

## CHAPTER III

### RESULTS

#### 3.1 Study of molecular background of silent $\alpha$ -thalassemia

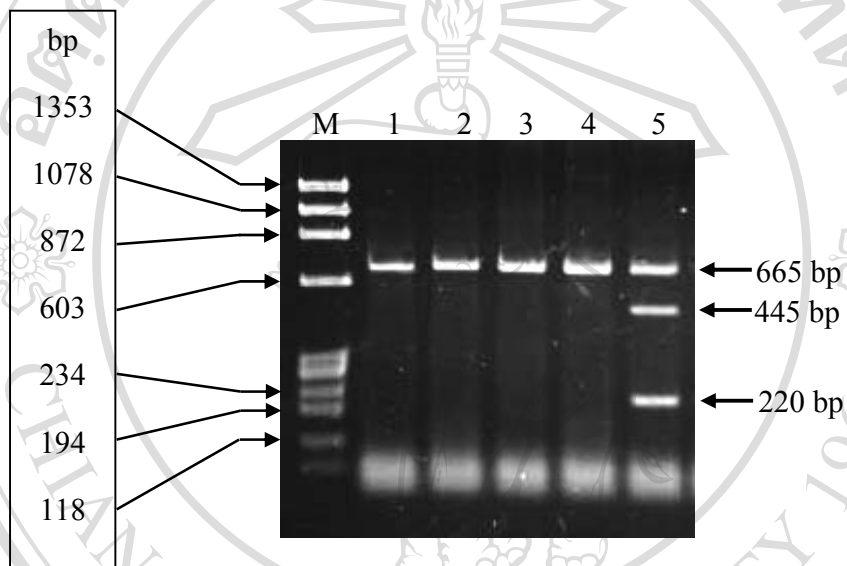
##### 3.1.1 Detection of $XmnI$ - $G\gamma$ polymorphism

The detection of the  $XmnI$ - $G\gamma$  polymorphism was performed. After PCR process, the amplified products were digested with the restriction endonuclease  $XmnI$ , the procedure of which was described previously in the Materials and Methods. It was found that the  $XmnI$ - $G\gamma$  polymorphism was absent in all 4 blood samples; i.e. nucleotide at the position -158 on the  $G\gamma$ -globin promoter was “T/T” which was normal. On the other hand, the results could also be reported as “homozygote for the absence of the  $XmnI$ - $G\gamma$  site”, the genotype of which was written to be “ $XmnI$ - $G\gamma$  : -/-”. The results of this study is shown in the Figure 3.1.

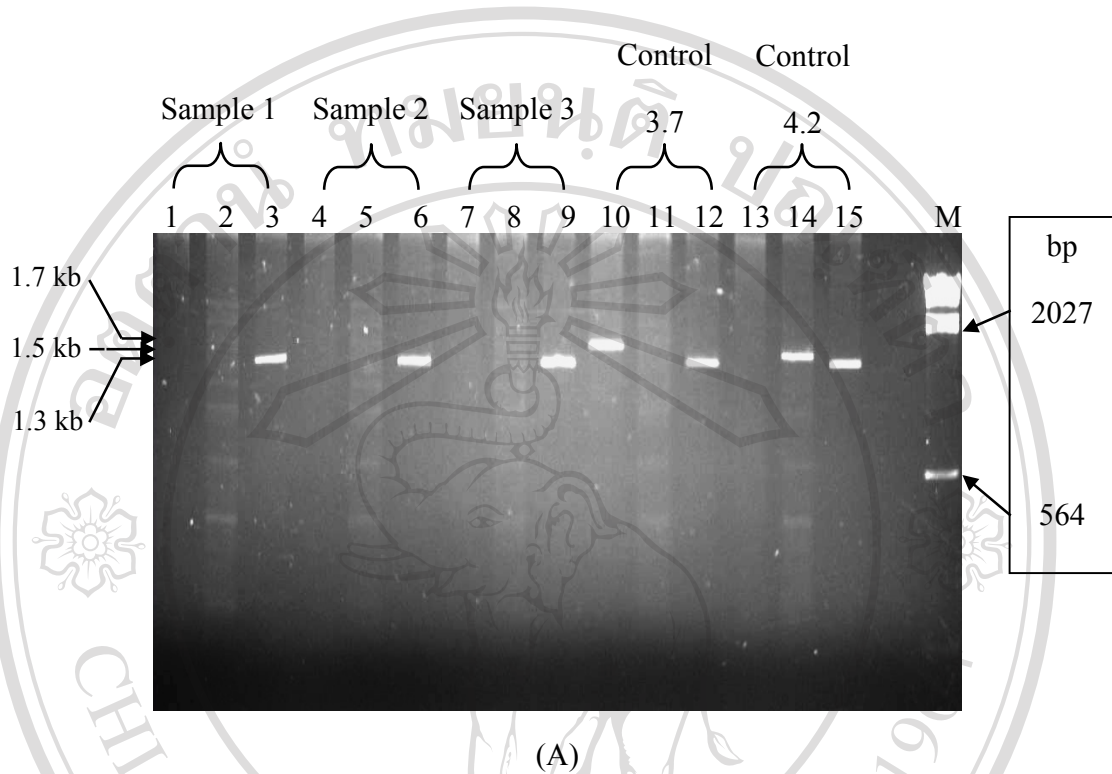
##### 3.1.2 Detection of $\alpha$ -thalassemia 2

In order to identify homozygous and heterozygous state of  $\alpha$ -thalassemia 2 with 3.7-kb or 4.2-kb deletions that might cause silent form of  $\alpha$ -thalassemia in the 4 samples analyzed, Gap-PCR was performed using the protocol described previously in the Materials and Methods section. The results showed that none of the 4 samples carried the

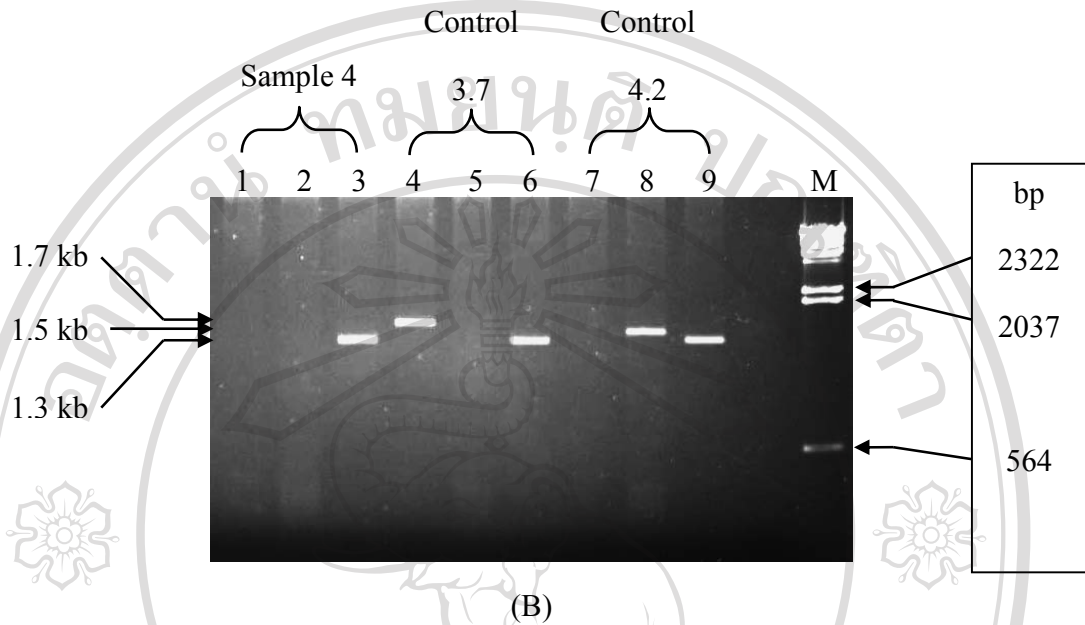
3.7-kb and 4.2-kb deletions. Thus silent phenotype of  $\alpha$ -thalassemia observed these 4 samples were not originated from the  $\alpha$ -thalassemia 2 determinant of these 2 deletions (Figure 3.2).



**Figure 3.1** *XmnI*-digested fragments separated on a 1.0% agarose gel. Lanes 1 to 4 are samples number 1-4 which were homozygous for the absence (*XmnI*- $G\gamma$  : -/-) and lane 5 is the sample heterozygous for the presence of the *XmnI*- $G\gamma$ .site (*XmnI*- $G\gamma$  : +/-) Lane M represents the  $\Phi$ X174 DNA (*Hae*III digest) standard size marker. Fragment sizes (in base pairs, bp) of the digested fragments are also shown.



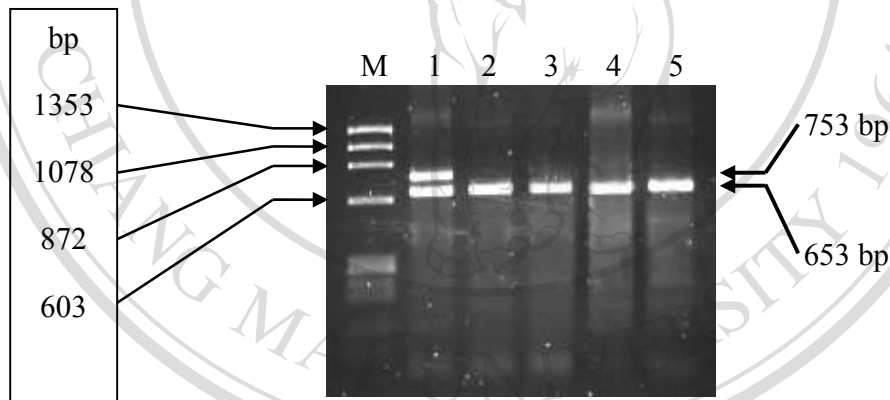
**Figure 3.2 (A)** Agarose gel electrophoresis of PCR products for detection of  $\alpha$ -thalassemia 2 with 3.7-kb and 4.2-kb deletions (details written in the next page)



**Figure 3.2** Agarose gel electrophoresis of PCR products for detection of  $\alpha$ -thalassemia 2 with 3.7-kb and 4.2-kb deletions. The amplified PCR products of samples 1, 2, 3 were demonstrated in panel (A) and sample 4 in panel (B) as indicated above each panel. Lanes 1, 4, 7, 13 in panel (A) and lanes 1, 4, 7 in panel (B) are products of primers 3.7A + 3.7B. Lanes 2, 5, 8, 11, 14 in panel (A) and lanes 2, 5, 8 in panel (B) are products of primers 4.2C + 4.2D. Lanes 3, 6, 9, 12, 15 in panel (A) and lanes 3, 6, 9 in panel (B) are products of primers GI + GII. Lane M represents  $\lambda$ -DNA/Hind III size standard marker. Note that all samples have no  $\alpha$ -thalassemia 2 of 3.7-kb and 4.2-kb deletion.

### 3.1.3 Detection of $\alpha$ -thalassemia 1

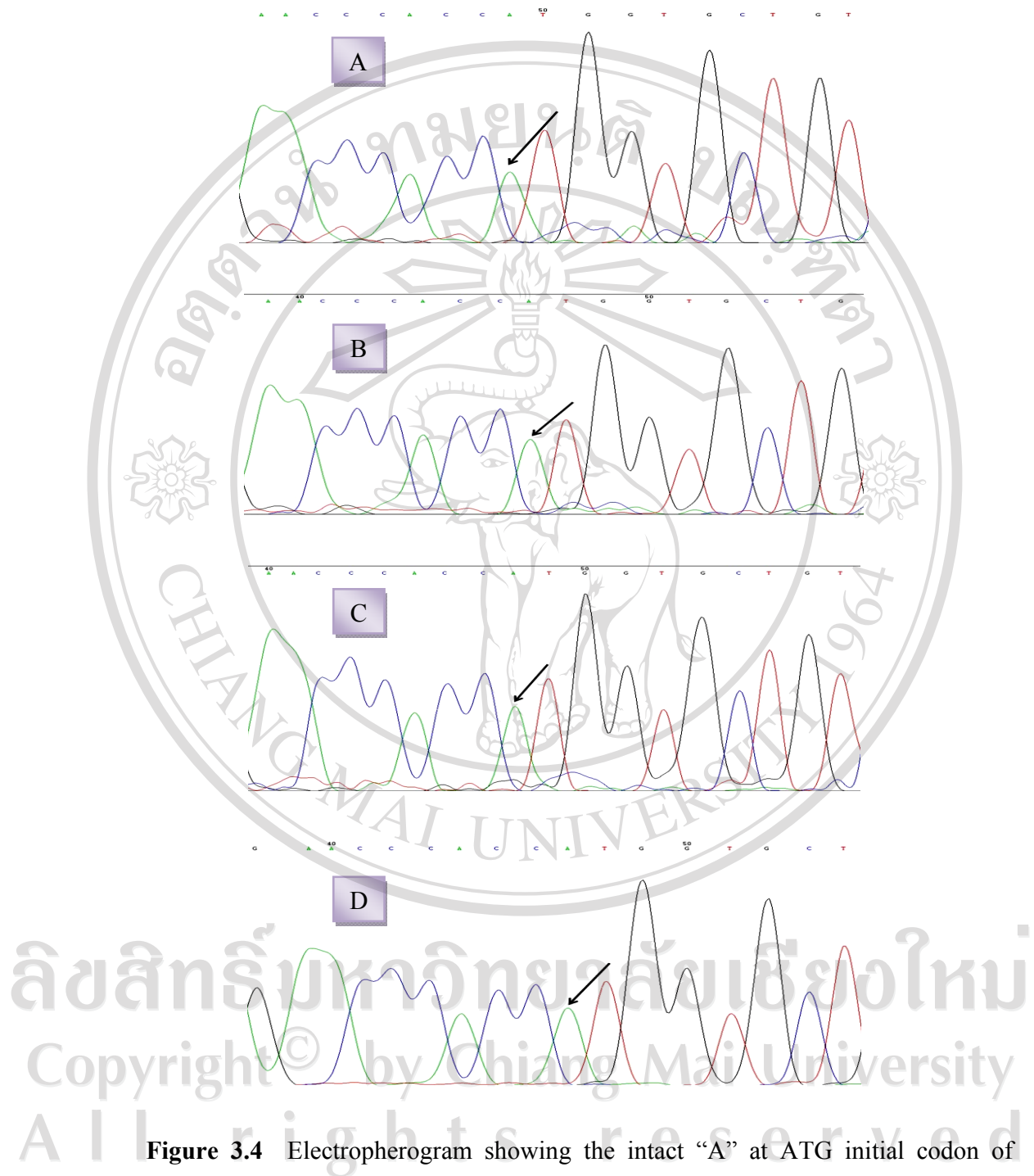
To detect  $\alpha$ -thalassemia 1 with SEA deletion, the Gap-PCR analysis was performed. The procedure was described in detail in the section 2.2.1.4. After PCR process, the amplified product was separated on 2.0 % agarose gel electrophoresis and stained with ethidium bromide. The gel was visualized under UV transilluminator. The results of this investigation showed that none of these four samples carried the deletional  $\alpha$ -thalassemia 1 of SEA type as shown in the Figure 3.3.



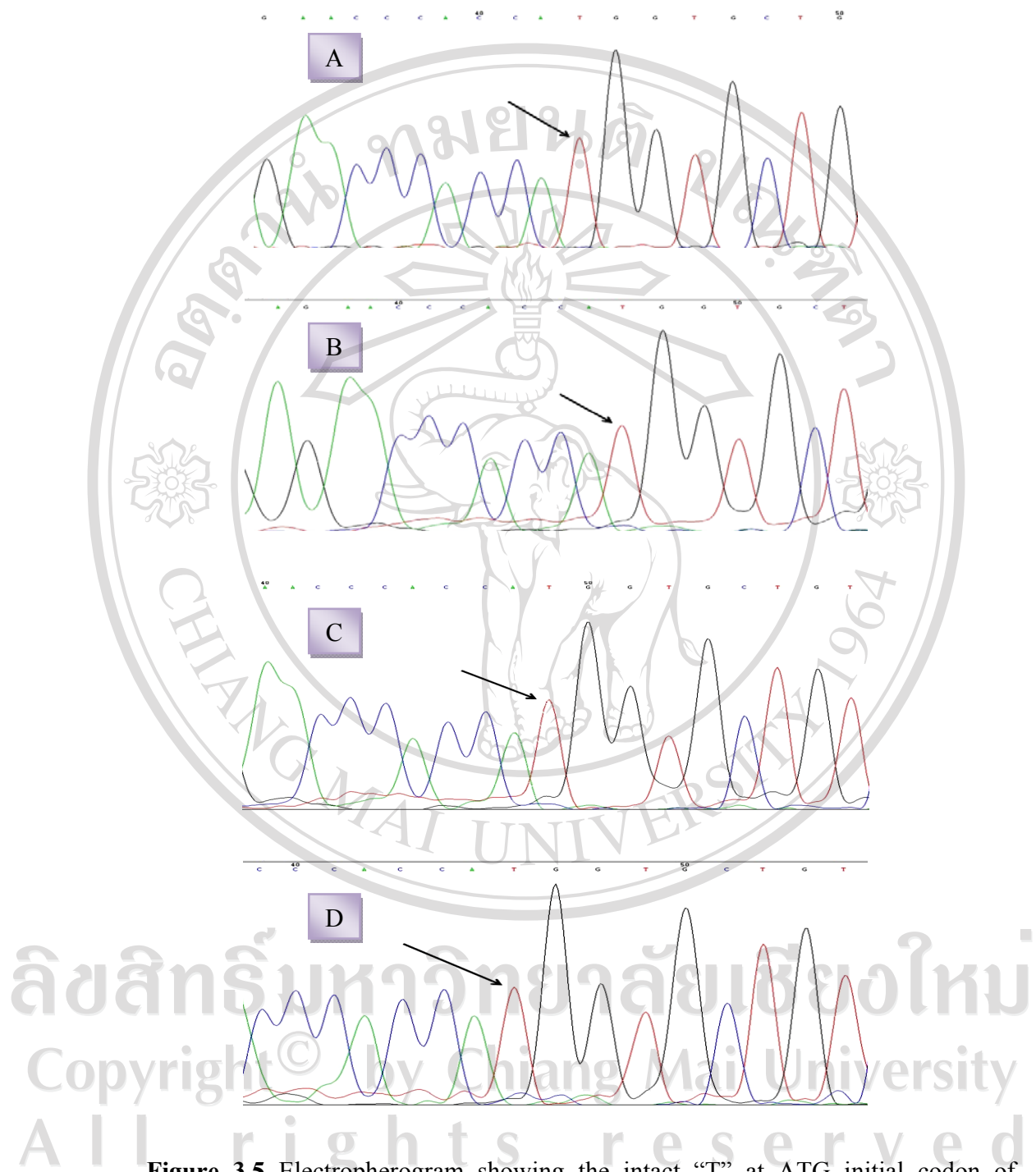
**Figure 3.3** Agarose gel picture of  $\alpha$ -thalassemia 1 (SEA type) after Gap-PCR process. Lane 1 is heterozygote for  $\alpha$ -thalassemia 1 (SEA type) in which both 653-bp and 753-bp PCR products are seen. Lane 2-5 are the tested samples of number 1-4, respectively where only 653-bp amplified fragments (interpreted a SEA deletion : -/-) are seen. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size markers.

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

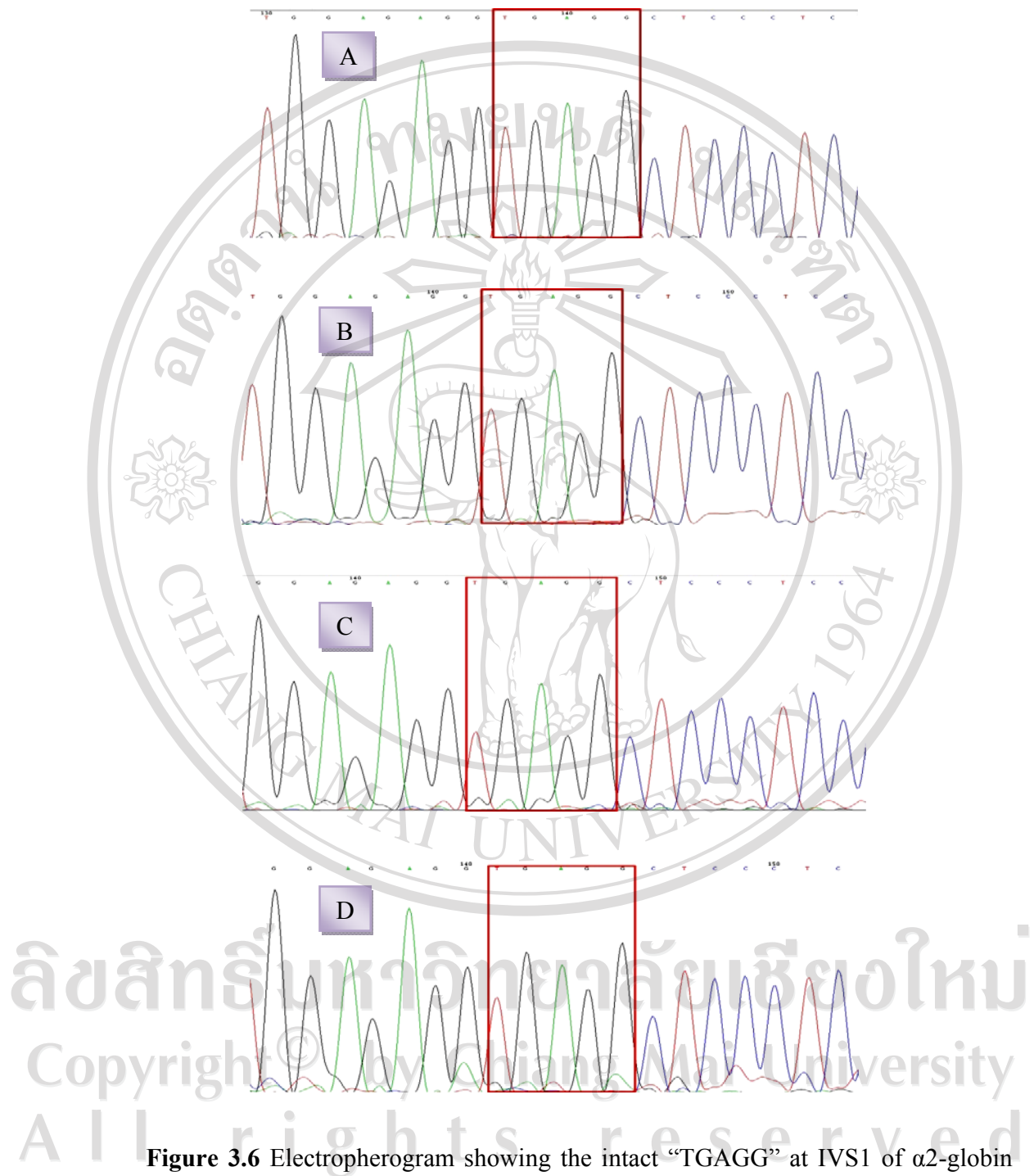
Determination of nucleotide sequences within and flanking both  $\alpha$ -globin genes was performed in all 4 samples. The procedure was detailed in the section 2.2.1.5. By using the 2 sequencing primers, the nucleotide sequences in the regions spanning nucleotide -55 from cap site (nucleotide 10441 of HUMHBA4) to nucleotide 155 after stop codon (nucleotide 11363 of HUMHBA4) for  $\alpha_1$ -globin gene and nucleotide -55 from cap site (nucleotide 6630 of HUMHBA4) to nucleotide 85 after stop codon (nucleotide 7475 of HUMHBA4) for  $\alpha_2$ -globin gene were determined. It was found that the nucleotide sequences on these regions are intact in 3 samples; e.g. no  $\alpha_2^{\text{NcoI}}$ , no  $\alpha_2^{\text{HphI}}$ , no A-G substitution at the initiation codon of  $\alpha_1$ -globin gene, no A-G substitution in IVSI-nt116 of  $\alpha_2$ -globin gene, no Hb Suandok and Hb Paksè, as shown in figures 3.4 to 3.10. However, the T-C substitution at the stop codon of the  $\alpha_2$ -globin gene (Hb Constant Spring) was found in one sample. Figures 3.10 showed the intact sequences at the positions reported to harbor mutations causing silent  $\alpha$ -thalassemia in other ethnic group.



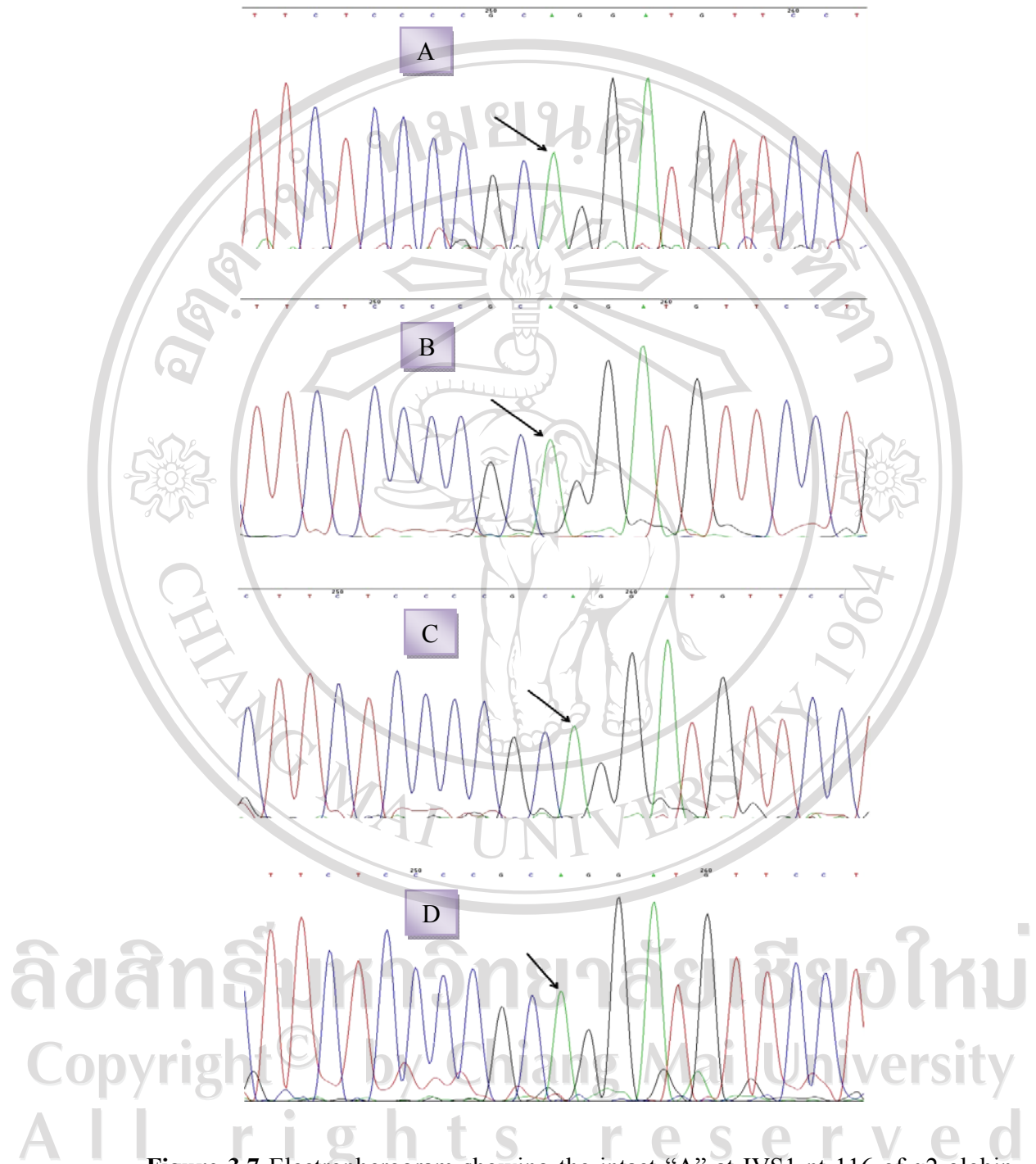
**Figure 3.4** Electropherogram showing the intact “A” at ATG initial codon of  $\alpha$ 1-globin gene (arrowed) of 4 samples analyzed (A-D, respectively). Note that the sequence of this point is normal.



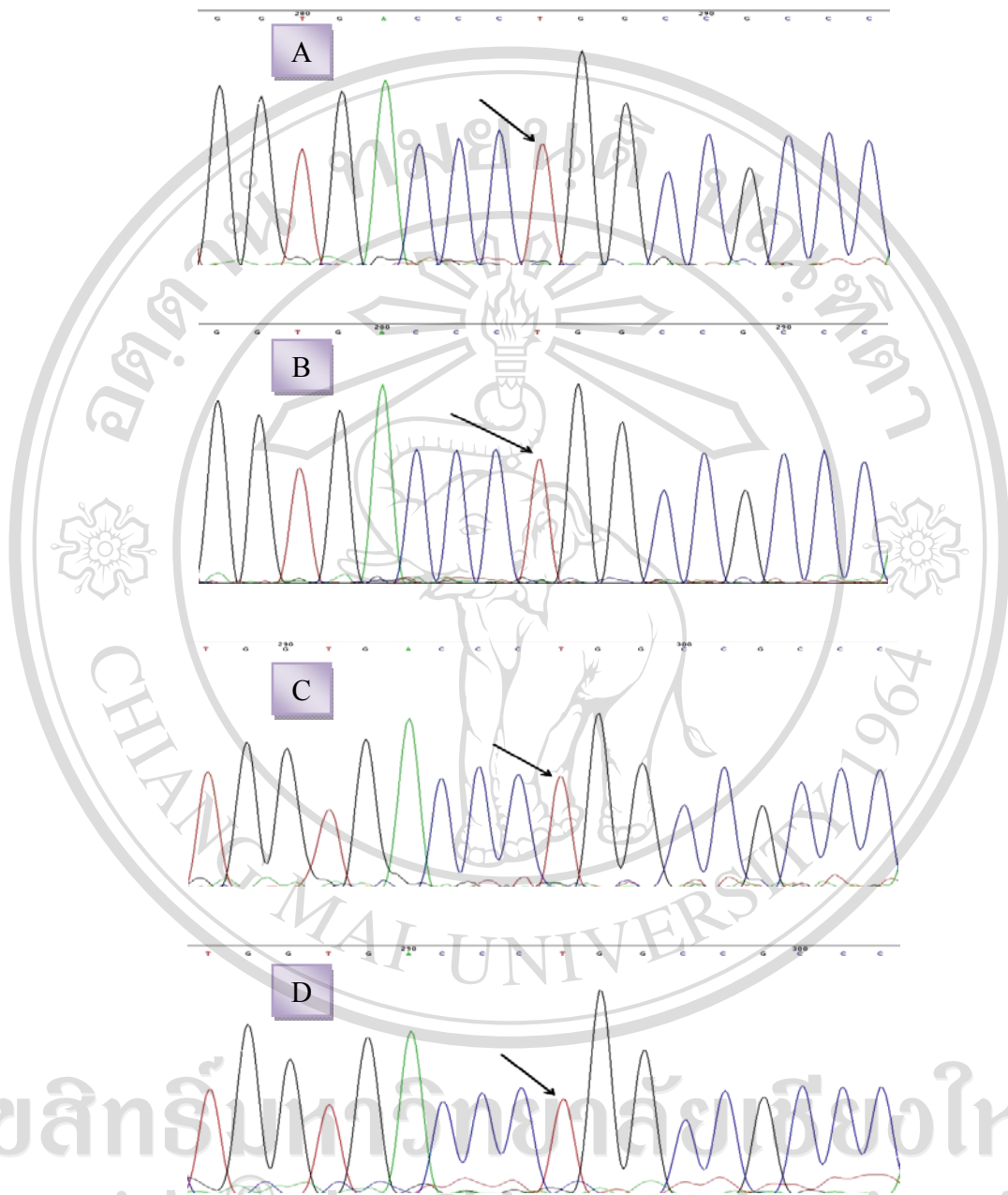




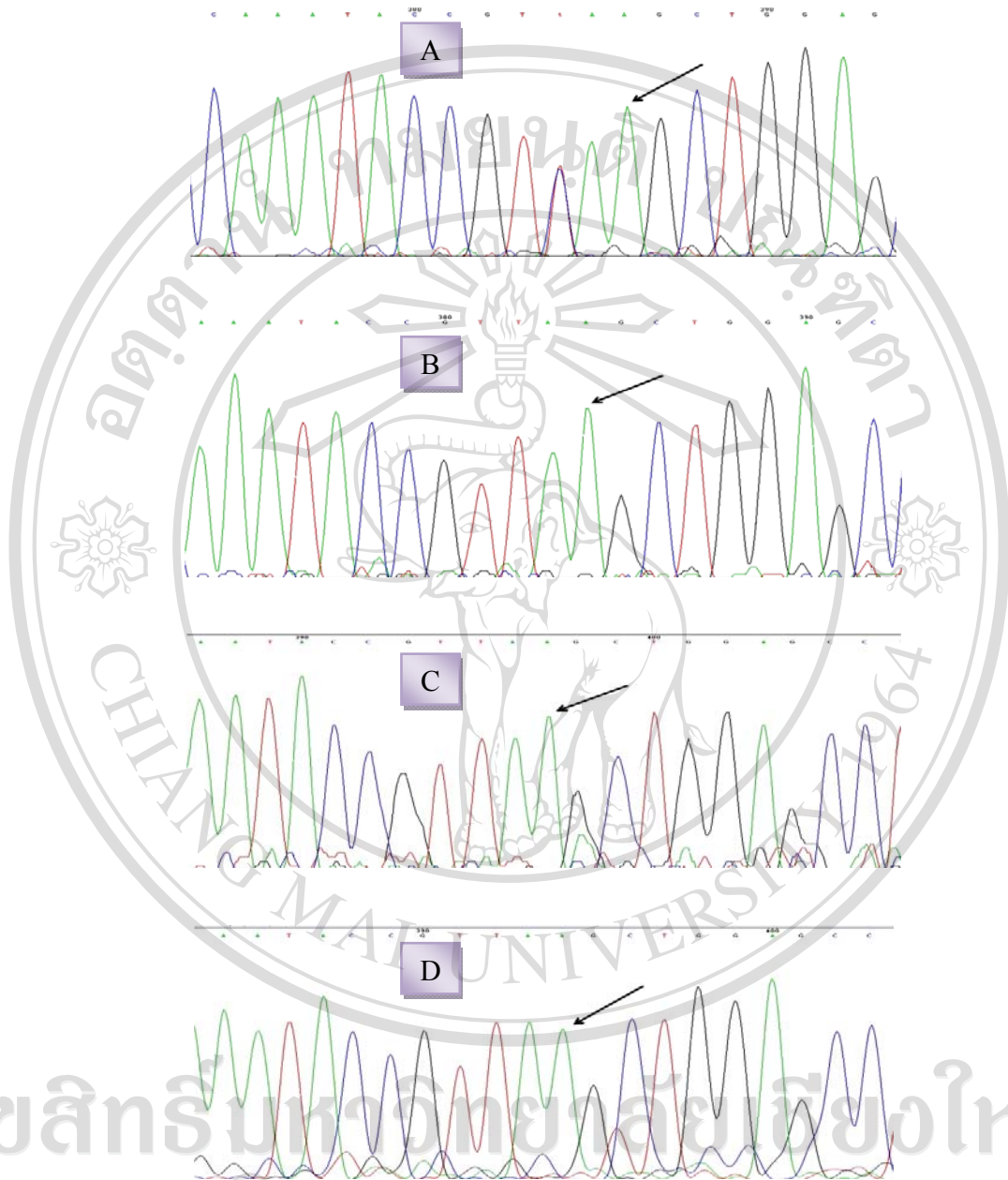
**Figure 3.6** Electropherogram showing the intact “TGAGG” at IVS1 of  $\alpha$ 2-globin gene (rectangled) of 4 samples analyzed (A-D, respectively). Note that the sequence of this point is normal and no *Hph*I cutting site present



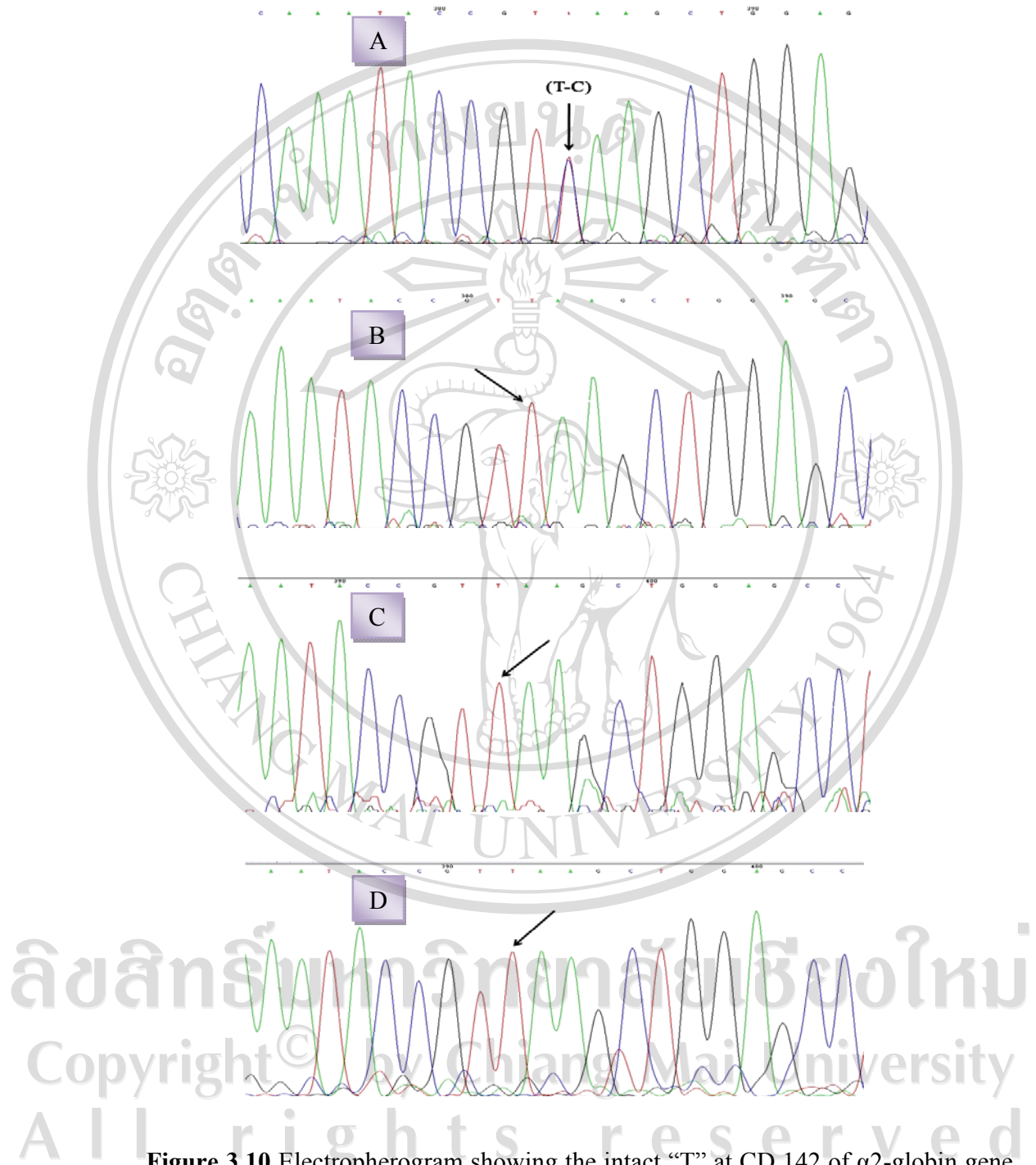
**Figure 3.7** Electropherogram showing the intact “A” at IVS1-nt 116 of  $\alpha 2$ -globin gene (arrowed) of 4 samples analyzed (A-D, respectively). Note that the sequence of this point is normal.



**Figure 3.8** Electropherogram showing the intact “T” at CD 109 of  $\alpha 2$ -globin gene (arrowed) of 4 samples analyzed (A-D, respectively). Note that the sequence of this point is normal and no codon for Hb Suan Dok ( $\alpha 2$ -globin gene with Cd 109: T-G; Leu-Arg) present



**Figure 3.9** Electropherogram showing the intact “A” at CD 142 of  $\alpha 2$ -globin gene (arrowed) of 4 samples analyzed (A-D, respectively). Note that the sequence of this point is normal and no codon for Hb Paksè ( $\alpha 2$ -globin gene with CD142: A-T; Stop-Tyr) present



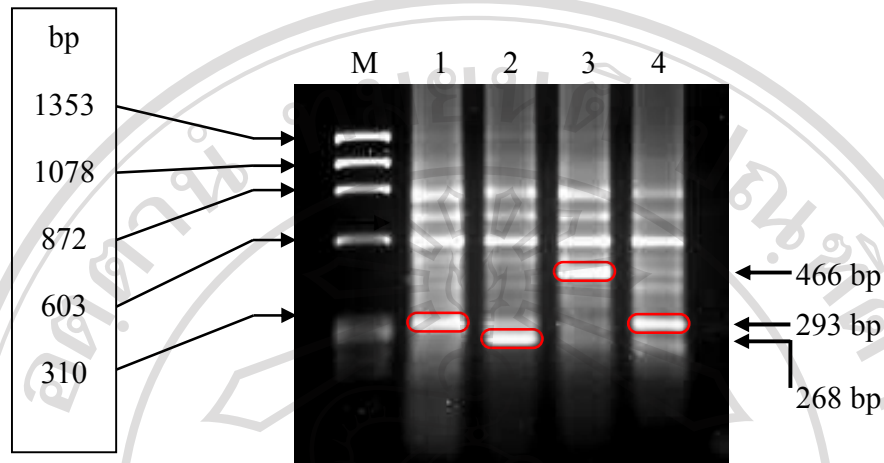
**Figure 3.10** Electropherogram showing the intact “T” at CD 142 of  $\alpha 2$ -globin gene (arrowed) of 3 samples analyzed (B-D, respectively) and T-C substitution at this position (arrowed) corresponding to the Hb Constant Spring present for the sample in panel A.

### **3.2 Optimization of multiplex allele-specific PCR for detection of double $\alpha/\beta$ -thalassemia heterozygote**

Since this protocol was initiated in this thesis, the optimization was then essential, i.e. to search for conditions that allow this newly developed method to be capable of detecting the mutations of interest without any false positive and false negative results. The search for optimal condition was then carried out and the results was demonstrated as follow:

#### **3.2.1 Test of $\beta$ -globin gene mutation-specific primers newly designed and synthesised**

The newly designed  $\beta$ -globin gene primers including Beta-common-multiplex, Beta-17-multiplex, Beta-E-multiplex and Beta-cds41/42-multiplex were tested to determine whether they worked satisfactorily. The procedure was described earlier in section 2.2.2.2. Samples heterozygous for  $\beta^E$ ,  $\beta^{17}$  and  $\beta^{41/42}$  alleles were used for this purpose. If amplified products size 268 bp, 293 bp and 466 bp were seen regardless of any nonspecific products, the test was interpreted to be successful. As seen in the Figure 3.11, all the expected PCR products are seen in the agarose gel picture. Thus it meant that the newly designed  $\beta$ -globin specific primers were in a good condition and were able to bind to their specific binding sites.

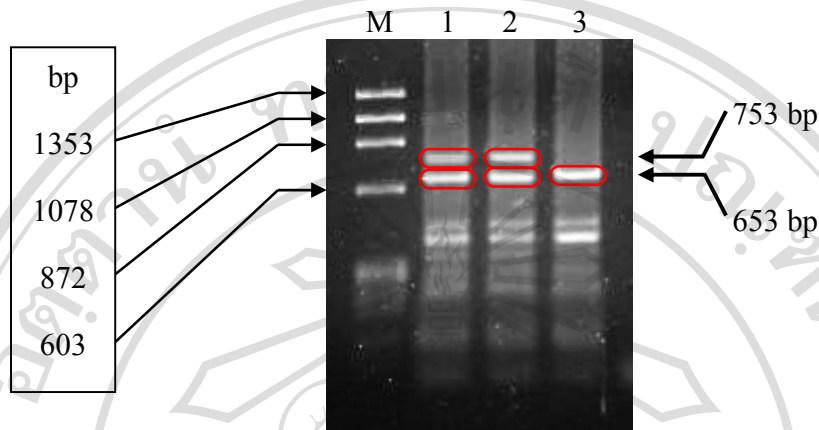


**Figure 3.11** Amplified products of three  $\beta$ -globin gene mutation-specific primers (highlighted). Lanes 1 and 4 are the 293-bp amplified products of primers Beta-common-multiplex + Beta-E-multiplex to detect  $\beta^E$ -allele. Lane 2 is the PCR products size 268 bp yielded from primers Beta-common-multiplex + Beta-17-multiplex to detect  $\beta^{17}$ -allele. Lane 3 is the 466-bp amplified products generated from primers Beta-common-multiplex + Beta-cds41/42-multiplex to detect  $\beta^{41/42}$  allele. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker

### 3.2.2 Test of $\alpha$ -thalassemia 1 (SEA type)-specific primers newly designed and synthesised

The  $\alpha$ -thalassemia 1(SEA type)-specific primers including “SEA-1-multiplex”, “SEA-2-multiplex” and “SEA-3-multiplex” were tested to determine their ability to correctly detect the  $\alpha$ -thalassemia 1 (SEA type). The step-by-step procedure was explained in the section 2.2.2.2. In the absence of the  $\alpha$ -thalassemia 1 (SEA type) allele, the amplified products size 653 bp (SEA-1-multiplex + SEA-2-multiplex) were produced. In homozygote for this defect, the amplified products generated from the primers SEA-1-multiplex and SEA-3-multiplex were synthesised. In heterozygote, the 653-bp and 753-bp amplified yields were produced. Thus, if the specific PCR products were seen on the agarose gel picture, even with some non-specific amplified products, the newly designed  $\alpha$ -thalassemia 1 (SEA type)-specific primers were concluded to be satisfied. As shown in the Figure 3.12, both heterozygote and absence of the SEA deletion are clearly seen. Thus these three primers were in good condition and could be used in subsequent experiments.

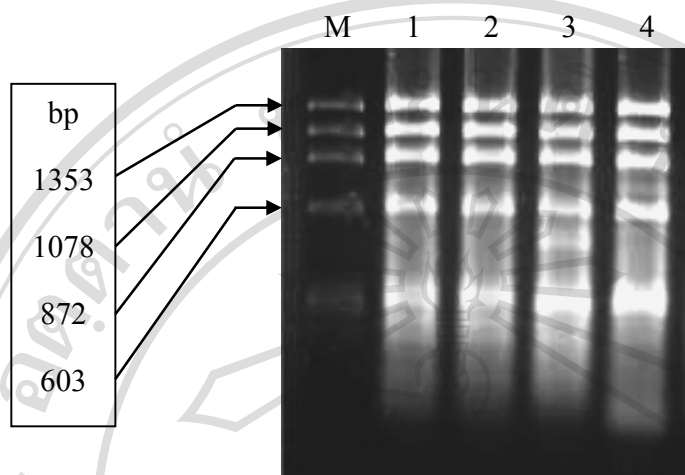




**Figure 3.12** Amplified product of  $\alpha$ -thalassemia (SEA type)-specific primers (highlighted). Lanes 1, 2 are heterozygote (both 653-bp and 753-bp fragments generated) and lane 3 is absent for the SEA-deletion as only the 653-bp fragment is seen. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker

### 3.2.3 Test of multiplex allele-specific PCR

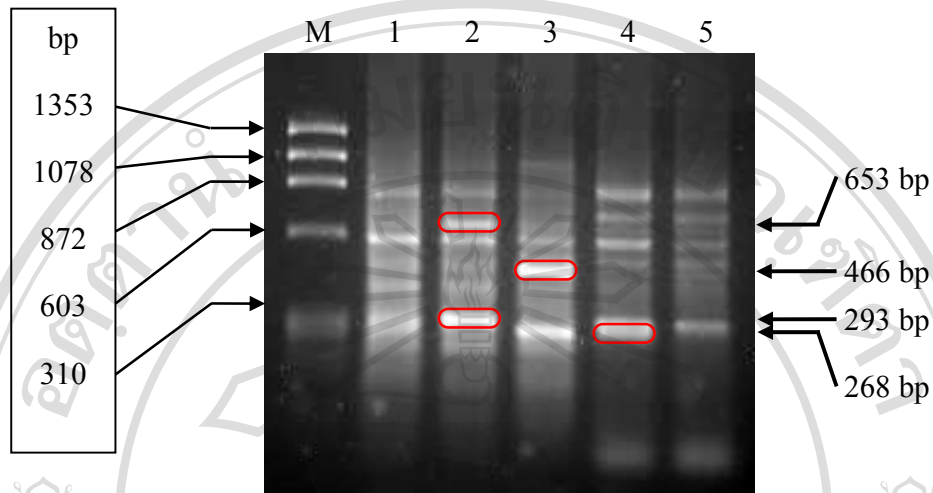
After the  $\alpha$ - and  $\beta$ -globin gene mutation-specific primers were proven to be in good condition and capable to detect the mutations they were specific for, the PCR system were set up with the use of all 7 primers; i.e. so-called “multiplex allele-specific PCR”. The procedure were dealt with in detail in the section 2.2.2.2. The amplified products were shown in Figure 3.13 and no specific amplified products were produced, except those of non-specific types. It was initially thought that the primer quantities could be too high and the optimization of the primer quantities was then required.



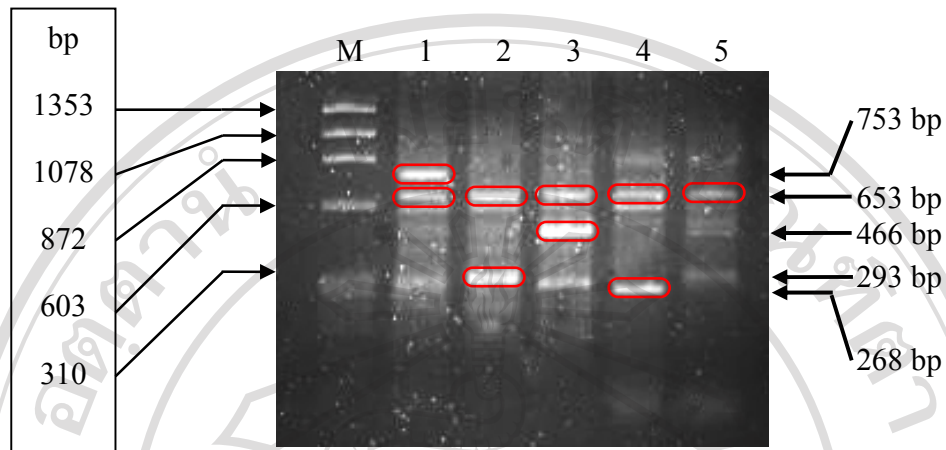
**Figure 3.13** Amplified product of the first-round multiplex allele-specific PCR. Only non-specific PCR fragments were generated in all samples. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard markers

### 3.2.4 Optimization of primers quantities

Since the first-round multiplex allele-specific PCR failed to demonstrate the specific bands at all. The overloading of primers into PCR reaction was supposed to cause this problem. Then the titration of primer quantities was carried out, the procedure of which also was described in the section 2.2.2.2. The success of PCR was determined by presence of specific PCR bands regardless of the presence of non-specific products. The results of multiplex allele-specific PCR using each primers at 50 ng and at 25 ng were shown in Figures 3.14 and 3.15, respectively. As shown in the Figure 3.14, the yields of amplification using 50 ng of each primer was better than the first-round experiment. However, considerable amount of non-specific products was still produced and SEA-specific products failed to be amplified in some specimens. Using 25 ng of each primer, in contrast, produced better results with all specific products generated and minimal signal of non-specific ones (Figure 3.15).



**Figure 3.14** Specific amplified products of multiplex allele-specific PCR using 50 ng of each primer (highlighted). Lane 1 is the known case of heterozygous  $\alpha$ -thalassemia 1 (SEA type) with no specific PCR products. Lane 2 is heterozygote for  $\beta^E$  allele in which specific products size 293 bp are seen. Lane 3 is heterozygote for  $\beta^{41/42}$  allele where the specific 466-bp fragments were produced. Lane 4 was heterozygote of  $\beta^{17}$  and the 268-bp specific fragments are seen. Lane 5 is the normal individual with only the 653-bp products. The 653-bp specific products of SEA-1-multiplex + SEA-2-multiplex served as the internal control which was inconsistently synthesised. Lane M represents the  $\Phi$ X174 *Hae*III digested DNA size standard marker



**Figure 3.15** Specific amplified products generated from the multiplex allele-specific PCR using 25 ng of each primer (highlighted). Lane 1 is heterozygote or  $\alpha$ -thalassemia 1 (SEA type) in which the specific 653-bp and 753-bp amplified fragment are seen. Lane 2 is heterozygote of  $\beta^E$  with the 293-bp PCR products. Lane 3 is the heterozygote of  $\beta^{41/42}$  allele in which the 466-bp products were produced. Lane 4 was heterozygote of  $\beta^{17}$  and the 268-bp specific fragments are seen. Lane 5 is the normal individual with only the 653-bp products, which were also used as internal control, are seen. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker.

### 3.2.5 Optimization of concentration of DMSO

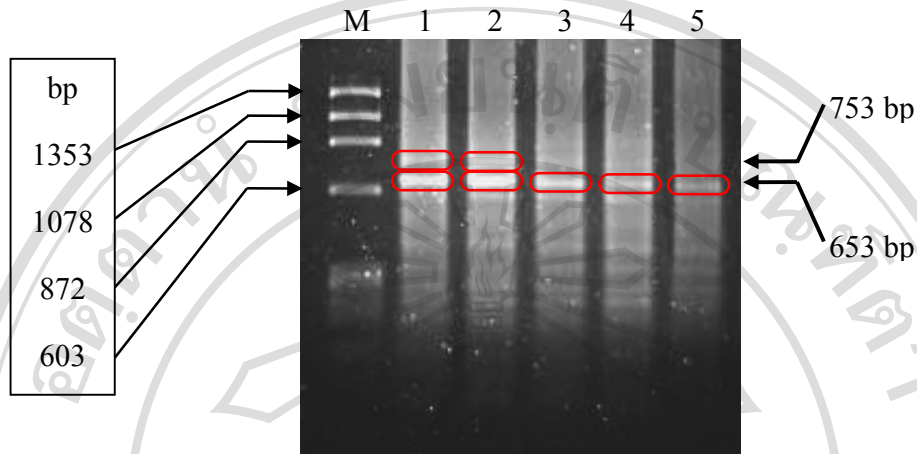
Dimethyl sulfoxide (DMSO) is an enhancer for the PCR reaction. The optimal amount of DMSO is then important for successful multiplex allele-specific PCR. Thus, to search for optimal amount of DMSO, the titration was performed on varieties of DMSO final concentration used in this experiment. The procedure for titration were described in the section 2.2.2.2. It was found that the optimal DMSO final concentration was 2%, the results of which is shown in Figure 3.16.

### 3.2.6 Optimization of final concentration of MgCl<sub>2</sub>

The optimal MgCl<sub>2</sub> concentration for the developed multiplex allele-specific PCR was determined by the procedure described in the section 2.2.2.2. By employing the DNA sample of heterozygous  $\alpha$ -thalassemia 1 (SEA type), the optimal amount of MgCl<sub>2</sub> was determined by visualizing the high intensity of specific amplified products as well as low amount of non-specific bands. As seen in Figure 3.17, the final MgCl<sub>2</sub> concentrations of 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM and 3.5 mM yielded different pattern of amplified products. At 2.0 mM final concentration of MgCl<sub>2</sub> yielded considerable intensity of specific bands, while other concentrations yielded unsatisfactory amplified products. Thus, MgCl<sub>2</sub> final concentration at 2.0 mM was chosen in subsequent experiments.



**Figure 3.16** Results of titration for optimal DMSO final concentration for use in the developed multiplex allele-specific PCR for detection of  $\alpha$ -thalassemia 1 (SEA type) in lanes 1 to 5 and of  $\beta^E$  in lanes 6 to 10. The DMSO final concentration titrated comprised 1%, 2%, 4%, 6% and 8%. The 293-bp fragment is specific for  $\beta^E$  and 753-bp fragments for SEA deletion. The 653-bp fragments generated from the SEA-1-multiplex + SEA-2-multiplex primers are the internal control. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker. Specific bands are rectangle.



**Figure 3.17** Result of titration for final concentration  $\text{MgCl}_2$  used in the developed multiplex allele-specific PCR using the DNA template from  $\alpha$ -thalassemia 1 (SEA deletion) at 25 ng. The  $\text{MgCl}_2$  final concentration of 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM and 3.5 mM were titrated and the products of which are shown in lanes labeled 1, 2, 3, 4, 5, respectively. Lane M indicates the  $\Phi\text{X174}$  *Hae*III digested DNA size standard marker. Note that at 2.0 mM  $\text{MgCl}_2$  (Lane 2), the 2  $\alpha$ -thalassemia 1 (SEA type)-specific products were clearly yielded. Specific bands are highlighted.

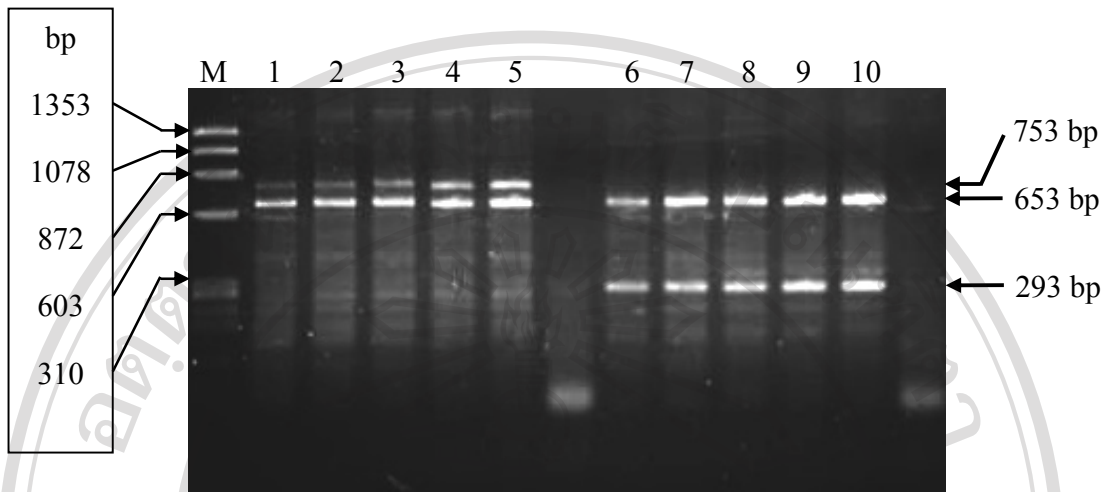


### 3.2.7 Optimization of quantities of dNTPs

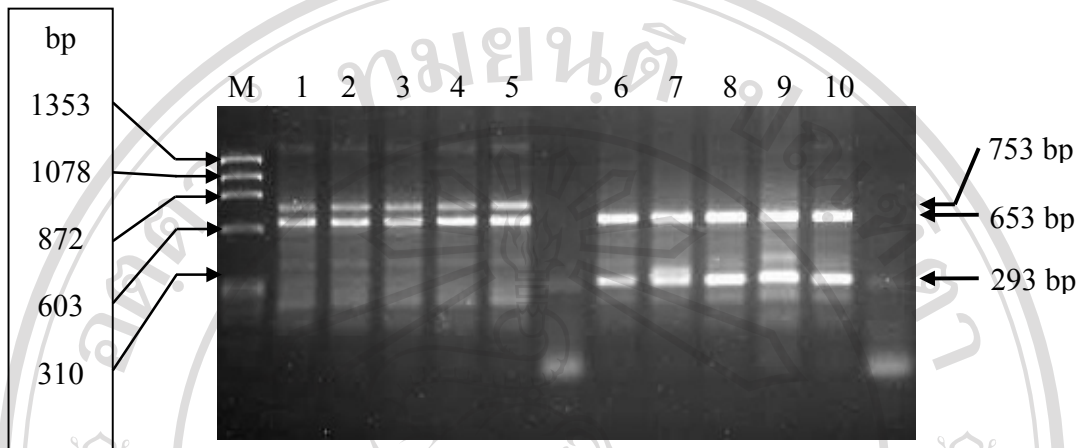
The optimal amount of dNTPs is usually important for the success of multiplex allele-specific PCR. Thus, to search for optimal amount of dNTPs, the titration was performed on varieties of dNTPs concentration used in this technique. The procedure for titration were described in section 2.2.2.2. The DNA sample of individual with heterozygous  $\alpha$ -thalassemia 1 (SEA type) and heterozygous  $\beta^E$  were employed for this experiment. The optimal quantities of dNTPs were determined by the high intensities of the 2 SEA-specific product bands, a clear separation of specific products on agarose gel electrophoresis and an absence of non-specific products. The dNTPs concentrations of 60  $\mu$ M, 80  $\mu$ M, 100  $\mu$ M, 120  $\mu$ M and 140  $\mu$ M were evaluated. It was found that the dNTPs concentration of 140  $\mu$ M yielded the products fulfilling the above criteria. Thus the final concentration of 140  $\mu$ M was used in the subsequent experiments (Figure 3.18).

### 3.2.8 Optimization of target DNA

The optimal amount of target DNA is essential for the success of developed multiplex allele-specific PCR. Thus, to search for optimal amount of DNA, the titration was performed on varieties amount of the Chelex<sup>TM</sup>-extracted DNA which was designed to be used for this new PCR technique. The procedure for titration were described in the section 2.2.2.2. The amount of the Chelex<sup>TM</sup>-extracted DNA of 1  $\mu$ l, 2  $\mu$ l, 3  $\mu$ l, 4  $\mu$ l and 5  $\mu$ l were titrated. It was found that quantity of the Chelex<sup>TM</sup>-extracted DNA of 5  $\mu$ l yielded the best results and then selected for the study throughout this thesis, as shown in figure 3.19.



**Figure 3.18** Results of titration of dNTPs final concentration to be used in the developed multiplex allele-specific PCR. Lanes 1-5 are samples of heterozygous  $\alpha$ -thalassemia (SEA type). Lanes 6-10 are samples of heterozygous  $\beta^E$ . The dNTPs concentration of 60  $\mu\text{M}$ , 80  $\mu\text{M}$ , 100  $\mu\text{M}$ , 120  $\mu\text{M}$  and 140  $\mu\text{M}$  were titrated, corresponding to lanes 1, 2, 3, 4, 5 and 6, 7, 8, 9, 10, respectively. The 293 bp fragment is specific for  $\beta^E$  and 753 bp for SEA deletion. The 653 bp is the internal control. Lane M is the  $\Phi\text{X174}$  *Hae*III digested DNA size standard marker. Note that lanes 5 and 10 yield the best results and then dNTPs at 140  $\mu\text{M}$  was chosen.



**Figure 3.19** Result of titration of amount of Chelex™-extracted DNA to be used in the developed multiplex allele-specific PCR. Sample of heterozygous  $\alpha$ -thalassemia (SEA deletion) was used (lanes 1-5) and that of heterozygous HbE in lanes 6-10. The amount of DNA of 1  $\mu$ l, 2  $\mu$ l, 3  $\mu$ l, 4  $\mu$ l and 5  $\mu$ l were titrated, corresponding to lanes 1, 2, 3, 4, 5 and 6, 7, 8, 9, 10, respectively. The 293 bp fragment is specific for HbE and 753 bp for SEA deletion. The 653 bp is the internal control. M indicates the  $\Phi$ X174 HaeIII digested DNA size markers. Note that lanes 4 and 10 shows the best results and then Chelex™-extracted DNA of 5  $\mu$ l was chosen.

### 3.2.9 Titration of SEA-2-multiplex primers

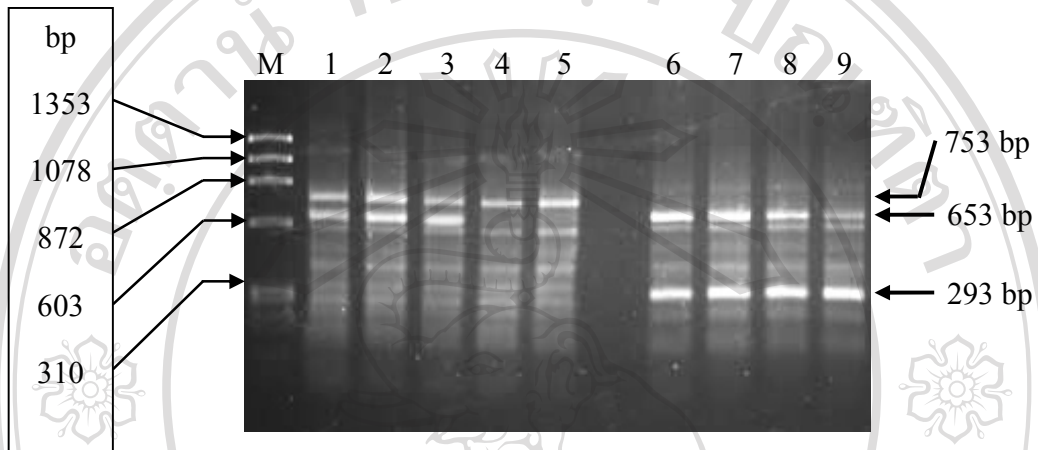
As the products generated from the primers SEA-1-multiplex + SEA-2-multiplex with size 653 bp are designed to serve as the internal control in this newly developed protocol, thus the optimal amount of the primer SEA-2-multiplex must be optimized; i.e. to assure that the internal control products are consistently synthesised in sufficient amount. The procedure for titration of amount of SEA-2 multiplex primer were described in 2.2.2.2 employing the concentration determined in the previous optimization steps. The amount of the primer SEA-2-multiplex titrated included 0.063 ng (0.008  $\mu$ M), 0.031 ng (0.004  $\mu$ M), 0.125 ng (0.176  $\mu$ M), 0.25 ng (0.035  $\mu$ M) and 0.5 ng (0.070  $\mu$ M). The result of this studies is shown in Figure 3.20. The results in lanes 2 and 7 corresponding to the primer's quantity of 0.25 ng are the best as demonstrated by the highest intensity with clear separation of the specific products and minimal amount of non-specific bands. The author then choosed 0.25 ng as the working amount of SEA-2-multiplex primer.

### 3.2.10 Validation of final optimized condition

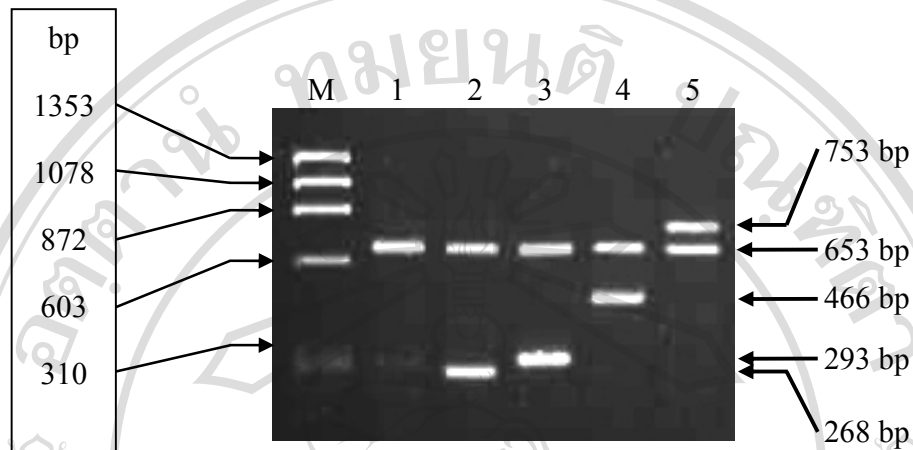
After conditions of all the essential ingredients of the newly developed multiplex allele-specific PCR for detecting, simultaneously,  $\alpha$ -thalassemia 1 (SEA type),  $\beta^E$ ,  $\beta^{17}$  and  $\beta^{41/42}$  alleles were successfully optimized, the set-up protocol was then tested again employing DNA sample from both single heterozygotes and double heterozygotes.

Significant validity of the developed technique was shown as it was capable to correctly

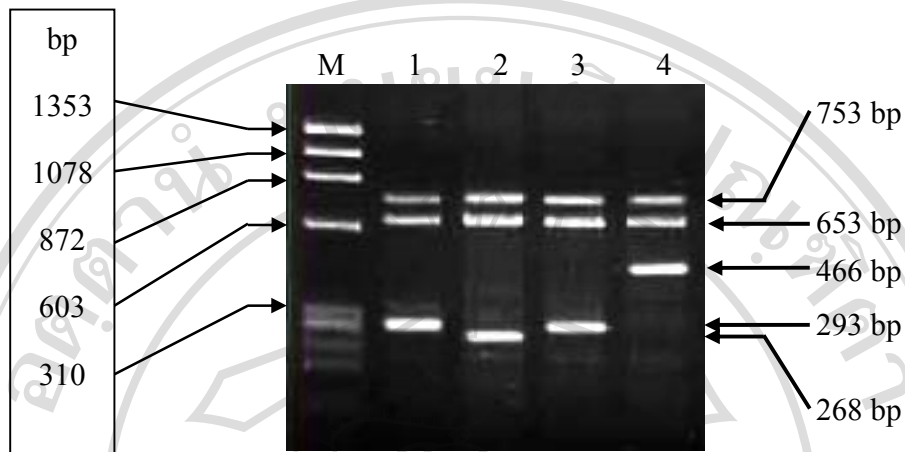
detect both single thalassemia heterozygotes (Figure 3.21) and double thalassemia heterozygote (Figure 3.22).



**Figure 3.20** Result of titration for optimal amount of SEA-2-multiplex primer for use in the developed multiplex allele-specific PCR. Results of heterozygous  $\alpha$ -thalassemia (SEA deletion) are shown in lanes 1-5. Results of heterozygous HbE are shown in lanes 6-9. The SEA-2-multiplex primer concentration of 0.125 ng, 0.25 ng, 0.5 ng, 1.0 ng and 1.5 ng, corresponding to lanes 1, 2, 3, 4, 5 were titrated for  $\alpha$ -thalassemia (SEA deletion). For heterozygous  $\beta^E$ , the SEA-2-multiplex primer concentration of 0.125 ng, 0.25 ng, 0.5 ng and 1.0 ng, corresponding to lanes 6, 7, 8, 9 were titrated. The 293-bp fragment is specific for HbE and 753-bp for SEA deletion. The 653-bp band is the internal control. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker. Note that the SEA-2-multiplex of 0.25 ng (lanes 2 and 7) was selected.



**Figure 3.21** Amplified products generated by the optimized multiplex allele-specific PCR to detect  $\beta^{17}$  (lane 2),  $\beta^E$  (lane 3),  $\beta^{41/42}$  (lane 4) and  $\alpha$ -thalassemia 1 (SEA type) (lane 5) alleles. condition for single mutation. Lane 1 is the PCR products of individual negative for those 4 mutations where 653-bp internal control band is seen. This control band of 653 bp in size are also seen in all lanes, otherwise the results are unreadable. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker.

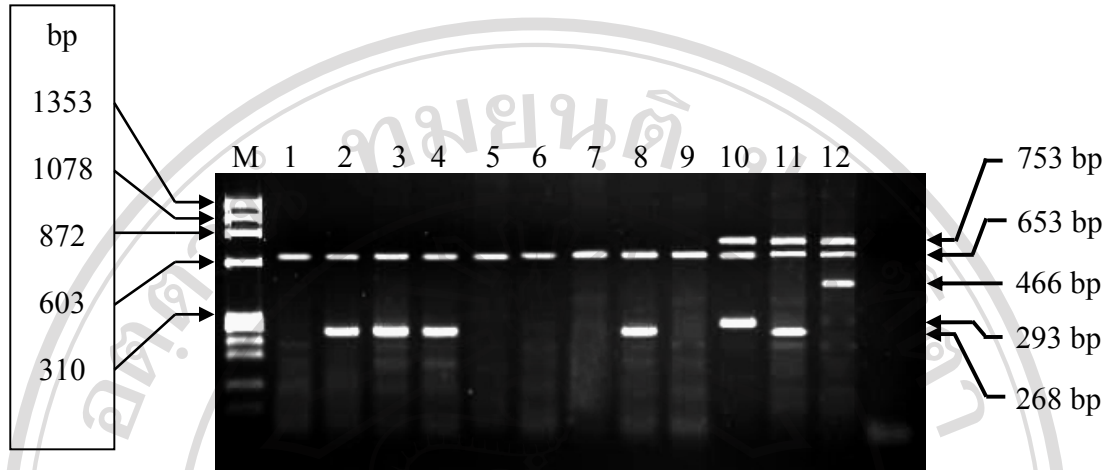


**Figure 3.22** Amplified products generated by the optimized multiplex allele-specific PCR to detect double heterozygote of  $\alpha$ -thalassemia 1 (SEA type) and  $\beta^E$  (lanes 1 and 3), double heterozygote of  $\alpha$ -thalassemia 1 (SEA type) and  $\beta^{17}$  (lane 2), double heterozygote of  $\alpha$ -thalassemia 1 (SEA type) and  $\beta^{41/42}$  (lane 4). PCR products of size 268 bp are  $\beta^{17}$ -specific, 293 bp  $\beta^E$ -specific, 466 bp  $\beta^{41/42}$  specific and 753 bp  $\alpha$ -thalassemia 1 (SEA type) specific. The 653-bp fragments are the internal control. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker.

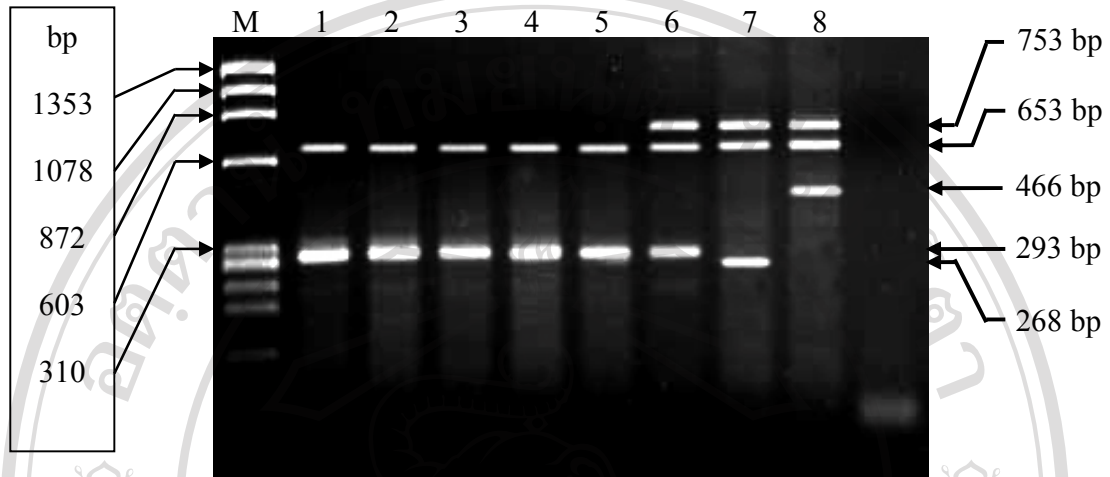
### 2.2.2.3 Determination of double heterozygous state using the developed multiplex allele-specific PCR

The samples initially diagnosed as  $\beta$ -thalassemia trait (BH in Table 1.1) and HbE trait (EH in Table 1.1) were tested for co-existence of  $\alpha$ -thalassemia employing the developed multiplex allele-specific PCR. After completion of the PCR process, 4 samples (lanes 2, 3, 4, 8) were single heterozygote for  $\beta^{17}$ , as shown in Figure 3.23. 5 samples (lanes 1-5) were single heterozygote for  $\beta^E$ , as shown in Figure 3.24. However, none were found to be heterozygote for  $\beta^{41/42}$  and for  $\alpha$ -thalassemia 1 (SEA type).





**Figure 3.23** Amplified products generated by the developed multiplex allele-specific PCR. Lanes 1-9 are samples diagnosed as  $\beta$ -thalassemia trait (BH in Table 1.1). Lanes 2-4 and lane 8 are only  $\beta^{17}$  heterozygote since only 283-bp fragments are seen. Lanes 1, 5, 6, 7, 9 are negative for both  $\alpha$ -thalassemia 1 (SEA type) and all 3 types of  $\beta$ -thalassemia mutations. Lanes 10-12 are known cases for double heterozygotes for  $\alpha$ -thalassemia/ $\beta^E$ ,  $\alpha$ -thalassemia/ $\beta^{17}$  and double  $\alpha$ -thalassemia/ $\beta^{41/42}$ , respectively. None of these samples are heterozygous for  $\alpha$ -thalassemia 1 (SEA type) as no 753-bp were generated.  $\alpha$ -globin specific amplified products size 653 bp are internal control and must be seen in all lanes. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker.



**Figure 3.24** Amplified products generated by the developed multiplex allele-specific PCR. Lanes 1-5 are samples diagnosed as HbE trait (EH in Table 1.1). Lanes 1-5 are only HbE ( $\beta^E$ ) heterozygote since only 293-bp bands are produced. Lanes 6-8 are known cases for double heterozygotes of  $\alpha$ -thalassemia/ $\beta^E$ ,  $\alpha$ -thalassemia/ $\beta^{17}$  and  $\alpha$ -thalassemia/ $\beta^{41/42}$ , respectively. None of these samples are heterozygous for  $\alpha$ -thalassemia 1 (SEA type) as no 753-bp were generated.  $\alpha$ -globin specific amplified products size 653 bp are internal controls and must be seen in all lanes. Lane M is the  $\Phi$ X174 *Hae*III digested DNA size standard marker.