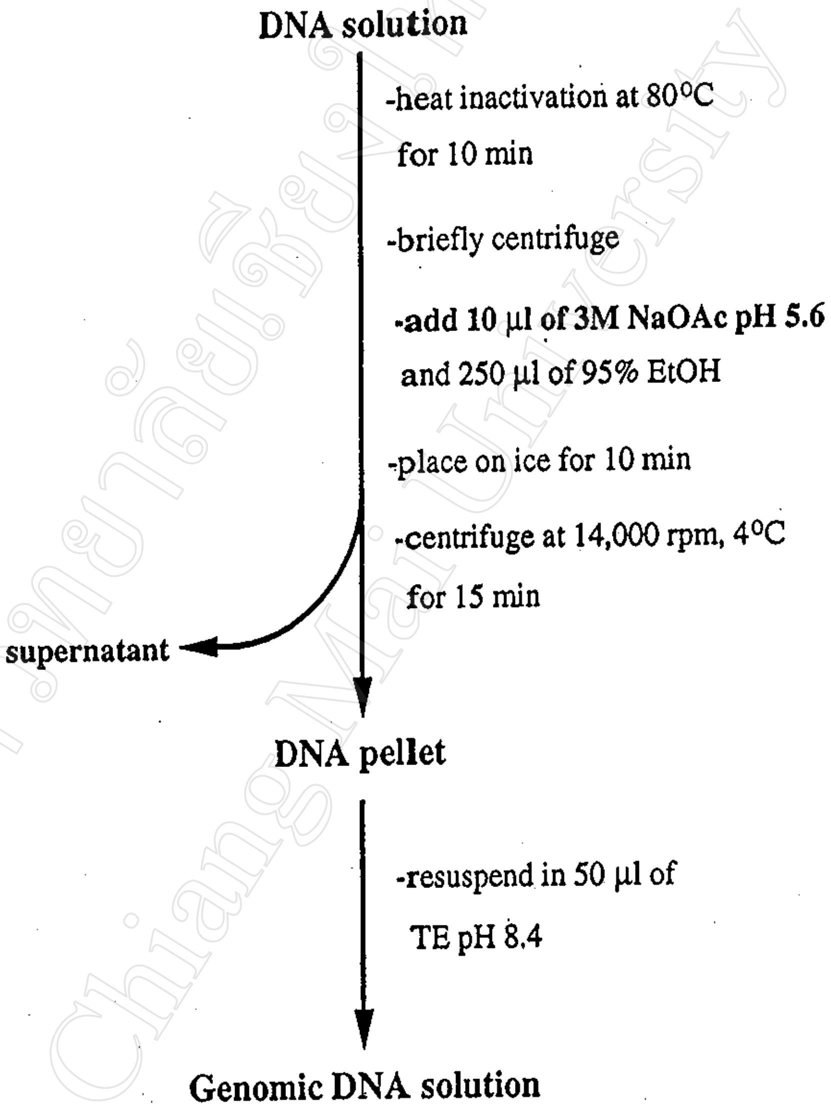

Figure 13.(continue) Diagrammatic representation of genomic DNA isolation from whole blood.

2.4.2 Preparation of gel electrophoresis.

2.4.2.1 Vertical agarose gel.

In this process, "Mini-PROTEAN®II Electrophoresis Cell" apparatus was mainly used, except for gel slab plates that were not from the main gel apparatus. The gel plate used was from used-up Mini-PROTEAN®II ready gel plate which was composed of a smaller-front glass plate and another larger plastic plate which had two 1.0 mm-thick spacers stuck on both left and right sides. Both plates were cleaned with detergent and water and dried at room temperature.

Agarose gel solution was prepared by combining agarose powder, Tris-Acetic acid-EDTA (TAE) buffer (1x final concentration) (Sambrook *et al.*, 1989) and distilled water into 300 ml glass bottle with a lid. The lid was loose to prevent inner pressure when the bottle was boiled in microwave oven. The gel was left to cool down to approximately 60-70°C at room temperature until the poring step.

Meanwhile, both gel plates were assembled according to the instruction manual from the company. For clean used-up ready gel plates, the smaller glass plate was placed along two spacer stuck on the larger plastic plate that was laid down on the table. The 3 edges, except the top, were sealed with transparant tape and then assembled in to the clamp assembly. All should be warmed using a hair dryer to blow the hot wind directly on to the surface of the plate and the clamp assembly. Note that, too cool a glass plate can be broken when too hot a gel mould is poured. The gel solution was pored smoothly with a pasture pipette and the air bubbles prevented until the solution reached the top of the front plate. If a higher gel percentage is desired, a hotter gel mould and slower pouring should be used in order to prevent air bubbles. Then, a comb was placed at the top of the plate sandwich. Another bul dog clip should be used to completely connect the comb to the inner surfaces of each plate. Finally, the gel was cooled down and set at room temperature for at least 10-15 min. Incubation at 4°C can be accelerated in the setting step without any interference.

After the gel has set, the bull dog clip and the comb should be gently removed from the gel sandwich. Transparant tape should also be removed from the bottom of the plate. Then, the gel sandwich set was attached to its core unit according to the instruction manual and the 1x TAE buffer, 4°C recommended, was poured into both reservoirs. Pre-run at 80-100 V for at least 5 min is recommened. 5 ml of PCR products were mixed with 1 ml of 6x loading buffer (Sambrook *et al.*, 1989) prior to loading into each well. The standard electricity applied to electrophoresis system was 150 V for 30 min.

2.4.2.2 Horizontal agarose gel.

Native-horizontal agarose gel was prepared as described by Sambrook and co-workers (1989). The gel dimension was 5.0 x 8.0 x 0.3 cm^3 with 1.0 mm-thicked 10 wells comb. The concentration of each native-horizontal agarose gel is indicated in the Figure legend. Standard electricity applied to the electrophoresis system was 55 V for 4 hr.

2.4.2.3 Polyacrylamide gel.

5% Polyacrylamide gel was prepared as described by Sambrook and co-workers (1989) and the details are given in Appendix 3. The gel plates and the comb used were the same as in vertical agarose gel. Standard electricity applied to electrophoresis system was 90 V for 90 min.

2.4.3 α_2- and α_1-globin genes amplification by Polymerase Chain Reaction.

2.4.3.1 Primers.

The sequences of α_2- and α_1-globin genes specific oligonucleotide primers were according to Molchanova and co-workers (1994). They were synthesized by Bio Service Unit (BSU), Department of Biochemistry, Mahidol University, Bangkok, Thailand. The sequences were as follows :

The forward primer (945), specific for both 5' ends of α-globin genes was 5'-CGC GCT CGC GGC CCG GCA C-3'

The reverse primer (2618), specific for 3' end of α_2-globin gene was 5'-GGG AGG CCC ATC GGG CAG GCG GAA C-3'.

The reverse primer (944), specific for 3' end of α_1-globin gene was 5'-GGG GGG GAG GCC CAA GGG GCA AGA A-3'.

Each stock solution was diluted to 10 mM final concentration with distilled-deionized water (the details are given in Appendix 3). The position of each primer specified for α_2- and α_1-globin genes and the length of the PCR products are shown in Figure 14.

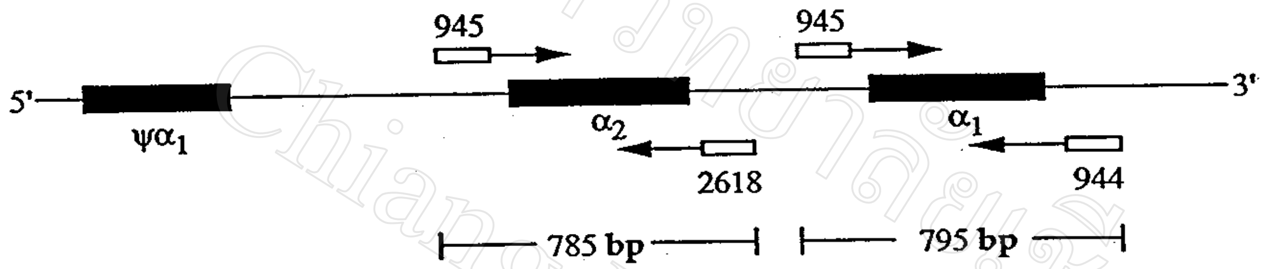

Figure 14. The position of each primers using for the specific amplification of α_2- and α_1-globin genes and the length of the PCR products

Forward primer, "945", was complementary to 5' end of both genes.

Reverse primers, "2618" and "944", were complementary to 3' end of α_2- and α_1-globin genes, respectively.

2.4.3.2. Optimization of α_2- and α_1-globin genes PCR.

In these experiments, α_2- and α_1-globin genes were amplified separately into 2 reaction tubes. The PCR reaction mixtures, 20 µl final volume each, were carried out in a 0.5 ml thin wall PCR tube and performed in "Omn-E" thermalcycler. PCR products were analysed on 2% VAGE at 150 V for 30 min. After staining in ethidium bromide staining solution and visualizing under long-wave length UV transluminator, the gels were photographed individually as indicated.

a) Native PCR system (Molchanova *et al.*, 1994).

First, α_2- and α_1-globin genes were amplified according to the methods described by Molchanova and co-workers (1994). Native reaction mixture and the three-step PCR protocol, totalling of 35 cycles, are shown in Table 3 and 4, respectively.

The reaction mixture, without DNA polymerase, was briefly mixed and overlayed with 30 µl of light-mineral oil to prevent the evaporation of the sample during PCR. After pre-heating at 99°C for 7 min, "hot start" was performed by adding 0.5 U of *Taq* DNA polymerase to the mixture. The other 0.5 U of *Taq* DNA polymerase was added to the reaction mixture at the 19th cycle.

Table 3. Native PCR reaction mixture (Molchanova *et al*.,1994). The preparation of native PCR reaction buffer is given in detail in Appendix 3.

Stock solutions	Final concentrations
10x PCR buffer :	
200 mM MOPS	20.0 mM
400 mM KCl	40.0 mM
100 % Tween-20	0.6%
1 mM each dNTPs	0.1 mM
100% Freshly-deionized formamide	7.5%
25 mM $MgCl_2$	2.0 mM
10 mM Forward primer (945)	0.02 mM
10 mM Reverse primer (2618 or 944)	0.5 mM
1,000 ng/µl Genomic DNA template	20.0 ng/µl
Distilled-deionized water	q.s.
0.5 U/µl Taq DNA polymerase	0.5 U/µl

Table 4. Native-three steps PCR protocol.(Molchanova *et al.*, 1994)

	Cycle 1-18	Cycle 19-34	Cycle 35
Stage 1	94°C : 59 sec*	94°C : 77 sec**	65°C : 7 min
Stage 2	68°C : 59 sec	68°C : 59 sec	
Stage 3	72°C : 90 sec	72°C : 90 sec	

* 1).The denaturation time was programmed to increase with 1 sec/cycle.

**2).The other 0.5 U of *Taq* DNA polymerase was added at the 19th cycle.

b) Titration of Formamide, $MgCl_2$, *Taq* DNA polymerase concentration and annealing temperature with native reaction mixture and native three-step PCR protocol.

1. Formamide, $MgCl_2$, Genomic DNA template and *Taq* DNA polymerase concentration were titrated individually in PCR optimization experiments as indicated in each Figure legend, while the other reaction mixture components and the native three-step cycle condition (as indicated in Table 3 and 4), showed no change.

2. The annealing temperature was titrated in PCR optimization experiments as indicated in the Figure legend while the other reaction mixture components were as indicated in Table 3 and 4.

2.4.3.3 α_2- and α_1-globin genes amplification by using *Taq* 2Δ buffer with "Touchdown" and "Semi-touchdown" PCR protocol.

In these experiments, α_2- and α_1-globin genes were separately amplified into 2 reaction tubes. The PCR reaction mixtures, 20 µl final volume each, were carried out in a 0.5 ml thin wall PCR tube and performed in an "Omn-E" Thermalcycler. PCR products were analysed on 2% VAGE at 150 V for 30 min. After staining in ethidium bromide staining solution and visualizing under long wavelength UV transluminator, the gels were photographed.individually

a) "Touchdown" PCR protocol.

The primers (according to Molchanova and co-workers (1994) were used in this optimization experiment. The other reaction mixture components such as native PCR reaction buffer, co-solvent and the cycle condition were changed. The modified reaction mixture is shown in Table 5.

The reaction mixture, without *Taq* DNA polymerase, was overlayed with 30 µl of light-mineral oil. After pre-heating the mixture at 99°C for 3 min, "hot start" was performed by adding 0.03 U of *Taq* DNA polymerase to the PCR mixture. "Touchdown" PCR protocol, totalling 31 cycles, is shown in Table 6.

Table 5. Modified PCR reaction mixture (from McKeown, 1994 and Molchanova and co-workers., 1994). Preparation of *Taq* 2Δ buffer is given in the detail in Appendix 3.

Stock solutions	Final concentrations
10 x Taq 2Δ buffer :	
450 mM Tris-HCl (pH 8.9, at 25°C)	45.0 mM
110 mM $(NH_4)_2SO_4$	11.0 mM
67 mM 2-Mercaptoethanol	6.7 mM
44 mM EDTA (pH 8.9)	4.4 mM
1 mg/ml BSA	113 mg/ml
1 mM each dNTPs	0.2 mM
100% DMSO	7.5 %
25 mM $MgCl_2$	1.5 mM
10 mM Forward primer (945)	0.25 mM
10 mM Reverse primer (2618 or 944)	0.25 mM
1,000 ng/μl Genomic DNA template	7.0 ng/μl
Distilled-deionized water	q.s.
0.5 U/μl Taq DNA polymerase	0.03 U/μl

Note : "Hot start" PCR was performed by adding 0.03 U of *Taq* DNA polymerase to the reaction mixture after heating at 99°C for 3 min.

Table 6. "Touchdown" PCR protocol.

	Cycle 1-10	Cycle 11-30	Cycle 31
Stage 1	94°C : 60 sec	94°C : 60 sec	70°C : 5 min
Stage 2	80°C* : 60 sec	67°C : 60 sec	
Stage 3	70°C : 120 sec	70°C : 120 sec	

*The annealing temperatures were programed to decrease 1.2°C/cycle for 10 cycles and reach the exact annealing temperature (67°C) at the 11th cycle.

b) "Semi-touchdown" PCR protocol.

Modified PCR reaction mixture as indicated in Table 6, without Taq DNA polymerase, was overlayed with 30 μl of light-mineral oil. After pre-heating the mixture at 99°C for 3 min, "hot start" was performed by adding 0.03 U of *Taq* DNA polymerase to the mixture. "Semi-Touchdown" PCR protocol, totalling 31 cycles, is shown in Table 7.

Table 7. "Semi-touchdown" PCR protocol. The temperature was programmed to decrease from 80°C to reach the exact annealing temperature at 67°C separately into 2 steps 5 cycles each (at 80°C and 73.5°C)

	Cycle 1-5	Cycle 6-10	Cycle 11-30	Cycle 31
Stage 1	94°C : 60 sec	94°C : 60 sec	94°C : 60 sec	70°C : 5 min
Stage 2	80°C : 60 sec	73.5°C : 60 sec	67°C : 60 sec	
Stage 3	70°C : 120 sec	70°C : 120 sec	70°C : 120 sec	

2.4.3.4 α_2- and α_1-globin genes amplification by using Vent$_R$® DNA polymerase and "Semi-touchdown" PCR protocol.

In attemping to sequence the α_1-and α_2-globin genes, they were amplified by using high fidellity Vent$_R$® DNA polymerase which occupied the 3'-->5' exonuclease activity, combining with "Semi-touchdown" PCR protocol. The reaction mixture as indicated in 2.4.3.2 (a), 50 ml each, was used except the 1x Vent$_R$® DNA polymerase buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 0.1% Triton x-100) and with finally 3 mM $MgSO_4$ were replaced.

The reaction mixture, without Vent$_R$® DNA polymerase, was overlayed with 30 µl of light-mineral oil. After pre-heating the mixture at 98°C for 3 min, "hot start" was performed by adding 0.03 U of Vent$_R$® DNA polymerase to the mixture. "Semi-touchdown" PCR protocol, totalling 31 cycles, is indicated in Table 7.

2.4.4 Gel electrophoresis and PCR products purification.

Because of the large volume of PCR products required for sequencing and restriction enzyme digestion experiments (200-300 µl of each α-globin gene amplification product), the products must first be run on two of 1% vertical agarose gels. Specific PCR products were then analyzed, separated from any other unwanted materials such as nonspecific products (smear) and the excess dNTPs and salts contained in the reaction mixture, and concentrated when all pieces of the gel containing specific bands were excised, collected into a 1.5 ml microfuge tube and purified by using a DNA purification kit, and finally resuspended in 50 µl of TE buffer pH 8.4.

PCR products from 2.4.3.3 (b) were collected into a new 1.5 ml microfuge tube. 0.2 vol of 6x loading buffer (Sambrook *et al.*, 1989) was added and then briefly mixed. 10 µl of the mixture was then loaded onto each well of the gel that had been pre-run at 100 V for at least 5 min. 150 ng of 100 bp. DNA ladder was used as a size marker.

The desired DNA bands were quickly excised, in order to minimize exposure to UV light, from the gel using a sterile blade after being visualized under long wavelength UV transluminator and then collected into a 1.5 ml microcentrifuge tube. PCR products were purified by using Wizard™ PCR Preps DNA Purification System as follows :

The agarose gel slices were incubated with 1 ml Resin (Promega, Madison, USA) and heated to 70°C in a water bath for 5 min or until the gel had melted completely. The tube was mixed throughly for 20 sec. The mixture was transferred into a 3 ml syringe barrel, without a syringe plunger, that had already been attached to the minicolumn (Promega, Madison., USA). The syringe plunger was then slowly inserted to push the slurry into the mincolumn. After detaching the plunger from the syringe, 2 ml of 80% ice-cooled isopropanol was pipetted into the syringe barrel to wash the syringe barrel and minicolumn by using the plunger in the same manner. Then, the minicolumn was detached from the syringe and placed on the top of 1.5 ml microfuge tube and then centrifuged at 14,000 rpm for 20 sec to dry the resin in the minicolumn. The minicolumn was transfered to a new 1.5 microfuge tube and 50 µl of TE buffer pH 8.4 was applied to the minicolumn, incubated for at least 5 min and centrifuged at 14,000 rpm for 2 min to elute the bounded DNA fragment. Finally, the purified DNA was transfered to a new microfuge tube and stored at -20°C for further experimented.

The DNA solution was quantitated by Spectrophotometer at 260 nm and calculated using the equation indicated above.

2.4.5 Photography.

Photographs of any gel were taken after the gels were stained in ethidium bromide staining solution (Sambrook *et al.*, 1989) and exposured to UV light on long-wave length UV transluminator. The camera's len was opened at 28 and a red filter was used. The exposured time was 1 min 30 sec. The films were processed with a developer for 5 min and then with fixer for at least 5 min as recommended by the company. The preparation of the stock of developer and fixer for film are given in detail in Apppendix 3.

2.4.6 α_2- and α_1-globin genes sequencing with Chain-termination cycle sequencing technique.

To sequence the α_2- and α_1-globin genes PCR products, a "ABI PRISM Dye Terminator Cycle Sequencing" ready reaction kit was used. This ready reaction kit combines the unique properties of AmpliTaq DNA polymerase, FS for dye terminator sequencing with the convenience of the ready reaction format. In this kit, the same formulation of reagents were included in a single tube and was ready to use. The reagents are suitable for performing fluorescence-based cycle sequencing reactions on single- or double-stranded DNA templates, or on PCR products.

2.4.6.1 Cycle sequencing reaction mixture.

a) Dye terminator premix.

Dye terminator ready reaction mix was composed of A-Dye, C-Dye, G-Dye, and T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-

HCl (pH 9.0), $MgCl_2$, Thermal stable pyrophosphatase, and Ampli*Taq* DNA polymerase, FS. For 20 ml cycle-sequencing reaction mixture, 8 μl of dye terminator-ready reaction mixture was used. The final concentration of each component in the stock ready reaction mixture was not indicated by the company.

b) Sequencing primers.

Sequencing primers were obtained from the method described by Molchanova and co-workers (1994). They were synthesized by Bio Service Unit (BSU), Department of Biochemistry, Mahidol University, Bangkok, Thailand.

The forward-sequencing primers for both α_2- and α_1-globin genes were :

Primer 946 : 5'- TCT TCT GGT CCC CAC AG -3'
Primer 947 : 5'- CCC GCC CGG ACC CAC A -3'
Primer 948 : 5'- GCG GGT TGC GGG AGG T -3'

The reverse-sequencing primer for α_2-globin gene was :

Primer 2168 : 5'- GGG AGG CCC ATC GGG CAG GAG GAA C -3'

And the reverse-sequencing primer for α_1-globin gene was :

Primer 944 : 5'- GGG GGG AGG CCC AAG GGG CAA GAA -3'

The positions of each sequencing primer specific for α_2- and α_1-globin genes DNA template are shown in Figure 15.

c) Co-solvent.

Dimethyl sulfoxide (DMSO) was used as co-solvent in the cycle sequencing reaction to reduce the primer Tm and the annealing temperature of the reaction. 5% DMSO is normally recommened, specially for the cycle sequencing of high GC-content DNA template.

d) α_2- and α_1-globin genes PCR products.

Purified α_2- and α_1-globin genes PCR products from Hb Chiang Mai's carrier and the normal haemoglobin samples from 2.4.4 were used as templates in the cycle sequencing reactions.

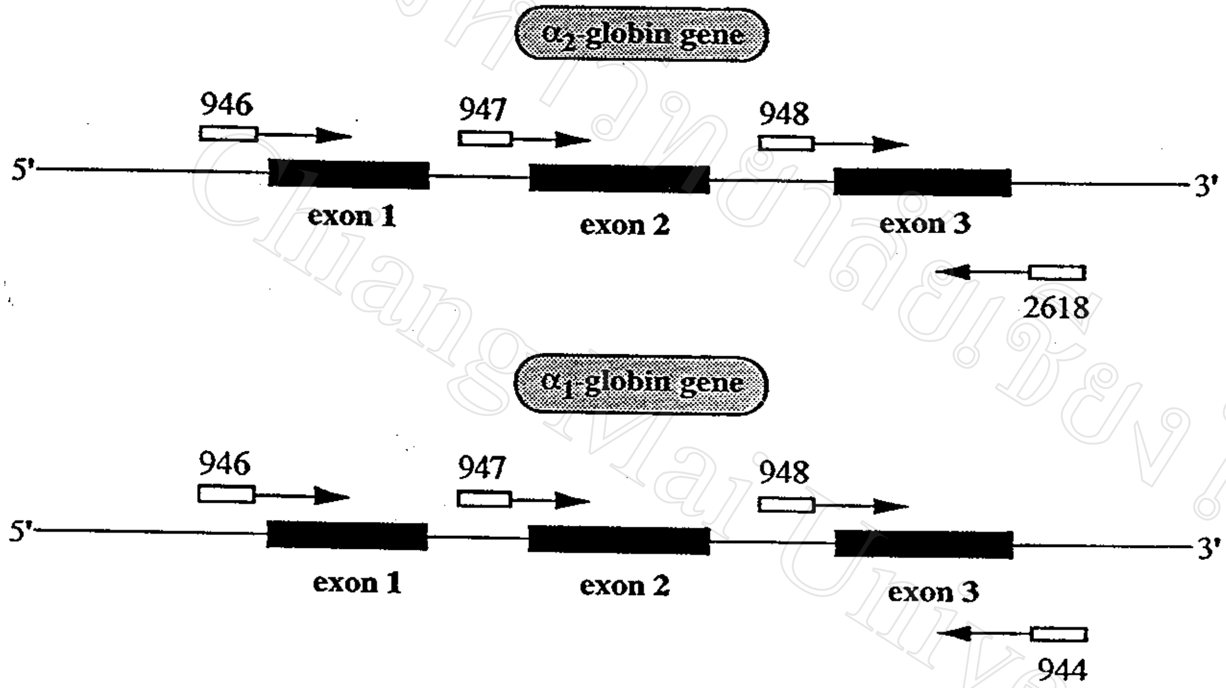

Figure 15. The position of each sequencing primers specific for α_2- and α_1- globin genes

2.4.6.2 α_2- and α_1-globin genes cycle sequencing with Chain-termination cycle sequencing technique.

In this process, the reaction mixture was by less exposure as little as possible to the light to prevent excitation of any fluorescent dye labelled dideoxynucleotide triphosphates. Cycle sequencing reaction mixture, 20 µl each, is shown in Table 8. It was added to 0.5 ml PCR tube, briefly mixed and overlayed with 30 µl of light mineral oil. Cycle sequencing was performed in an "Omn-E" thermalcycler. The native three-step PCR protocol, totalling 26 cycles, are shown in Table 9.

Table 8. Cycle sequencing reaction mixture.

Reagents	Final volume or concentrations
Terminator ready reaction mix	8 μl
α-globin genes PCR product	200 ng
DMSO	5%
Sequencing primer (946 or 947 or 948 or 944 or 2618)	3.2 pmole
Distilled-deionized water	q.s.

Table 9. Three-step PCR protocol used for cycle sequencing.

	Cycle 1-25	Cycle 26
Stage 1	94°C:60 sec	74°C:5 min
Stage 2	55°C:60 sec	
Stage 3	74°C:80 sec	

2.4.7 Purification of the cycle sequencing extension products.

In this process, the tube was protected from exposured to light to prevent the excitation of the fluorescents dye tagged on the products. The excess un-incorporated dye-terminators from the cycle sequencing step were removed by ethanol precipitation as follows :

a) For each 20 µl cycle sequencing reaction, a 1.5 ml micro-centrifuge tube was prepared by adding 2.0 µl of 3M NaOAc pH 5.6 and 50 µl of ice-cooled 95% EtOH. The tube was mixed and kept at -20°C.

b) The entire 20 µl of the cycle sequencing reaction was transfered to a tube from 2.4.7 a), mixed and placed on ice for at least 10 min. Then, the tube was centrifuged at 10,000 rpm for 30 min, 4°C.

c) As much as possible of ethanol was carefully aspirated with a micropipett .

d) The pellet was rinsed 1-2 times with 250 µl of 70% ice-cooled ethanol. Then, the pellet was dried at room temperature for at least 3 hours or at 70°C for at least 15 min.

2.4.8 Preparing and loading the samples into the sequencer.

The pellet from 2.4.7(d) was resuspended in 25 µl of "Template Suppression Reagent" (TSR., Perkin Elmer, Cetus, USA). The tube was mixed and briefly centrifuged before heating at 95°C for 2 min. After denaturation, the sample solution was transfered to a special tube with a rubber cap. Then, placed on ice until injected into the ABI 310 sequencer.

2.4.9 ABI "PRISM" 310 DNA sequencer.

The samples from 2.4.8 were placed on the tray of the sequencer machine. The names, comments and time for injection of each sample were entered to the injection list from the application software and then the sequencer was started. The first 30 min was used to fill up the "POP6" gel matrix to the microcolumn and approximately 10 μl of each sample solution was subsequently filled. One and a half hour was enough for sequencing each 700 bp DNA fragment. At the end of sequencing process, the raw data collected by the application software was automatically analysed and the electrophoregram was shown when recalled.

The sequencing data received from 4 sequencing experiments, with 3 forward primers and 1 reverse primer, were aligned by using "Excel 6.0" application software. The point mutation, compared with the normal α-globin genes sequences (the complete sequences of α_2- and α_1-globin genes were derived from GeneBank HUMHBA4) and with the sequence data of normal sample, when observed, was indicated.

2.4.10 Confirmation of single base substitution in Hb-Chiang Mai's α-globin genes by restriction enzyme digestion.

According to the sequencing data, two single base substitution were observed at the nucleotide number 7,330 (C-->G) and 10,853 (G-->C) (located in exon 3 of α_2- and in exon 2 of α_1-globin genes of Hb Chiang Mai's carrier, respectively (positions are listed according to sequences data from GeneBank, HUMHBA4). These two single base substitutions generated the new two palindromic sequences recognized by the specific restriction enzymes, *Stu* I and *Alw44* I, respectively. To confirm those two single base substitutions observed from the sequencing data, the purified Hb Chiang Mai's α_2- and α_1-globin gene PCR products were used as templates for the restriction enzymes digestion experiments.

Figure 16 shows the 785 bp of Hb Chiang Mai's α_2-globin gene PCR product, the cleavage site and the length of the *Alw44* I digested fragment generated. And Figure 17 also shows the 795 bp of Hb Chiang Mai's α_1-globin gene PCR product, the cleavage site and the length of the *Stu* I digested fragment generated.

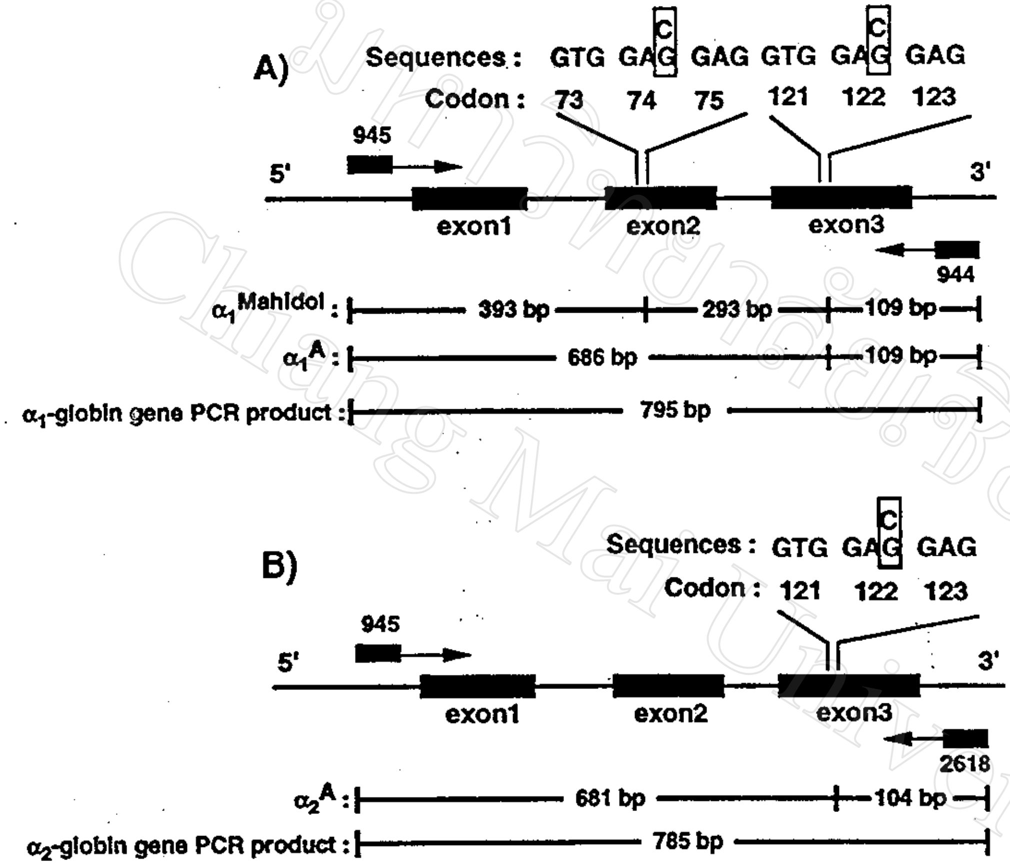

Figure 16. The clevage sites and the length of Hb Chiang Mai's α_1-globin gene digested with *Alw44* I.

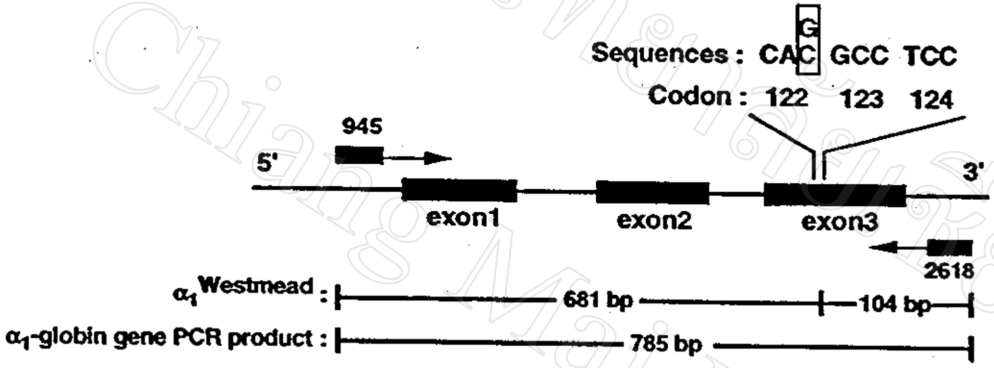

Figure 17. The clevage sites and the length of Hb Chiang Mai's α_2-globin gene digested with *Stu* I.

2.4.10.1 Confirmation of single base substitution in Hb Chiang Mai's α_1-globin gene by using *Alw44* I.

Purified 785 and 795 bp of α_2- and α_1-globin gene PCR products of Hb Chiang Mai and normal samples derived from 2.4.4 were used as the DNA templates for *Alw44* I digestion experiment. The restriction enzyme digestion reaction mixture is indicated in Table 10.

The reaction mixture was briefly mixed, and incubated at 37°C for 4 hr. *Alw44* I was inactivated by further incubation at 70°C for 10 min.

The entirely 20 µl of DNA digested products were precipitated by adding 2 µl of 3M NaOAc pH 5.6 and 50 µl of 95% EtOH and then briefly mixing. The tube was then left on ice for 10 min and then centrifuged at 10,000 rpm, -20°C for 30 min. The precipitate was washed with 250 µl of 70% EtOH and dried by incubation at 70°C for 10 min. The precipitate was dissolved in 5 µl of TE buffer pH 8.4 (further incubation at 70°C for 2-3 min may help the solubilization of DNA). It was then mixed with 1 µl of 6x loading buffer before running gel electrophoresis.

Undigested and digested PCR products were analysed on 2.5% horizontal agarose gel at 55 V for 4 hr. After being visualized under a long wave length UV transluminator, the gels were photographed.

Table 10. *Alw44* I digestion reaction mixture. Purified α_2- and α_1-globin genes PCR products of Hb Chiang Mai and normal samples were used as DNA templates for these restriction enzyme digestion experiments.

Reagents	Final volume or concentration
PCR products	1 mg
10x Restriction enzyme buffer	1x
10 mg/ml Actylated BSA	0.1 mg/ml
12 U/µl *Alw44* I	1 U
Distilled-deionized water	q.s.

2.4.10.2. Confirmation of single base substitution in Hb Chiang Mai's α_2-globin gene by using *Stu* I.

Purified 785 and 795 bp of α_2- and α_1-globin gene PCR products of Hb Chiang Mai and normal haemoglobin samples were used as the DNA templates for *Stu* I digestion experiment. The restriction enzyme digestion reaction mixture is indicated in Table 11.

The reaction mixture was briefly mixed, and incubated at 37°C for 4 hr. *Stu* I was inactivated by further incubation at 70°C for 10 min.

The entire 20 μl of DNA digested products were precipitated by adding 2 μl of 3M NaOAc pH 5.6 and 50 μl of 95% EtOH and briefly mixed. The tube was then left on ice for 10 min and then centrifuged at 10,000 rpm, -20°C for 30 min. The precipitate was washed with 250 μl of 70% EtOH and dried by incubation at 70°C for 10 min. The precipitate was dissolved in 5 μl of TE buffer pH 8.4 (further incubation at 70°C for 2-3 min may help the solubilization of DNA). It was then mixed with 1 μl of 6x loading buffer before running gel electrophoresis.

Undigested and digested PCR products were analysed on 2.5% horizontal agarose gel at 55 V for 4 hr. After being visualized under a long wave-length UV transluminator, the gels were photographed.

Table 11. *Stu* I digestion reaction mixture. Purified α_2- and α_1-globin genes PCR products of Hb Chiang Mai and normal samples were used as DNA templates for these restriction enzyme digestion experiments.

Reagents	Final volume or concentration
PCR products	1 mg
10x Restriction enzyme buffer	1x
10 mg/ml Actylated BSA	0.1 mg/ml
20 U/µl *Stu* I	1 U
Distilled-deionized water	q.s.