CHAPTER III

RESULTS

3.1 Optimization of single ARMS-PCR

3.1.1 Optimization of annealing temperature

The determination of optimal annealing temperature in the single ARMS-PCR for detection of codons 41/42 were described in 2.5.1 in which the annealing temperature of 59^0C, 61^0C, 63^0C, 65^0C, 67^0C and 69^0C were titrated. The amplified products were shown in figure 3.1. The signal intensity of the PCR products of different annealing temperature was clearly identical. This means that this ARMS-PCR could be successfully performed at least within the annealing temperature range between 59^0C to 69^0C. The author, however, chose 65^0 C as an annealing temperature for the study in the thesis.

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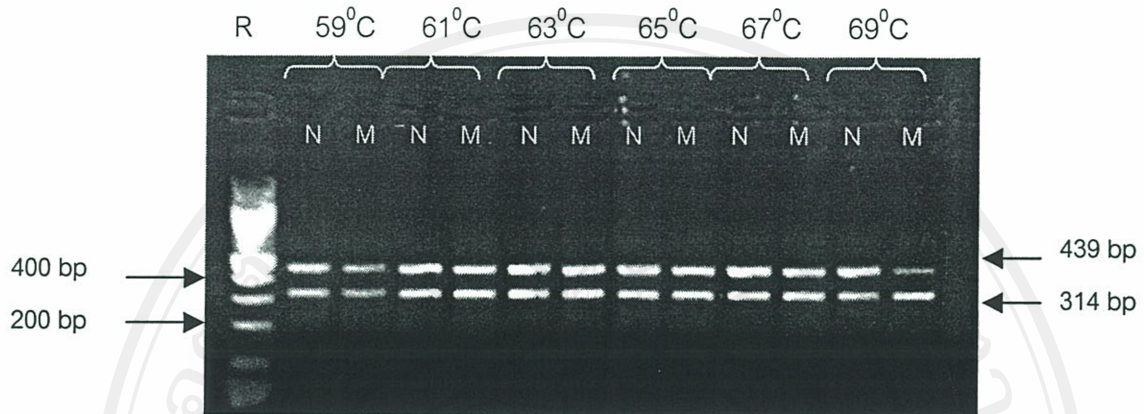

Figure 3.1 The amplified products of codons 41/42 generated from the single ARMS-PCR at different annealing temperature (59°C – 69°C). 439-bp was the PCR fragment for codons 41/42 detection and 314-bp the internal control. M represents mutant reaction. N represent normal reaction. R indicates the pME-80J3 *Eco*1471 and *Pvu*I digested DNA size marker.

3.1.2 Titration of primer quantities used in single ARMS-PCR

The investigation of optimal quantities of the primers was described in 2.5.2 and the efficiency of the ARMS-PCR was evaluated from the signal intensity of the PCR products on 2.0% agarose gel electrophoresis. The results of titration for optimal amount of primers for codon 17, codons 41/42, codons 71/72 and Hb E were shown in figures 3.2 to 3.5, respectively. It was found that 0.2 μM was optimal for normal and mutant codon 17 primers, 0.15 μM for codons 41/42 primers. However, for codons 71/72, 0.1 μM normal primer and 0.15 μM mutant primer were optimal. In addition, 0.1 μM normal primer and 0.15 μM mutant primer were sufficient for successful single ARMS-PCR for Hb E.

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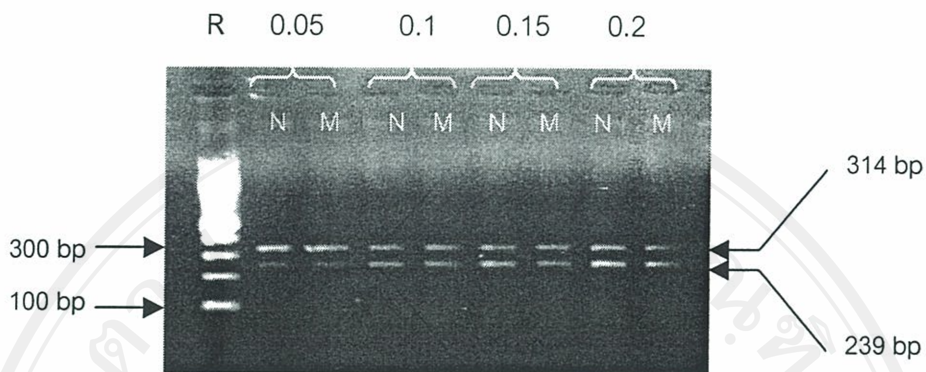

Figure 3.2 Titration of primer concentration used in single ARMS-PCR of condon 17. Primer concentrations of 0.05 μM to 0.2 μM were titrated as shown in the row above the figure. Lane R indicated the pME-80J3 *Eco*1471 and *Pvu*I digested DNA size marker. 314-bp is internal control and 239-bp is PCR fragment of codon 17. N represents normal reaction while M is for mutant reaction.

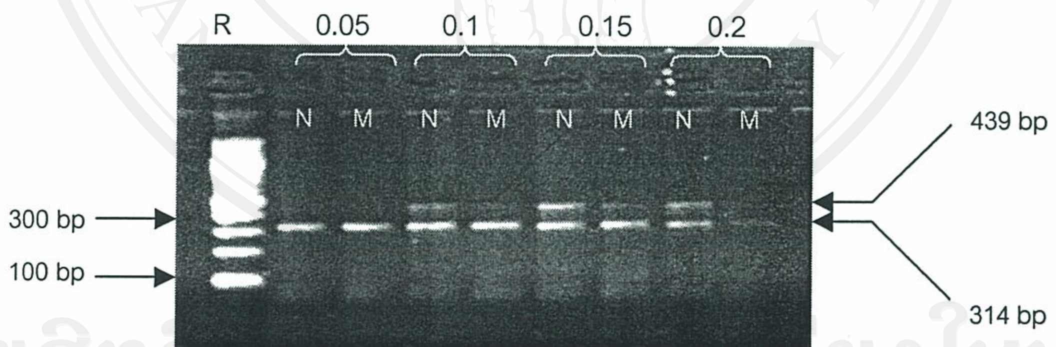

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Figure 3.3 Titration of primer concentration used in single ARMS-PCR of condons 41/42. Primer concentrations of 0.05 μM to 0.2 μM were titrated as shown in the row above the figure. Lane R indicated the pME-80J3 *Eco*1471 and *Pvu*I digested DNA size marker. 314-bp is internal control and 439-bp are PCR fragment size of codon 41/42. N represents normal reaction while M is for mutant reaction.

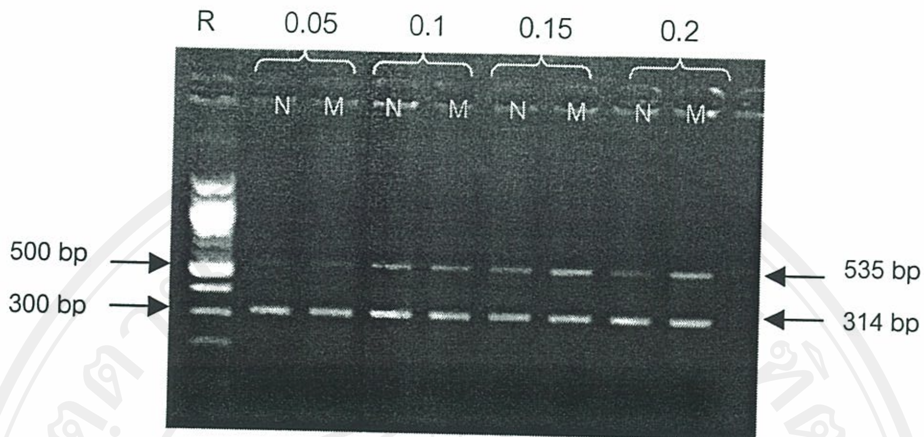

Figure 3.4 Titration of primer concentration used in single ARMS-PCR of condons 71/72. Primer concentrations of 0.05 μM to 0.2 μM were titrated as shown in the row above the figure. Lane R indicated the pME-80J3 *Eco*1471 and *Pvu*I digested DNA size marker. 314-bp is internal control and 535-bp are PCR fragment size of codon 71/72. N represents normal reaction while M is for mutant reaction.

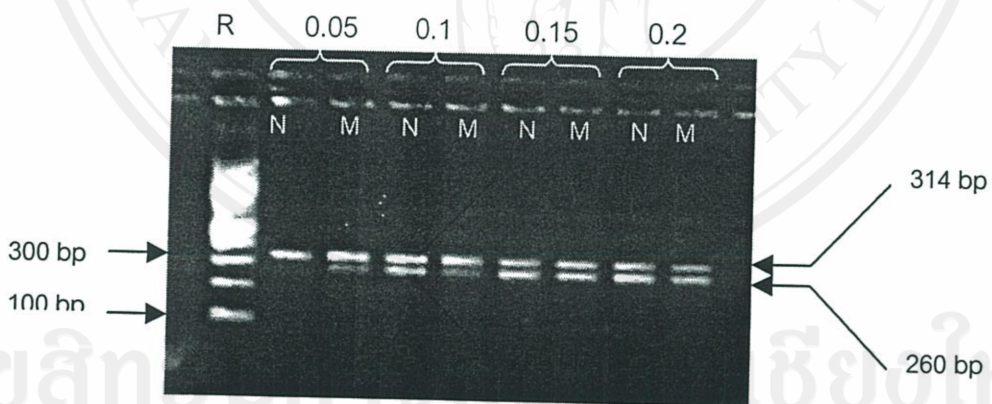

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Figure 3.5 Titration of primer concentration used in single ARMS-PCR of HbE. Primer concentrations of 0.05 μM to 0.2 μM were titrated as shown in the row above the figure. Lane R indicated the pME-80J3 *Eco*1471 and *Pvu*I digested DNA size marker.314-bp is internal control and 260-bp are PCR fragment size of HbE. N represents normal reaction while M is for mutant reaction.

3.2 Final evaluation of single ARMS-PCR for detection of β-thalassemia mutations

In the final evaluation of single ARMS-PCR, the PCR system mentioned in 2.4.1 with the optimized annealing temperature of 65^0 C and the optimized amount of ARMS-PCR primers in 3.1.2 was carried out with the DNA samples of known mutations, all of which had already been successfully characterized by the standard nucleotide sequencing. As shown in the figures 3.7-3.11, all the β-thalassemia results determined by the single ARMS-PCR did completely agree with those generated by the standard nucleotide sequencing.

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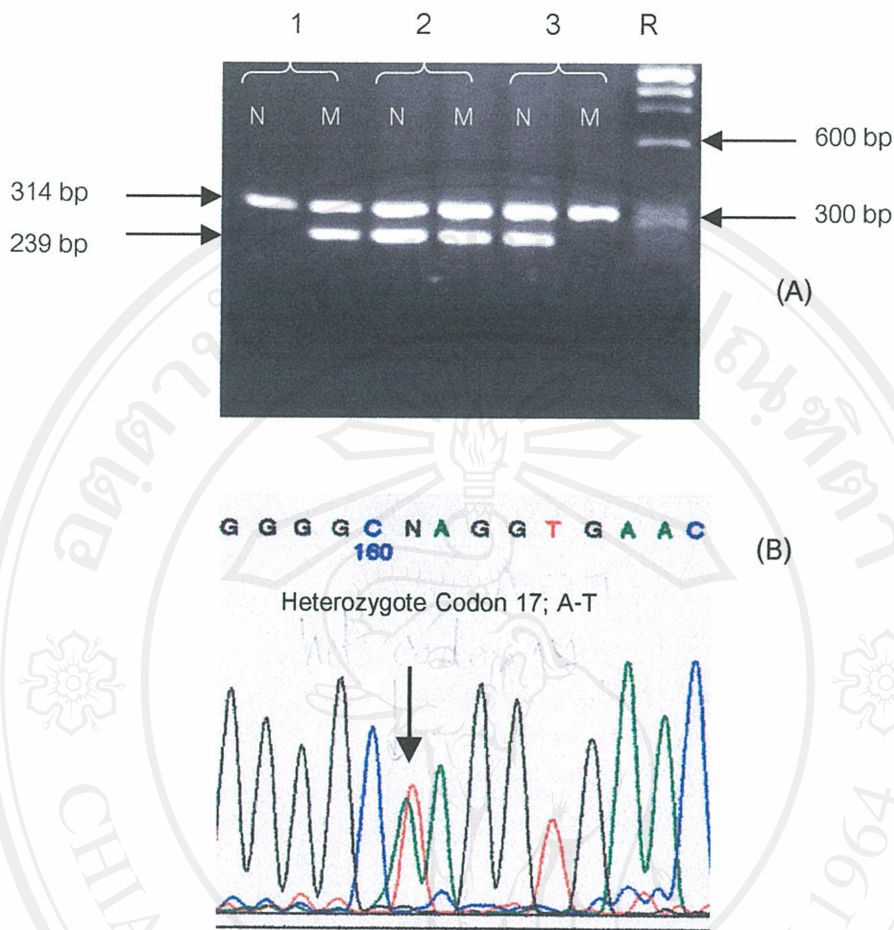

Figure 3.7 Detection of codon 17 by single ARMS-PCR. (A) The amplified products on 2.0% agarose gel in which number 1, 2 and 3 represent known DNA samples and "R" represents ϕX174 *Hae* III digested DNA size marker. "N" is the reaction in which common and normal primers were utilized, while "M" is the reaction where common and mutant primers were employed. The 314-bp fragment is the internal control and the 239-bp band is the specific PCR product. Case #1 is homozygote, case #2 heterozygote and case #3 negative for this mutation. The corresponding nucleotide sequencing of this mutation in case #2 are shows in (B).

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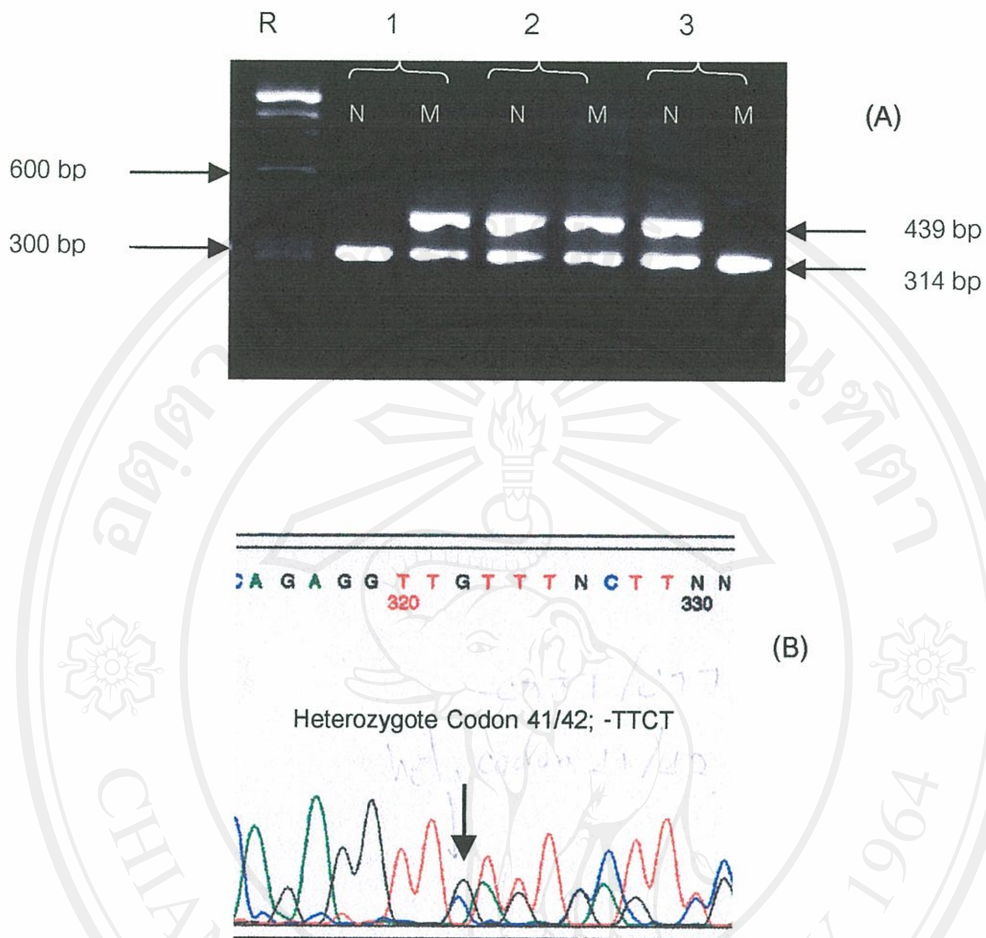

Figure 3.8 Detection of codon 41/42 by single ARMS-PCR. (A) The amplified products on 2.0% agarose gel in which number 1, 2 and 3 represent known DNA samples and "R" represents ϕX174 *Hae* III digested DNA size marker. "N" is the reaction in which common and normal primers were utilized, while "M" is the reaction where common and mutant primers were employed. The 314-bp fragment is the internal control and the 439-bp band is the specific PCR product. Case #1 is homozygote, case #2 heterozygote and case #3 negative for this mutation. The corresponding nucleotide sequencing of this mutation in case #2 are shows in (B).

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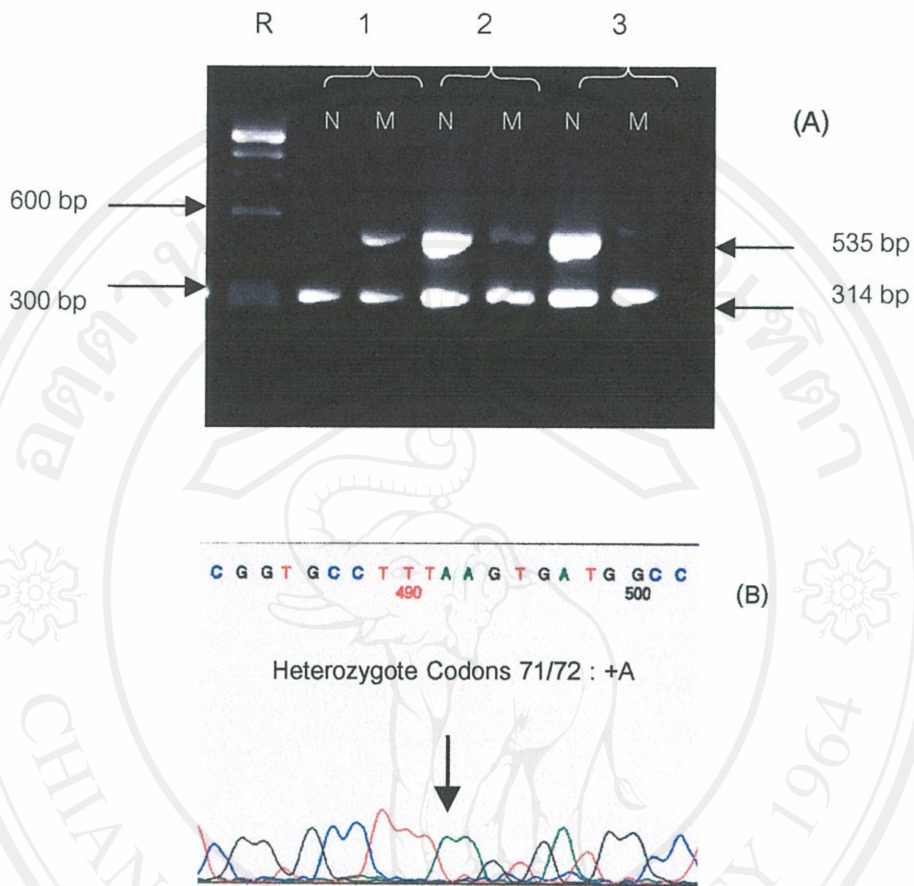

Figure 3.9 Detection of codon 71/72 by single ARMS-PCR. (A) The amplified products on 2.0% agarose gel in which number 1, 2 and 3 represent known DNA samples and "R" represents ϕX174 *Hae* III digested DNA size marker. "N" is the reaction in which common and normal primers were utilized, while "M" is the reaction where common and mutant primers were employed. The 314-bp fragment is the internal control and the 535-bp band is the specific PCR product. Case #1 is homozygote, case #2 heterozygote and case #3 negative for this mutation. The corresponding nucleotide sequencing of this mutation in case #2 are shows in (B).

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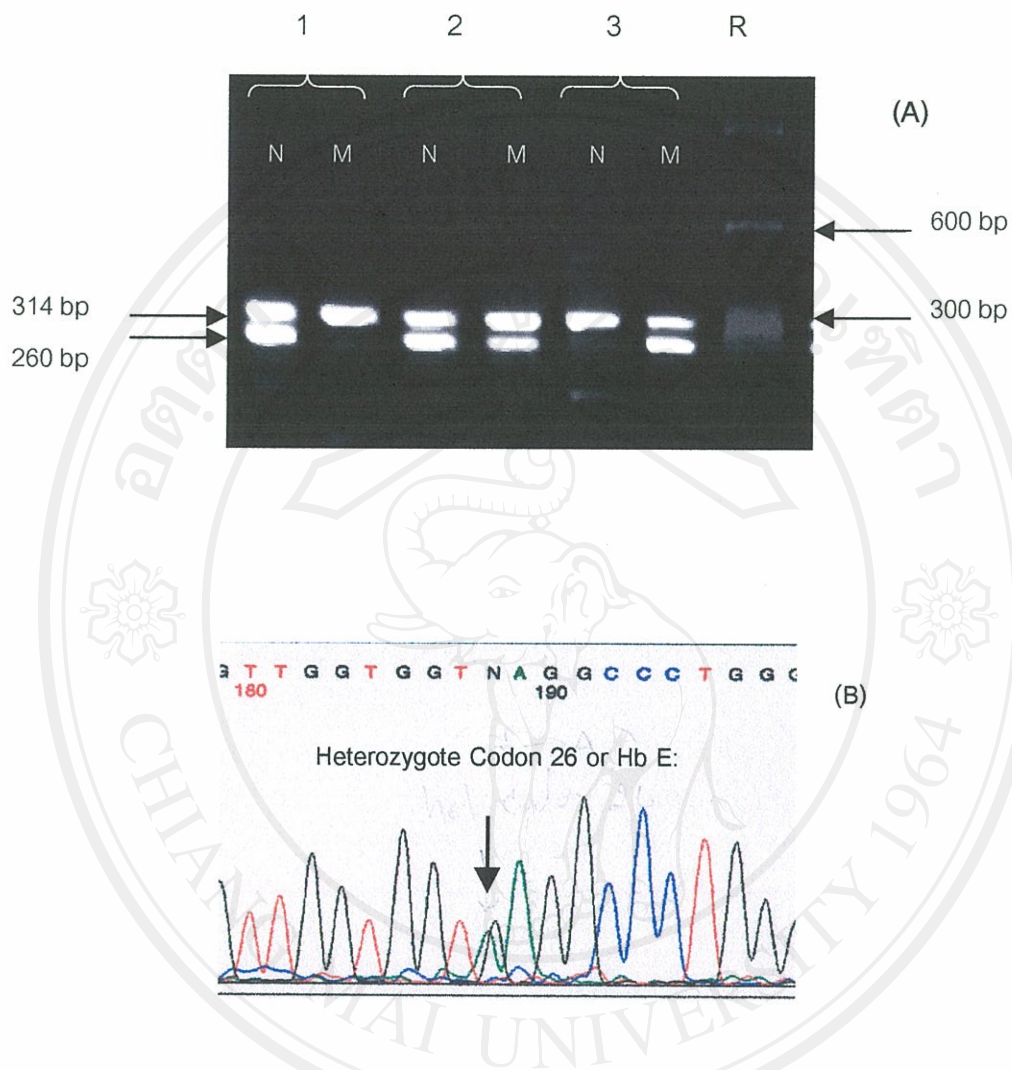

Figure 3.10 Detection of Hb E by single ARMS-PCR. (A) The amplified products on 2.0% agarose gel in which number 1, 2 and 3 represent known DNA samples and "R" represents ϕX174 *Hae* III digested DNA size marker. "N" is the reaction in which common and normal primers were utilized, while "M" is the reaction where common and mutant primers were employed. The 314-bp fragment is the internal control and the 260-bp band is the specific PCR product. Case #1 is negative, case #2 heterozygote and case #3 homozygote for this mutation. The corresponding nucleotide sequencing of this mutation in case #2 are shows in (B).

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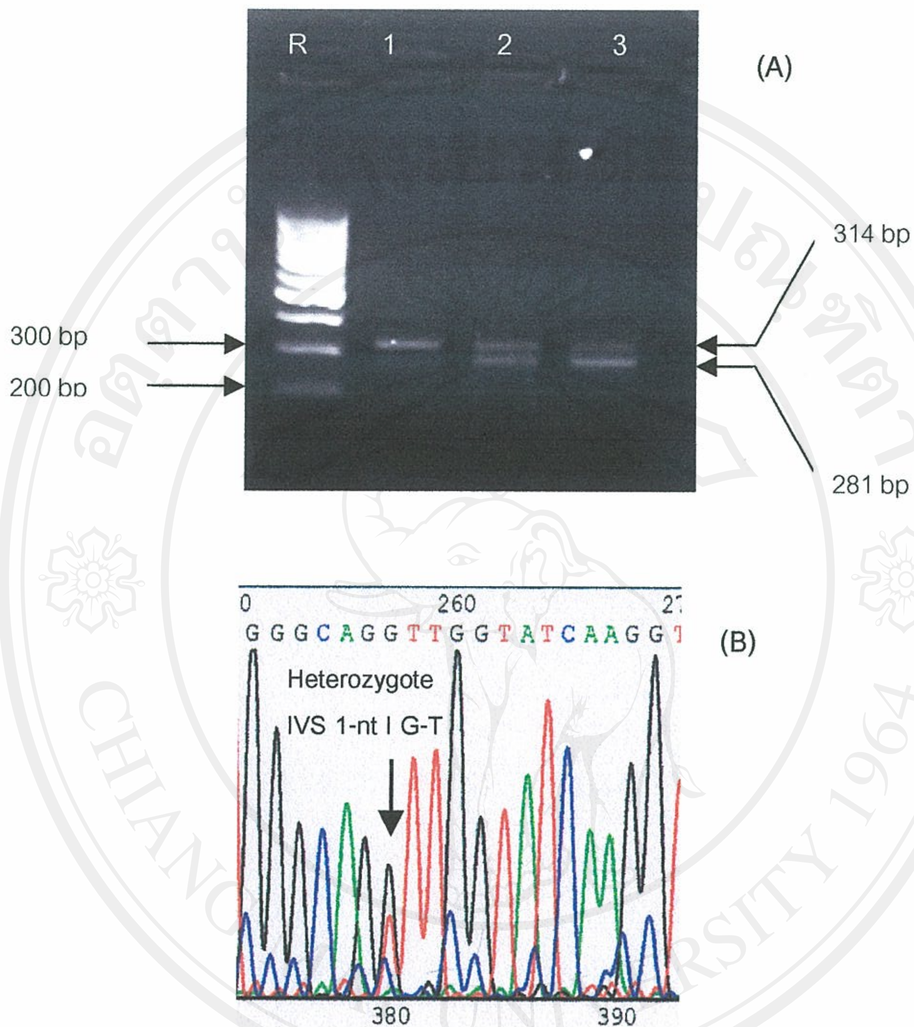

Figure 3.11 Detection of IVS1-nt I by single ARMS-PCR. (A) The amplified products on 2.0% agarose gel in which number 1, 2 and 3 represent known DNA samples and "R" represents pME-80J3 *Eco* 1471 and *Pvu* I digested DNA size marker. Only reaction where common and mutant primers were employed. The 314-bp fragment is the internal control and the 280-bp band is the specific band. Case #1 is negative, cases #2, #3 positive. The corresponding nucleotide sequencing of this mutation in case #2 are shows in (B) where heterozygous state of case #2 is revealed.

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3.3 Optimization of multiplex ARMS-PCR

3.3.1 Optimization of dNTP concentration

The optimal amount of dNTP is usually important for the success of ARMS-PCR including the multiplex ARMS-PCR. Thus, to search for optimal amount of dNTP, the titration was performed on varieties of dNTPs concentration used in multiplex-PCR. The procedure for titration were described in 2.5.3.1. It was found that the dNTP concentration of 300 μM was optimal as shown in figure 3.12.

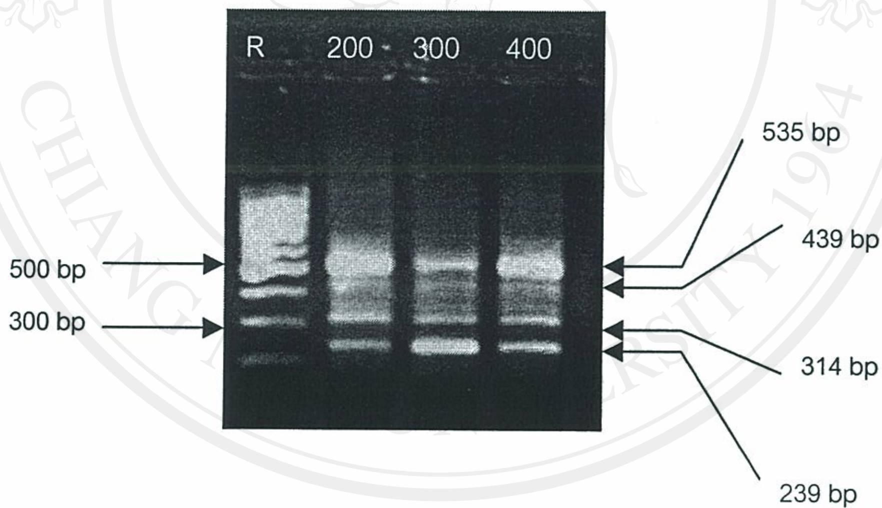

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Figure 3.12 The result of dNTP concentration titration in multiplex ARMS-PCR for wild-type primers of codons17, 41/42 and 71/72. The dNTP concentration of 200, 300, 400 μM were tritrated. Lane "R" indicates pME-80J3 *Eco* 1471 and *Pvu* I digested DNA size marker. The 314-bp fragment is the internal control. The 535-bp band is specific for codons 71/72 , 439-bp band for codons 41/42 and 239-bp band for codon 17.

3.3.2 Optimization of $MgCl_2$ concentration

The $MgCl_2$ concentration optimal for multiplex ARMS-PCR was determined by the procedure described in 2.5.3.1 using 300 μM dNTPs concentration determined in the previous section. By evaluating the signal of the PCR product on 2.0 % agarose gel, the optimal amount of $MgCl_2$ for this kind of PCR was determined. As seen in figure 3.13, the $MgCl_2$ concentrations of 1.8, 2.1, 2.3, 2.5 mM yield identical and discrete amplified bands of all three β-thalassemia alleles, whereas at 1.5 mM $MgCl_2$, the product seems to be low. The author, however, decided to use the $MgCl_2$ at 2.1 mM for the study throughout this thesis.

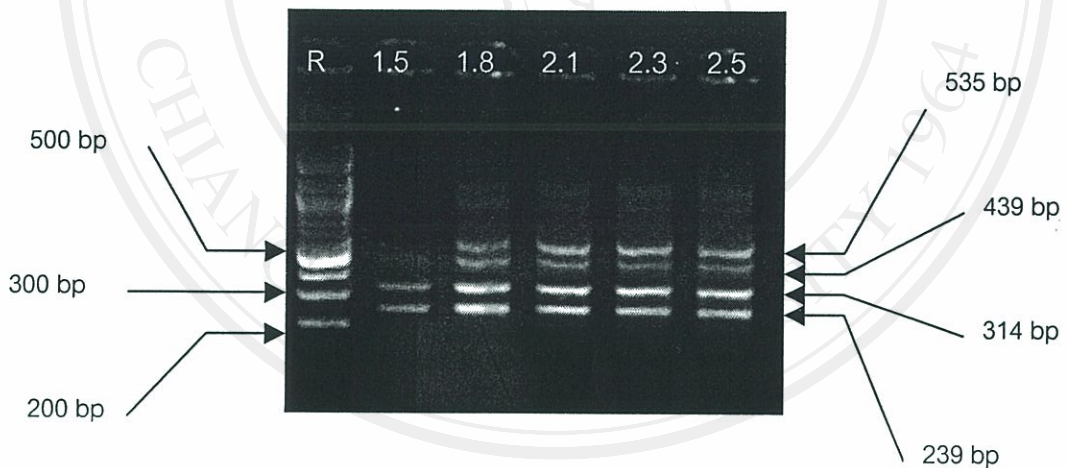

Figure 3.13 The result of $MgCl_2$ concentration titration in multiplex ARMS-PCR for wild-type primers of codons17, 41/42 and 71/72. The $MgCl_2$ concentration of 1.5, 1.8, 2.1, 2.3 and 2.5 mM were tritrated. Lane "R" indicates pME-80J3 *Eco* 1471 and *Pvu* I digested DNA size marker. The 314-bp fragment is the internal control. The 535-bp band is specific for codons 71/72 , 439-bp band for codons 41/42 and 239-bp band for codon 17.

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3.3.2 Optimization of the amount of β-specific common primer

The procedure for optimization of amount of β-specific common primers were described in 2.5.3.2 using 300 μM dNTPs and 2.1 mM $MgCl_2$. The result of this studies is shown in figure 3.14. where the intensity of PCR signal are almost the same in all concentration of common primer. The author choosed 0.4 μM as the working amount of β-specific common primer throughout the thesis.

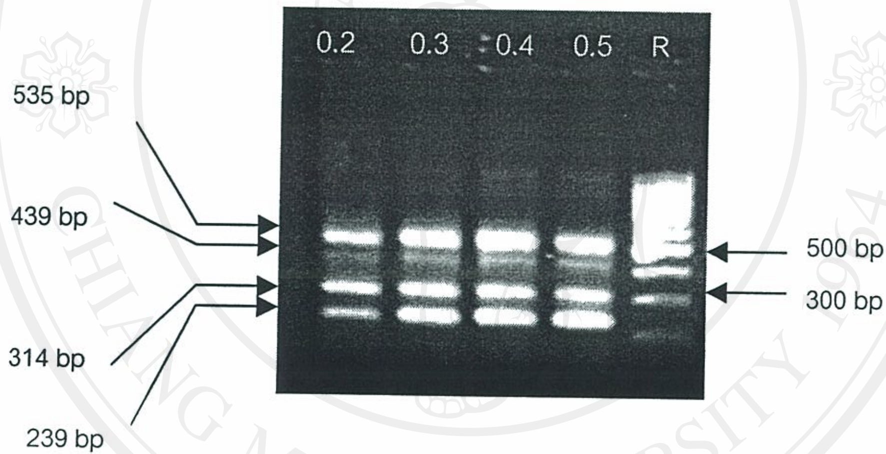

Figure 3.14 The result of titration for optimal concentration of common primer used in multiplex ARMS-PCR with wild-type primers for codons17, 41/42 and 71/72. The common primer concentration of 0.2, 0.3, 0.4 and 0.5 μM were tritrated. Lane "R" indicates pME-80J3 *Eco* 1471 and *Pvu* I digested DNA size marker. The 314-bp fragment is the internal control. The 535-bp band is specific for codons 71/72 , 439-bp band for codons 41/42 and 239-bp band for codon 17.

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3.4 Final evaluation of multiplex ARMS-PCR for detection of β-thalassemia mutations

After optimal amount of dNTPs (300 μM), $MgCl_2$ (2.1 mM), annealing temperature of 65^0C and common primer (0.4 μM) were obtained, the multiplex ARMS-PCR was then used for the identification of β-thalassemia mutation in DNA samples that had been characterized for mutations already. As described in 2.4.2, four combinations of primers were prepared for ARMS-PCR; hence making it multiplex ARMS-PCR .The four combinations of primers included 1) codon 17 (A-T) and codons 41/42 (-TTCT), 2) codon 17 (A-T) and HbE, 3) condons 41/42 (-TTCT) and HbE, 4) condon 17 (A-T), codons 41/42 (-TTCT) and codons 71/72 (+A). The started amount of wild type and mutant specific ARMS-PCR primers used in the single ARMS-PCR were adjusted to obtain better yields in multiplex ARMS-PCR. The four multiplex ARMS-PCR results for β-thalassemia mutation detection was demonstrated in figures 3.15 to 3.18. The β-thalassemia mutations detected from multiplex ARMS-PCR were perfectly identical to those characterized by the direct nucleotide sequencing.

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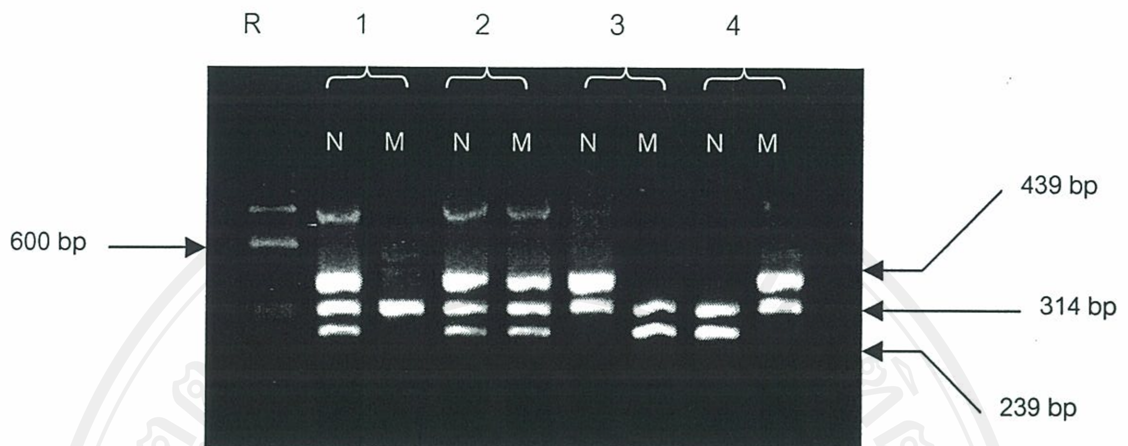

Figure 3.15 The results of multiplex ARMS for detection of codons 41/42 and codon 17. Lane R indicated the ϕX174 *Hae* III digested DNA size marker. No 1 represents negative case where no PCR products seen in M lane (mutant reaction) but the 439-bp and 239-bp bands are present only in N lane (normal reaction). No 2 is compound heterozygote for codon 17 and codons 41/42 where the 439-bp and 239-bp band are present in the both M and N lanes. No 3 represents homozygote for codon 17 in which the 239-bp band is present only in M lane (mutant reaction). No 4 represent homozygote for codons 41/42 [439-bp band is present only in M lane (mutant reaction)].

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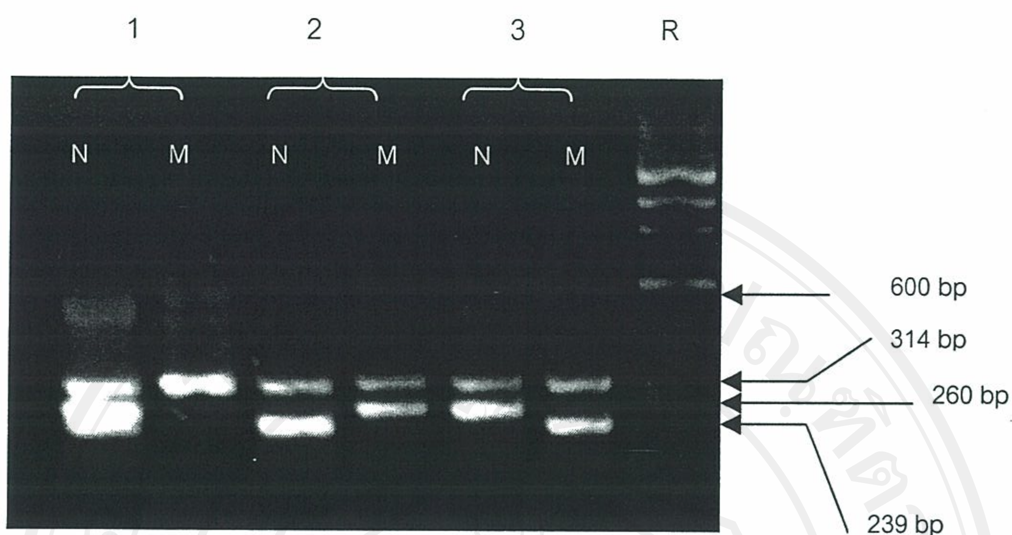

Figure 3.16 The results of multiplex ARMS for detection of codon 17and Hb E. Lane R indicated the ϕX174 *Hae* III digested DNA size marker. No 1 represents negative case where no PCR products seen in M lane (mutant reaction) but the 239-bp and 260-bp bands are present only in N lane (normal reaction). No 2 is homozygote for Hb E in which the 260-bp band is present only in M lane (mutant reaction). No 3 represents homozygote for codon 17 in which the 239-bp band is present only in M lane (mutant reaction).

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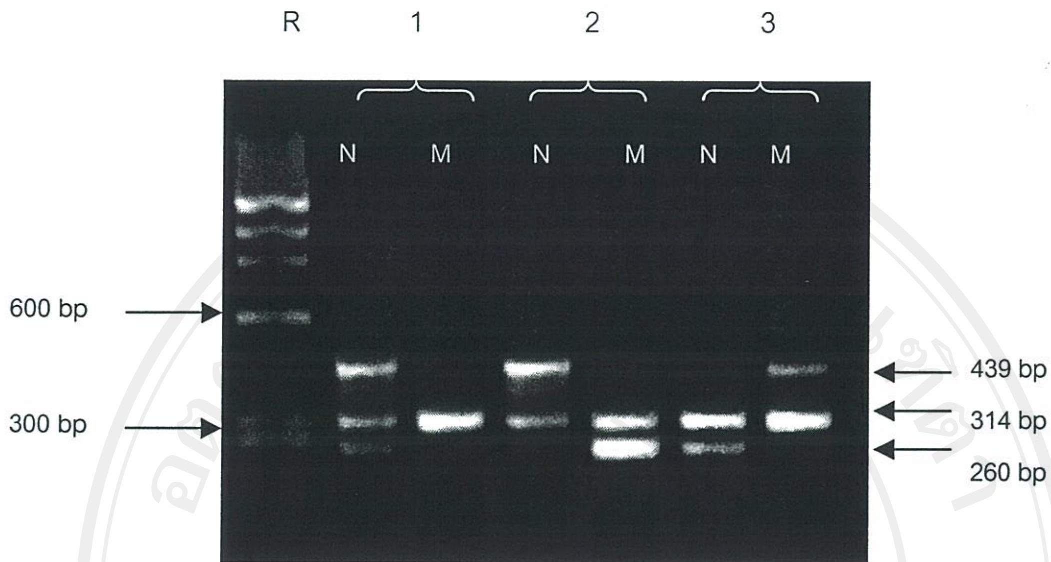

Figure 3.17 The results of multiplex ARMS for detection of codons 41/42 and Hb E. Lane R indicated the ϕX174 *Hae* III digested DNA size marker. No 1 represents negative case where no PCR products seen in M lane (mutant reaction) but the 439-bp and 260-bp bands are present only in N lane (normal reaction). No 2 is homozygote for Hb E in which the 260-bp band is present only in M lane (mutant reaction). No 3 represents homozygote for codons 41/42 in which the 439-bp band is present only in M lane (mutant reaction).

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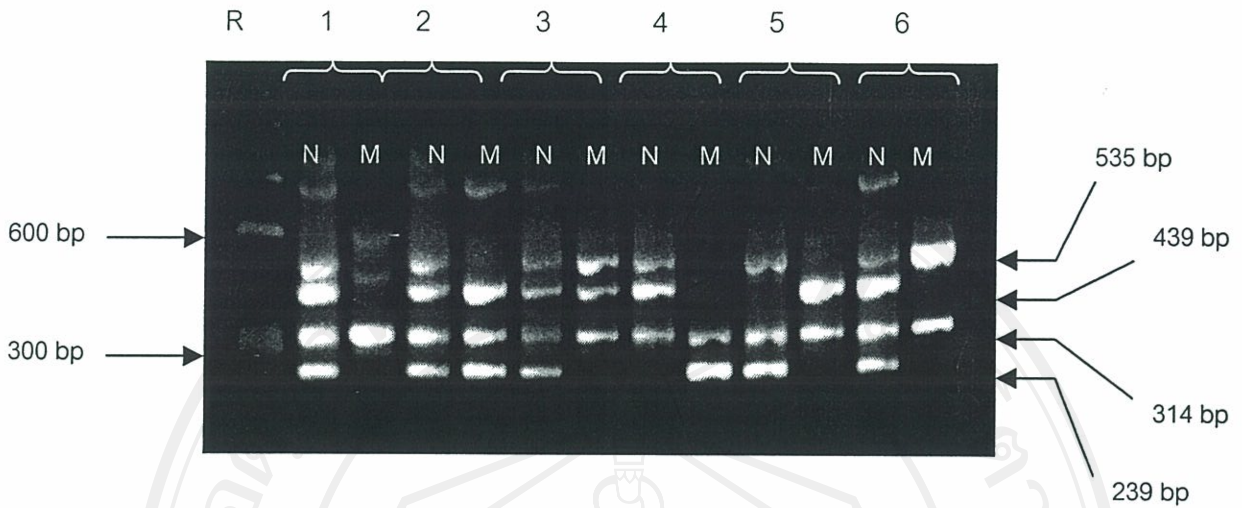

Figure 3.18 The results of multiplex ARMS for detection of codons 41/42, codon 17 and codons 71/72. Lane R indicated the ϕX174 *Hae* III digested DNA size marker. No 1 represents negative case where no PCR products seen in M lane (mutant reaction) but the 535-bp, 439-bp and 239-bp bands are present only in N lane (normal reaction). No 2 is compound heterozygote for codon 17 and codons 41/42 where the 439-bp and 239-bp band are present in the both M and N lanes. No 3 represents compound heterozygote for codons 41/42 and codons 71/72 where the 439-bp and 535-bp band are present in the both M and N lanes. No 4 is homozygote for codon 17 in which the 239-bp band is present only in M lane (mutant reaction). No 5 represent homozygote for codons 41/42 [439-bp band is present only in M lane (mutant reaction)]. No 6 is homozygote for codons 71/72 in which the 535-bp band is present only in M lane (mutant reaction).

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3.5 Evaluation of the optimal numbers of white blood cells for ARMS-PCR

The process for evaluation of the optimal number of white blood cells for ARMS-PCR were described in 2.6. The optimized single ARMS-PCR using the wild type primer of codons 41/42 (-TTCT) as described in 3.2 and optimized multiplex ARMS-PCR with the combination of wild type primers of condon 17, codons 41/42 and codons 71/72 as described in 3.4 were performed in this study. WBC count to be evaluated ranged from approximately 130 to 13,200 cells/cu.mm. corresponding WBC numbers from approximately 5,200 to 528,000 cells. It was found for the single ARMS-PCR that the lowest levels of WBC numbers that can yield amplified products enough to visualize by naked eyes after agarose gel electrophoresis was 21,000 to 33,000 cells and that for the multiplex ARMS-PCR was 10,500 to 16,500 cells. The detail of the data is shown in the table 3.1 and figures 3.19 (A) and (B). As demonstrated in figure 3.19 (A), the amount of DNA template from diluted blood at 1:16 corresponding to WBC number of 25,000 cells was enough for the single ARMS-PCR, whereas the 1:32-diluted blood corresponding to WBC number of 12,500 cells was enough for multiplex ARMS-PCR as indicated in figure 3.19 (B).

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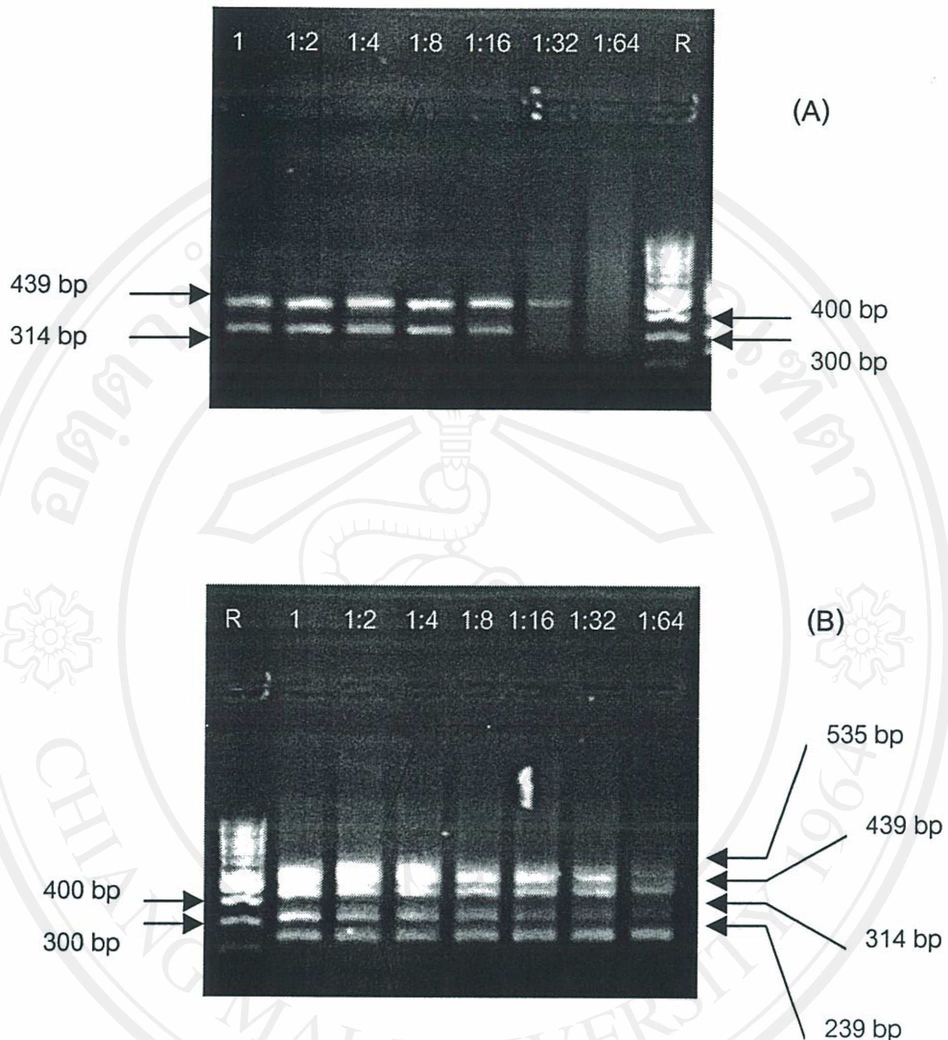

Figure 3.19 The results of evaluation of the optimal numbers of white blood cells for single ARMS-PCR (A) and multiplex ARMS-PCR (B) using normal reaction of single ARMS-PCR for codons 41/42 (A) and normal reaction of multiplex ARMS-PCR to detect . The 314-bp band is the internal control, The 239-bp, 439-bp and 535-bp are the specific bands for codons 17, 41/42 and 71/72, respectively. Lane R indicates the pME-80J3 *Eco* 1471 and *Pvu* I digested DNA size marker. The enough PCR yield was still produced up to 1:16 WBC dilution in single ARMS, while 1:32-dilution of WBC still was enough to generate detectable the signal in multiplex ARMS.

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Table 3.1 Effects of WBC counts and WBC numbers for success of ARMS-PCR The single ARMS-PCR was performed using the optimized normal reaction for codons 41/42. The multiplex ARMS-PCR was undertaken using the optimized normal reaction for codons 17, 41/42, 71/72.

WBC number (cells)	Single ARMS-PCR	Multiplex ARMS-PCR
Undilute (336,000-528,000)	Positive	Positive
1/2 (168,000-264,000)	Positive	Positive
1/4 (84,000-132,000)	Positive	Positive
1/8 (42,000-66,000)	Positive	Positive
1/16 (21,000-33,000)	Positive	Positive
1/32 (10,500-16,500)	Negative	Positive
1/64 (5,250-8,250)	Negative	Negative

3.6 Assessment of application potential of ARMS for β-thalassemia heterozygote screening

The subjects were classified into four groups as described in the chapter II section 2.2. Detection of β-thalassemia mutations in these subjects was accomplished using the single and multiplex ARMS-PCRs to detect four β-thalassemia mutations which were codon 17, codons 41/42, codons 71/72 and IVS I nt 1. It was found that none of the subjects in groups I, II and III were positive for these four mutations. However, in group IV in which the β-thalassemia was suspected, 16 cases were heterozygous for codons 41/42 , 9 heterozygous for codon 17, 2 heterozygous for IVS I-nt 1, 1 heterozygous for codons 71/72 and 2 without the evidence of these four β-thalassemia mutations (Appendix D).

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3.7 Assessment of application potential of ARMS-PCR technique in Hb E screening

The ARMS-PCR for Hb E was applied in a cohort of fifty pregnant women presenting with Hb A_2 levels identified by HPLC ranging from 14.4 to 99.5%. It was clearly shown that all of the studied cases carried Hb E allele with 41 heterozygotes (Hb E = 14.4 to 33.7%), 9 homozygotes (Hb E = 81.5 to 99.5%) (Appendix E).

3.8 Assessment of application potential of ARMS-PCR for prenatal diagnosis (PND)

3.8.1 PND for β-thalassemia major

12 couples at risk for β-thalassemia major (after being screened with OFT, followed by HPLC) were recruited in this study. By using both multiplex ARMS-PCR and single ARMS-PCR, all four analyzed β-thalassemia mutations were encountered in these couples. The codons 41/42 was found in 16 cases, codon 17 in 9 cases, IVS I-nt 1 in 2 cases and codons 71/72 in 1 case (see Appendix F for the detail). The investigation of β-thalassemia mutations in 12 fetal blood samples revealed that 8 samples having the DNA analysis results similar to those generated by HPLC (Figure 3.20 and 3.21 (A) and (B)). However, the DNA analysis showed the results that were not concordant with HPLC in the rest 4 fetal blood samples. In these 4 cases, HPLC revealed no evidence of β-thalassemia heterozygote and ARMS-PCR could detect the β-thalassemia mutations previously identified in the parents (codons 41/42 (2), codon 17 (1) and codons 71/72 (1)). The presence of β-thalassemia mutations in all 4 uncorrelated samples were, finally, confirmed by the direct nucleotide sequencing.

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3.8.2 PND for β-thalassemia/Hb E disease

14 couples at risk for β-thalassemia/Hb E disease (after being screened with OFT, followed by HPLC) were recruited in this study. Identification by HPLC method, the 14 subjects were characterized for Hb E and another 14 samples were characterized for β-thalassemia heterozygote. By using both multiplex ARMS-PCR and single ARMS-PCR, Hb E was found in 14 subjects, the rest four analyzed β-thalassemia mutations were encountered in another 13 subjects, the codons 41/42 in 5 cases, codon 17 in 5 cases and IVS 1-nt I in 3 cases (see Appendix F for the detail). However, none of these four mutations were found in one case whose Hb A_2 level was 8.2%. After nucleotide sequencing was performed in this case, Hb Tak was indicated. In the analysis of 14 fetal blood samples, 13 had the diagnosis in total agree with that generated from HPLC and inherited both β-thalassemic alleles from the parents. However, in another fetal blood sample, Hb identification showed no Hb A and none of the four analyzed β-thalassemia mutations were observed by both ARMS-PCR techniques. Nucleotide sequencing of this sample was later able to identify Hb Tak, raising the diagnosis of compound heterozygote of Hb Tak/Hb E in this fetus (Figure 3.22 (A) and (B)).

3.8.3 PND for β-thalassemia/Hb E disease by DNA analysis from CVS

5 couples (after being screened with OFT, followed by HPLC) were recruited for the study. As shown by HPLC, half of the subjects had Hb E allele and another half were β-thalassemia heterozygotes in which 4 cases were found to have codon 17 and 1 case with codons 41/42. After being genotype for the β-thalassemia mutations and Hb E, all 5 CVS possessed the DNA diagnosis results completely agree with those generated by standard nucleotide sequencing, all of which are inherited from the parents (Appendix F).

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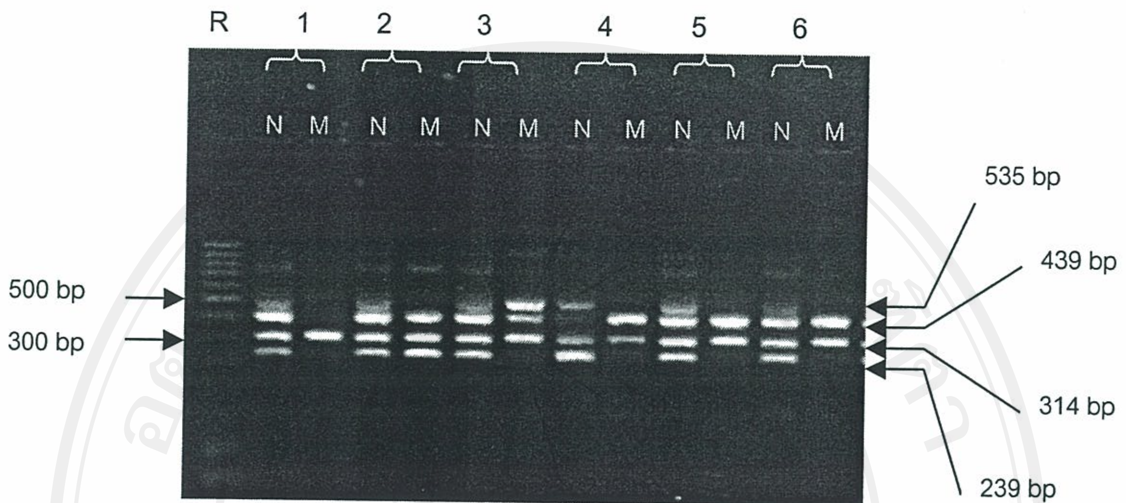

Figure 3.20 The results of multiplex ARMS-PCR for detection at codons 17, 41/42 and 71/72 in PND cases. Lane R indicates the pME-80J3 *Eco*1471and *Pvu*I digested DNA size marker. No 1 represents normal control (535-bp, 439-bp and 239-bp are present only N lane (normal reaction)). No 2 represents compound heterozygote for codons 17 and 41/42 (439-bp and 239-bp bands are present in both lanes). No 3 represents compound heterozygote for codons 41/42 and 71/72 (439-bp and 535-bp bands are present in the both lanes). No 4, 5 and 6 are samples from the family at risk for β-thalassemia major. No 4 is fetal blood sample which is homozygote for codons 41/42 (439-bp band is present only M lane (mutant reaction)). No 5 and No 6 are the parents heterozygous for codons 41/42 (439-bp bands are present in the both lanes).

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Figure 3.21 (A) The Hb types of fetal blood identified by HPLC demonstrate β-thalassemia major case ; no Hb A was presented.

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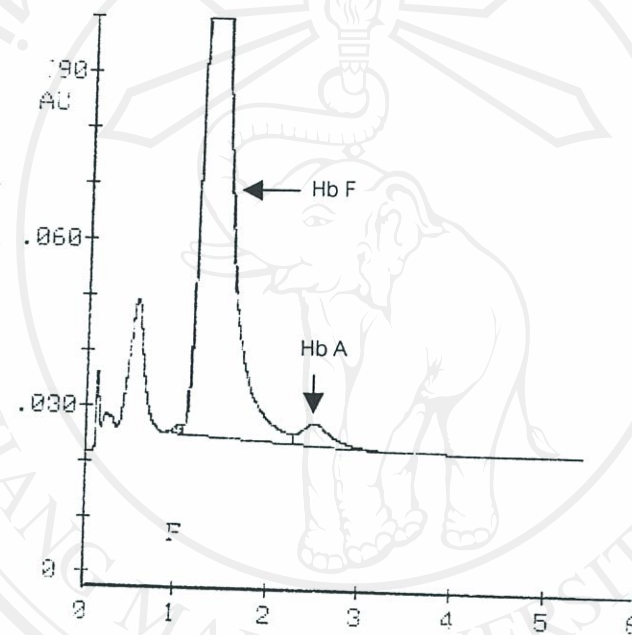

Figure 3.21 (B) The Hb types of fetal blood identified by HPLC demonstrate normal case ; Hb A was presented.

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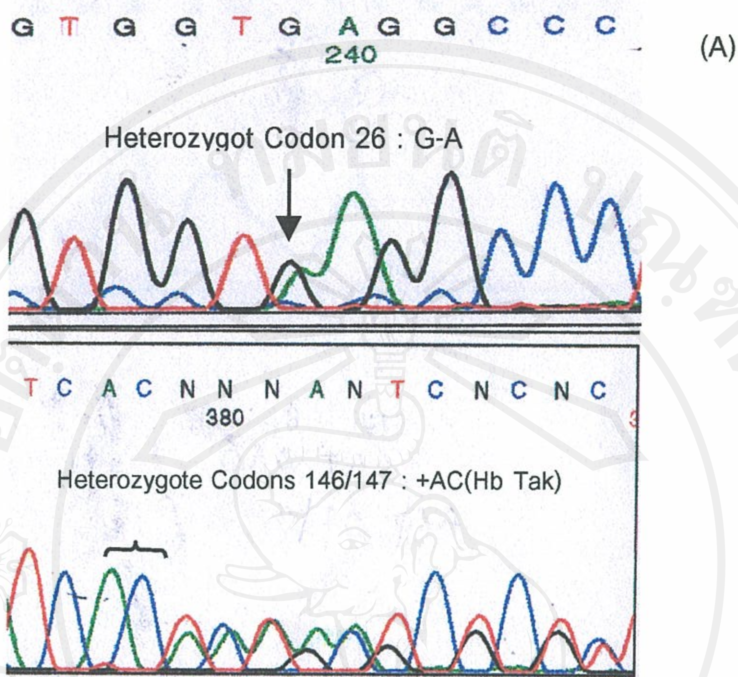

(A)

Figure 3.22 (A) Direct nucleotide sequencing results of Hb Tak and Hb E.

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(B)

Figure 3.22 (B) The Hb typing fetal blood identified by HPLC demonstrate compound heterozygote Hb Tak/ Hb E case ; no Hb A was presented.

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