CHAPTER III

RESULTS

3.1. Genomic DNA isolation from whole blood.

The absorbance at 260 and 280 nm and the final concentration of the genomic DNA solution isolated from the whole blood of Hb Chiang Mai and the normal haemoglobin sample are shown in Table 12.

3.2. Optimization of α_2- and α_1-globin gene amplification reactions.

α_2- and α_1-globin gene amplification by polymerase chain reaction reported by Molchanova and co-workers (1994) are not repeatable. The optimization of some parameters such as the titration of formamide (Figure 18), $MgCl_2$ (Figure 19), DNA template (Figure 20), *Taq* DNA polymerase (Figure 21) and the annealing temperatures (Figure 22), did not improved the amplification system. As shown in Figure 23, the amplification system was not improved by the two-step PCR even with 70, 72, 74 and 76°C annealing/extension temperatures or by using "Touchdown" PCR protocol in which the annealing temperature was programmed to decrease from 80 to 67°C for the first 10 cycles (1.2°C/cycle) and at 67°C for further 20 cycles.

Table 12. The absorbance at 260 and 280 nm and final concentration of genomic DNA isolation from Hb Chiang Mai and normal haemoglobin sample.

Samples	OD (nm)		260/280	Concentration
	260	280	Ratio	(ng/μl)
Hb Chiang Mai	0.129	0.082	1.57	1,290.0
Normal	0.119	0.095	1.25	1,190.0

As shown in Figure 24, the native PCR reaction buffer (Molchanova *et al.*, 1994) was replaced with *Taq* 2Δ buffer. 785 and 795 bp of α_2- and α_1-globin genes PCR products were obtained with two-step PCR protocol (70°C annealing/extension temperature). "Touchdown" PCR protocol (the annealing temperature was programmed to decrease from 80 to 67°C for the first 10 cycles (1.2°C/cycle) and at 67°C for a further 20 cycles) and with the "Semi-touchdown" PCR protocol (PCR protocol divided into 3 stages : 5 cycles with the annealing temperature at 80°C ; next 5 cycles with the annealing temperature at 73.5°C ; and the last 20 cycles with the annealing temperature at 67°C). As shown in Figure 25, 5-7.5% DMSO was necessary for the amplification system. Without DMSO, those specific amplification products were not enhanced.

For the purpose of sequencing of α_2- and α_1-globin gene, *Taq* DNA polymerase was replaced by high fidelity Vent$_R$® DNA polymerase and the $MgCl_2$ final concentration was adjusted to 3 mM final concentration. The specificity and intensity of the PCR products were nearly the same (data not shown).

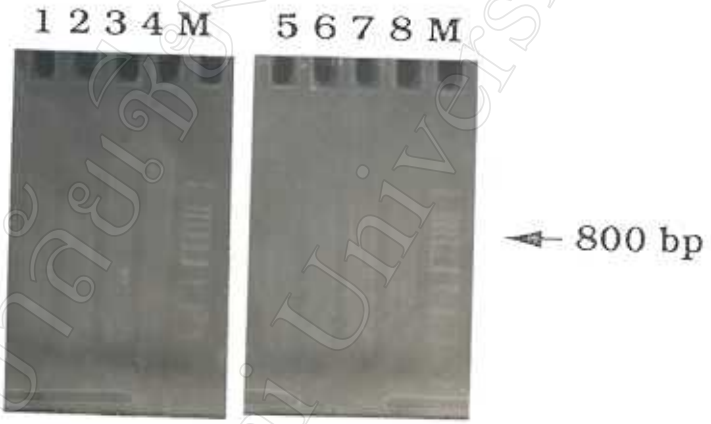

Figure 18. The effect of formamide concentrations on native α_2- and α_1-globin genes amplification system (Molchanova *et al.*, 1994). Formamide was titrated for 0 (lane 1 and 5), 5 (lane 2 and 6), 7.5 (lane 3 and 7) and 10% final concentration (lane 4 and 8).The native reaction mixture (7.5% final concentration) is shown in lane 3 and 7 for α_2-and α_1-globin genes respectively. "M" = 100 bp DNA ladder.

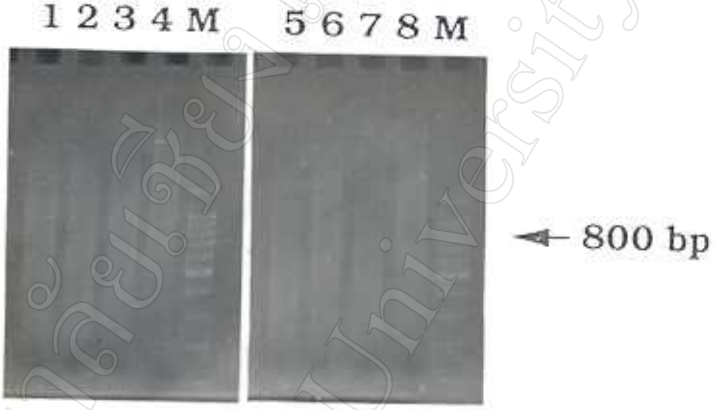

Figure 19. The effect of $MgCl_2$ concentrations on the native α_2-and α_1-globin genes amplification system (Molchanova *et al.*., 1994). $MgCl_2$ was titrated for 1.5 (lane 1 and 5), 2.0 (lane 2 and 6), 3.0 (lane 3 and 7) and 4.0 mM final concentration (lane 4 and 8). The native reaction mixture (2.0 mM final concentration) is shown in lane 2 and 6 for α_2- and α_1-globin genes respectively. "M" = 100 bp DNA ladder.

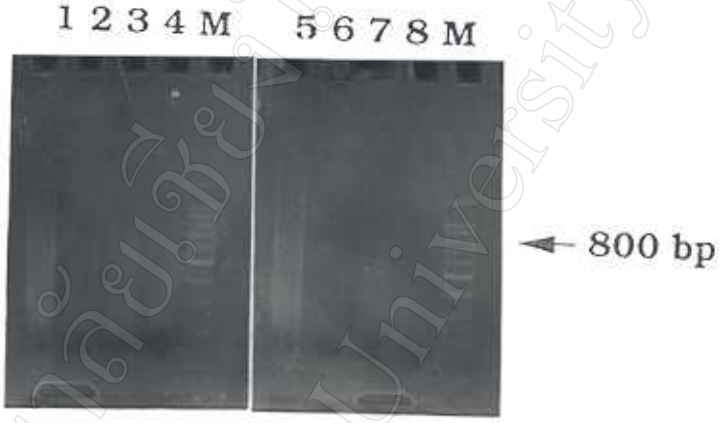

Figure 20. The effect of genomic DNA template concentrations on the native α_2- and α_1-globin genes amplification system (Molchanova *et al.*, 1994). Genomic DNA template was titrated for 20 (lane 1 and 5), 12 (lane 2 and 6), 7 (lane 3 and 7) and 5 ng/µl final concentration (lane 4 and 8). The native reaction mixture (20 ng/µl final concentration) is shown in lane 1 and 5 for α_2-and α_1-globin genes respectively. "M" = 100 bp DNA ladder.

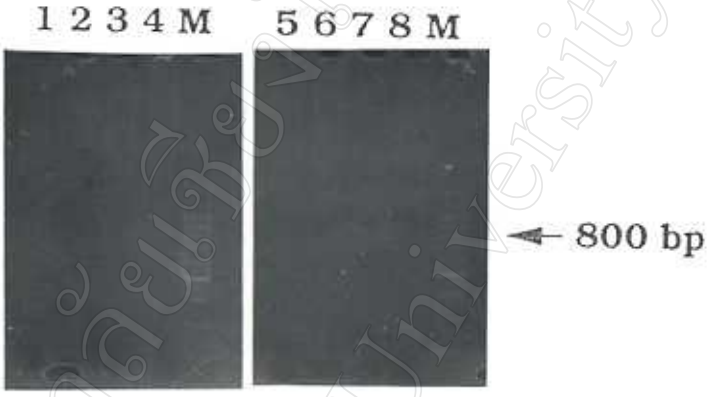

Figure 21. The effect of *Taq* DNA polymerase concentrations on the native α_2- and α_1-globin genes amplification system (Molchanova *et al.*, 1994). *Taq* DNA polymerase was titrated for 0.025 (lane 1 and 5), 0.05 (lane 2 and 6), 0.1 (lane 3 and 7) and 0.3 U/µl final concentration (lane 4 and 8). The native reaction mixture (0.1 U/µl final concentration) is shown in lane 3 and 7 for α_2-and α_1-globin genes respectively. "M" = 100 bp DNA ladder.

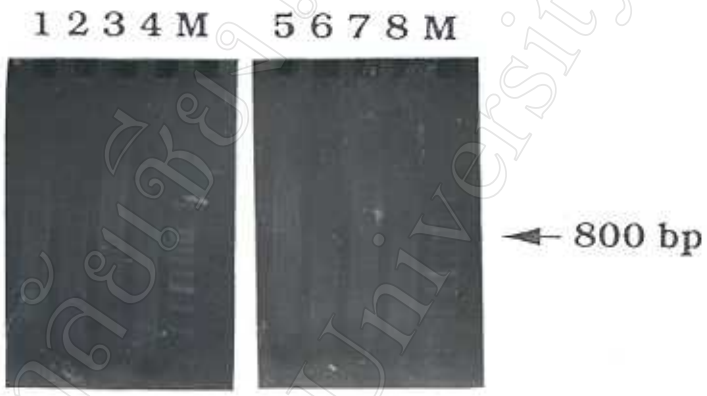

Figure 22. The effect of the annealing temperatures on the native α_2- and α_1-globin genes amplification system (Molchanova *et al.*, 1994). The annealing temperature used in the native three-step PCR protocol was titrated for 70 (lane 1 and 5), 68 (lane 2 and 6), 63 (lane 3 and 7) and 58°C (lane 4 and 8). The native three-step PCR protocol with 68°C annealing temperature is shown in lane 2 and 6 for α_2-and α_1-globin genes respectively. "M" = 100 bp DNA ladder.

Figure 23. The effect of Two-step PCR and "Touchdown" PCR protocols on the native α_2- and α_1-globin genes amplification reaction (Molchanova *et al.*, 1994). The annealing/extension temperature required for two-step PCR was titrated for 70 (lane 1 and 6), 72 (lane 2 and 7), 74 (lane 3 and 8) and 76°C (lane 4 and 9). The annealing temperature required for "Touchdown " PCR was programmed to decrease from 80 to 67°C for the first 10 cycles (1.2°C/cycle) and at 67°C for further 20 cycles (lane 5 and 10). "M" = 100 bp DNA ladder.

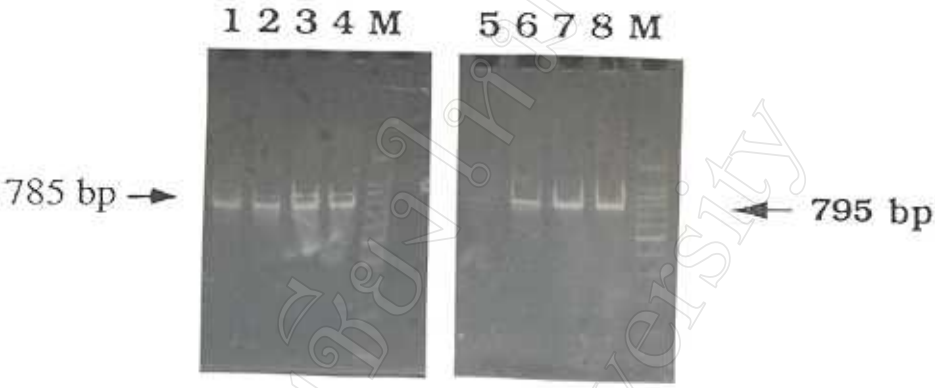

Figure 24. The improvement of the specific α_2- and α_1-globin genes amplification products of Hb Chiang Mai's carrier and the normal sample. The reaction mixtures as indicated in Tabe 5 were used. Lane 1 and 5 : with native three-step PCR protocol (68°C annealing temperature) ; lane 2 and 6 : with two-step PCR protocol (70°C annealing/ extension temperature) ; lane 3 and 7 : with "Touchdown" PCR protocol (the annealing temperature decrement of 1.2°C/cycle (from 80 to 67°C) for the first 10 cycles and at 67°C for further 20 cycles) ; lane 4 and 8 : with "Semi-touchdown" PCR protocol : (the annealing temperature of : 80°C for the first 5 cycles ; 73.5°C for the next 5 cycles ; and 67°C for the last 20 cycles). "M" = 100 bp DNA ladder.

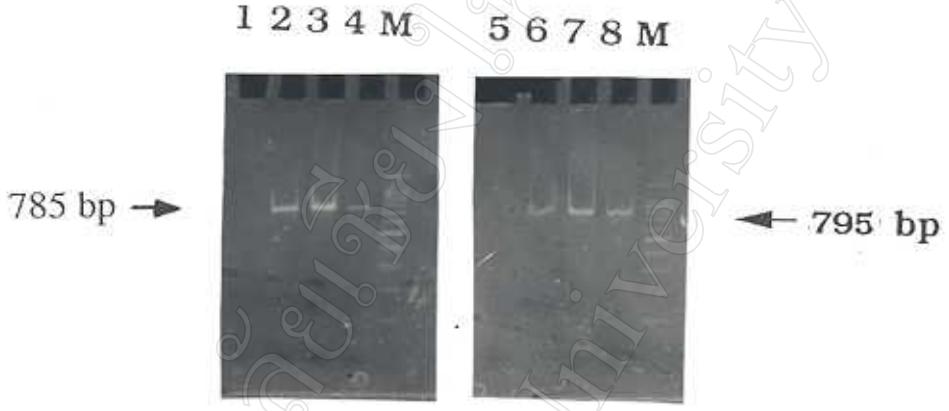

Figure 25. The effect of DMSO on the modified α_2-and α_1-globin genes PCR system. DMSO was titrated for 0 (lane 1 and 5), 5 (lane 2 and 6), 7.5 (lane 3 and 7) and 10% final concentration (lane 4 and 8). The specific 785 and 795 bp of Hb Chiang Mai's α_2- and α_1-globin PCR products enhanced by 7.5% DMSO are shown in lane 3 and 7, respectively. "M" = 100 bp DNA ladder.

3.3 Vertical Agarose Gel Electrophoresis (VAGE).

Agarose gel electrophoresis in the vertical configuration, so called Vertical Agarose Gel Electrophoresis (VAGE), is reported. 1.0 mm-thick agarose gel were prepared by using the used-up mini-PROTEAN®II Cell ready gel plates. The time used for the preparation step was less than 20 min. Agarose gel was left to cool down and set up either at room temperature for at least 10 min or at -20°C for at least 5 min. Moreover, the prepared gel slabs kept in plastic bag at 4°C as long as 1 month can be used and no interference was observed (data not shown).

As shown in Figure 26, the effect of staining time for 1 mm-thick vertical agarose gel used as the gel matrix for PCR products purification on the recovering yield. The staining time for 3 mm-thick agarose gel is normally 5 min followed by 5 min destaining ; however, the time required for 1 mm-thick vertical agarose gel was less. The PCR product was divided into 3 aliquots (50 ml each) and electrophoresis was run on 1 of 2% of 3 mm-thick horizontal agarose gel and 2 of 2% of 1 mm-thick vertical agarose gel. The specific band was quickly exised when the gel was analysed under a long wavelength UV transluminater after : 5 min staining followed by 5 min destaining for 3 mm-thick horizontal agarose gel ; 5 min staining for 1 of 1 mm-thick vertical agarose gel and ; 1 min staining for 1 of 1 mm-thick vertical agarose

gel. PCR products were then purified as indicated. 5 µl of unpurified and purified PCR products were run on 3% vertical agarose gel at 100 V for 50 min. From the results, it can be concluded that long time staining for 1 mm-thick vertical agarose gel caused a loss of yield after purification. For the purpose of DNA purification, 1 min staining of 1 mm thick vertical agarose gel, without destaining, was recommened.

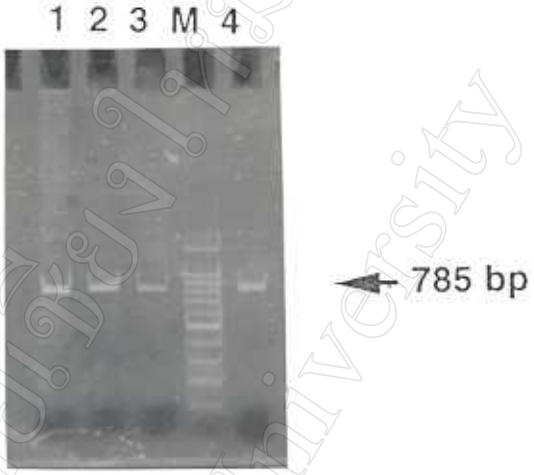

Figure 26. The effect of staining time with ethedium bromide for 1 mm-thick vertical agarose gel using as the gel matrix for PCR products purification on the DNA recovering yield. 5 µl of 785 bp of α_2-globin gene PCR products, lane 1 : unpurified ; lane 2 : purified from 3 mm-thick agarose gel with 5 min staining followed by 5 min destaining ; lane 3 : purified from 1 mm-thick vertical agarose gel with 5 min staining ; lane 4 : purified from 1-mm thick vertical agarose gel with 1 min staining, were loaded on to 3% vertical agarose. "M" = 100 bp DNA ladder.

3.4 Chain-termination cycle sequencing of α_2- and α_1-globin genes PCR products.

Because the Ampli*Taq* DNA polymerase, FS had already been included in the cycle sequencing reaction mixture, "Hot Start PCR" performed by adding the polymerase enzyme to the reaction mixture after pre-heating at 99°C for 3 min was then not possible. However, the longer readable range of the sequencing data was retrieved when "Hot Start PCR" performed by adding the sequencing primer to the reaction mixture after preheated was used (data not shown). 5% DMSO included in the reaction mixture may be a facter for longer readable range too.

The sequencing data of α_2-globin gene PCR products showed a 7 bp deletion (5'...CTCGGCC...3' ; observed only in α_1-globin genes) between nucleotide number 11049-11055 (positions are listed according to the sequence from GeneBank, HUMHBA4), which was not observed in α_2-globin gene (Figure 27 a and b). This stated that the α_2- and α_1-globin genes were specifically amplified.

According to the sequencing data retrieved from Hb Chiang Mai's α_1-globin gene, the single base substitution at the nucleotide number 10853 (position is listed according to the sequence from GeneBank, HUMHBA4) was observed as equally high peaks of G and C and was nearly in the same position (Figure 28 a), compared with normal α_1-

globin gene (Figure 28 b). This single base substitution caused the amino acid subtitution of Asp-->His ($\alpha_1$74 (EF3) Asp-->His) or from the acidic residue to the neutral one. This agreed with the data derived from Sitthipreechacharn (1994) who detected that Hb Chiang Mai was slower moving to the anode in cellulose-acetate gel electrophoresis at pH 8.5 comparing with normal Hb A.

Moreover, according to the sequencing data retrieved from Hb Chiang Mai's α_2-globin gene, the single base substitution at the nucleotide number 7330 (position is listed according to the sequences from GeneBank HUMHBA4) was observed as a single G peak (Figure 29 a), compared with a single C peak from the normal α_2-globin gene (Figure 29 b). This single base substitution caused the amino acid subtitution of His-->Gln ($\alpha_2$122 (H5) His-->Gln) or from the baisdic residue to the basidic one. This may be the cause of the inability to detect this haemoglobin variant in cellulose-acetate gel electrophoresis at pH 8.5 as reported by Sitthipreechacharn (1994).

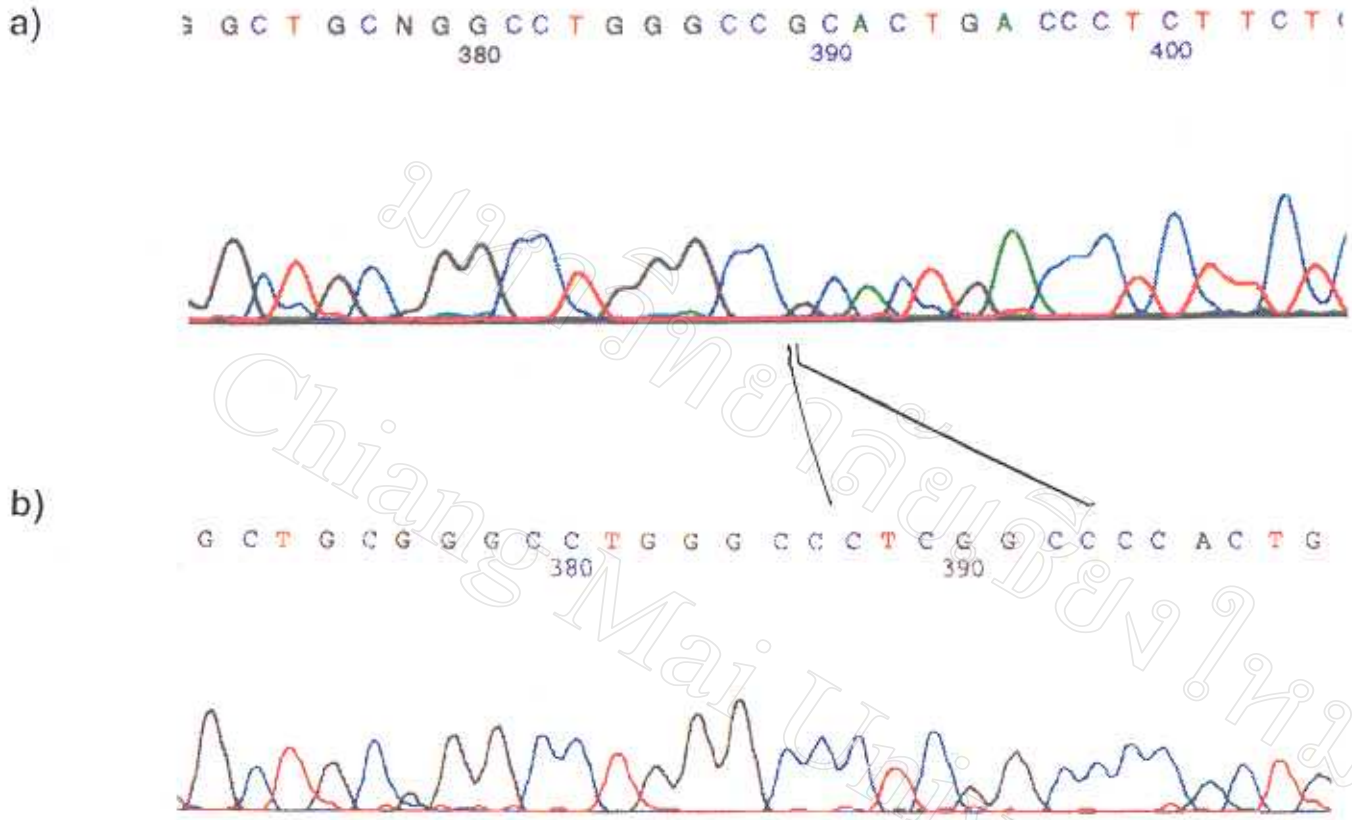

Figure 27. The electrophoregrams of the sequencing data of Hb Chiang Mai's α-globin gene PCR products. 7 bp deletion between nucleotide number 11049-11055 (positions are listed according to data derived from GeneBank, HUMHBA4) was observed in α_2-globin gene (a) and not observed in α_1-globin gene. (b).

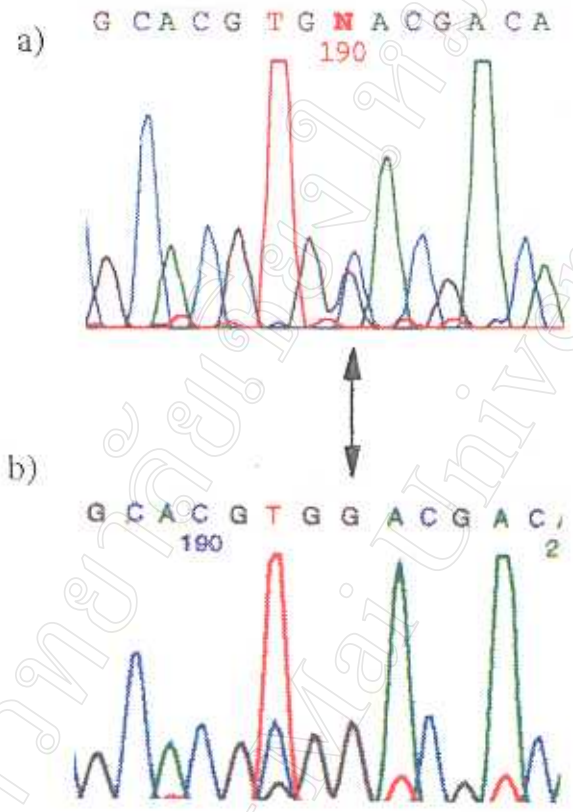

Figure 28. The electrophoregrams of the sequencing data of Hb Chiang Mai's α_1-globin gene. The single base substitution at the nucleotide number 10853 (position is listed according to the sequence from GeneBank, HUMHBA4) was shows as the equally high peaks of G and C nearly at the same position (a) compared with the single G peak at the same nucleotide of the normal α_1-globin gene (b).

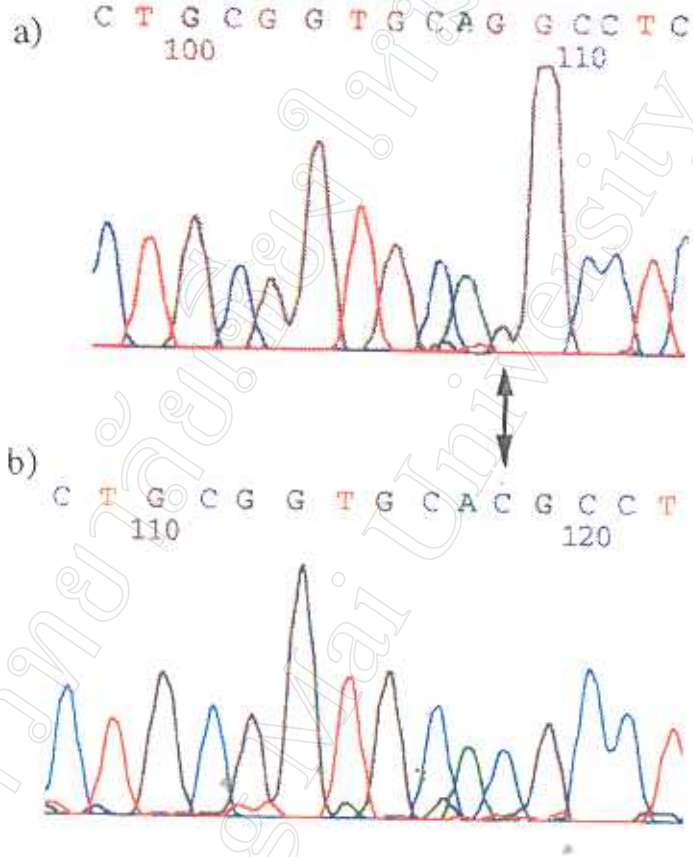

Figure 29. The electropherograms of the sequencing data of Hb Chiang Mai's α_2-globin gene. The single base substitution at the nucleotide number 7330 (position is listed according to the sequence from GeneBank, HUMHBA4) was showing as the single G (a) compared with the single C peak at the same nucleotide of the normal α_2-globin gene (b).

3.5. Confirmation of single base substitution in exon 2 of Hb Chiang Mai's α_1-globin gene by using *Alw44* I digestion experiment.

To directly confirm that single base substitution occurred in exon 2 of Hb Chiang Mai's α_1-globin gene, the restriction enzyme, *Alw44* I, which detected the palindromic sequences of "5'...GTG CAC...3'" was applied to the digestion experiment.

According to the palindromic sequences recognized by *Alw44* I that already existed in exon 3 of both normal α_2- and α_1- globin genes, 785 and 795 bp of the normal's α_2- and α_1-globin gene PCR products and 795 bp of Hb Chiang Mai's α_1-globin gene PCR products were estimated to be completely digested to 686/109 bp for α_1-globin gene and to 681/104 bp for α_2-globin gene, respectively. However, the single base substitution occurring in exon 3 of Hb Chiang Mai's α_2-globin gene generated the new palindromic sequence that was not recognized by *Alw44* I, and showed no digested products at all.

The single base substitution at nucleotide number 10853 (position is listed according to the sequences from GeneBank HUMHBA4) which corresponded to the first nucleotide of amino acid residue 74 of Hb Chiang Mai's α_1-globin gene was further detected in the same *Alw44* I digestion experiment as 393/293 bp digested fragments.

As shown in Figure 30, the single base substitution of G-->C at nucleotide number 10853 (GeneBank HUMHBA4) was confirmed by the *Alw44* I digestion experiment. The disappearance of 795 bp of α_1-globin gene PCR product, after digestion, indicates that the partial digestion of these DNA fragments had not occurred. 393/293 bp digested fragments (lane 2) was indicated the single base substitution of G-->C located in exon 2 of Hb Chiang Mai's α_1-globin gene, which was not observed in normal's α_1-globin gene (lane 4). However, the 686 bp digested fragments (lane 2) indicated that this single base substitution occurred only on one chromosome (heterozygous). 681/104 bp digested fragments of normal's α_2-globin gene (lane 8) and 686/109 bp of Hb Chiang Mai's and normal's α_1-globin gene (lane 2 and 4) indicating that the palindromic sequence recognized by *Alw44* I was normally located in exon 3 of those α-globin genes.

As shown in Figure 30 (lane 6), Hb Chiang Mai's α_2-globin gene PCR product was not digested with *Alw44* I. This indicated that, 1) the single base substitution of G-->C which was observed in Hb Chiang Mai's α_1-globin gene, had not occurred in exon 2 of its α_2-globin gene. 2) the palindromic sequence normally recognized by *Alw44* I (in exon 3 of α_2-globin gene) was changed (the other single point mutation).

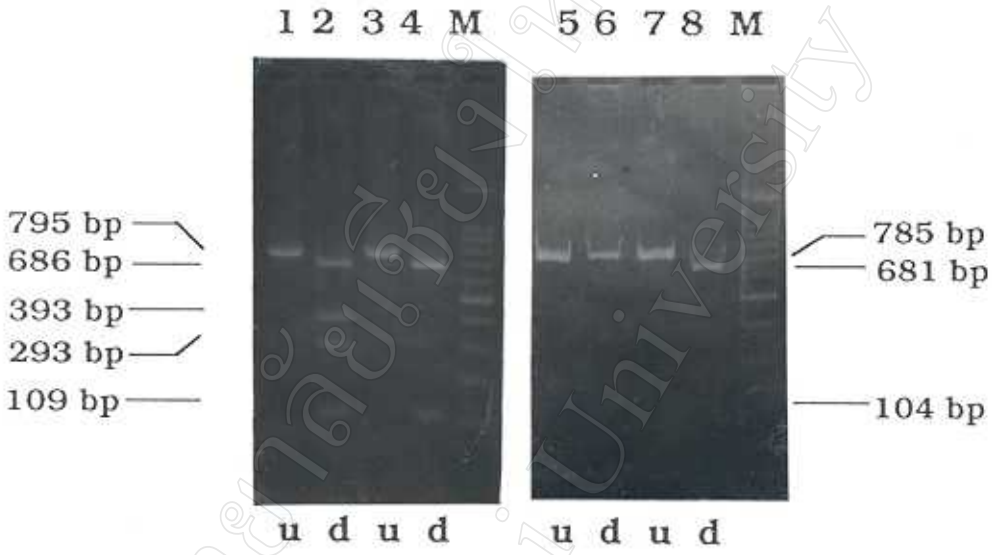

Figure 30. 785 and 795 bp of α_2- and α_1-globin gene PCR products from Hb Chiang Mai and normal sample, undigested (u) and digested (d) with *Alw44* I . The digested PCR products were precipitated and then resuspended in TE buffer pH 8.4 prior to analysis on 2.5% agarose gel at 55 V for 4 hr. Lane 1 and 2 : Hb Chiang Mai's α_1-globin gene ; Lane 3 and 4 : normal's α_1-globin gene ; Lane 5 and 6 : Hb Chiang Mai's α_2-globin gene ; Lane 7 and 8 : normal's α_2-globin gene. "M" = 100 bp DNA ladder.

3.6 Confirmation of single base substitution in exon 3 of Hb Chiang Mai's α_2-globin gene by using *Stu* I digestion experiment.

To directly confirm the single base substitution occurred in exon 3 of α_2-globin gene of Hb Chiang Mai's carrier, the restriction enzyme, *Stu* I, which detected the palindromic sequences of "5'...AGG CCT...3'" was applied to the digestion experiments.

As shown in Figure 31, the single base substitution of C-->G at nucleotide number 7330 (GeneBank HUMHBA4) was confirmed by the *Stu* I digestion experiment. The disappearance of 785 bp of α_2-globin gene PCR product, after digestion, is indicates that the partial digestion of these DNA fragments had not occurred. 681/104 bp digested fragments (lane 2) was indicated a single base substitution of C-->G located in exon 3 of Hb Chiang Mai's α_2-globin gene, which was not observed in normal's α_2-globin gene (lane 4).

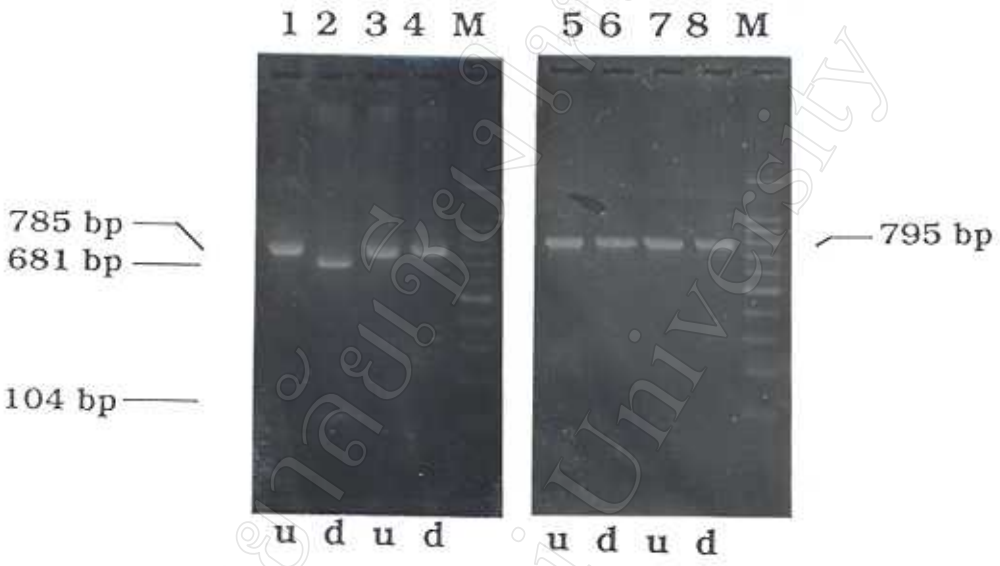

Figure 31. 785 and 795 bp of α_2- and α_1-globin gene PCR products from Hb Chiang Mai and normal sample, undigested (u) and digested (d) with *Stu* I. The digested PCR products were precipitated and then resuspended in TE buffer pH 8.4 prior to analysis on 3% agarose gel. at 55 V for 4 hr. Lane 1 and 2 : Hb Chiang Mai's α_2-globin gene ; Lane 3 and 4 : normal's α_2-globin gene ; Lane 5 and 6 : Hb Chiang Mai's α_1-globin gene ; Lane 7 and 8 : normal's α_1-globin gene. "M" = 100 bp DNA ladder.