CHAPTER 3

RESULTS

Two parts of results were established in this thesis, with regards to the described methods, including those for development of the in-house multiplex allele-specific PCR and those for development of the in-house direct PCR from whole-blood without DNA isolation. The details of these results will be described in the following sections.

3.1 Development of the in-house multiplex allele-specific PCR

3.1.1 Determination of locations of allele-specific primers in the corresponding globin genes

The nucleotide sequences of α- and β-globin gene specific primers were successfully aligned with the GenBank reference sequences; "AE006462" and "HUMHBB", respectively (Figures 3.1, 3.2). Having aligned the primer sequences with those in the reference database, it was found that the sequences of all primers were in complete agreement with those in the reference sequences. With regards to the locations of α2- and α1-globin genes at nt 162875-163709 and nt 166674-167521, respectively, in the AE006462, the locations of 7 α-globin gene specific primers were revealed as shown in Table 6. With regards to the location of the β-globin gene at nt 62137-63741 in the HUMHBB, the locations of 5 β-globin gene specific primers were also picked up as

shown in Table 3.1. Sizes of PCR products generated from each primer pair were then deduced from those numerical locations and summarized in the Table 3.2.

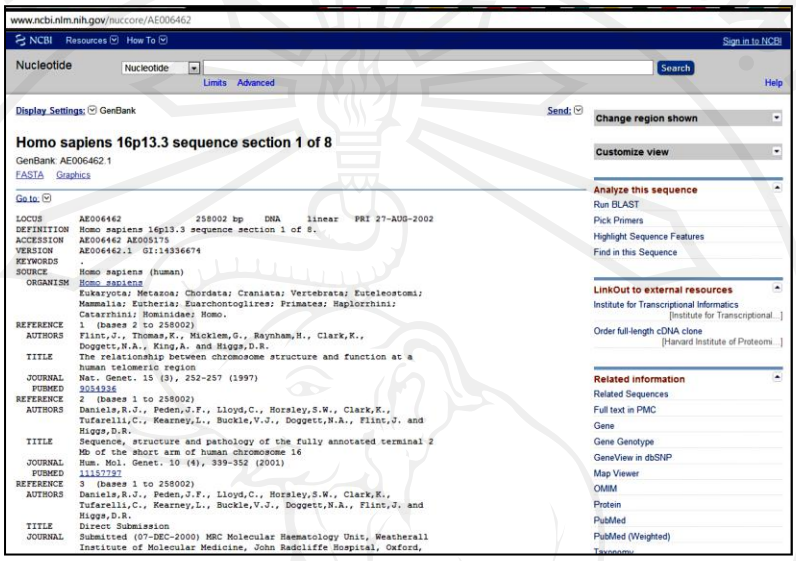

Figure 3.1 Part of the "AE006462" GenBank database as source of α-globin gene sequences used in this thesis [http://www.ncbi.nlm.nih.gov/nuccore/AE006462]

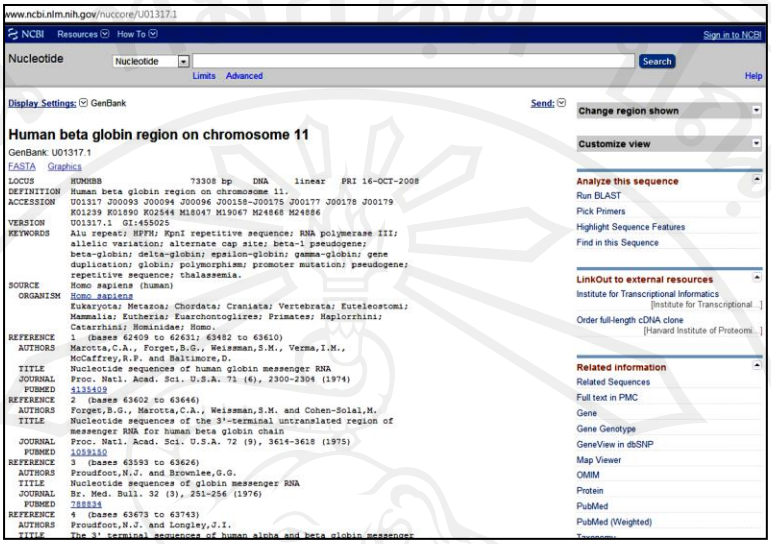

Figure 3.2 Part of the "HUMHBB" GenBank database as source of β-globin gene sequences used in this thesis **[**http://www.ncbi.nlm.nih.gov/nuccore/U01317.1]

Table 3.1 Numerical positions of α- and β-globin gene specific primers in the GenBank database

Primer names	GenBank access codes	GenBank coordinates
SEA1	AE006462	155228-155250
SEA2	AE006462	155857-155879
SEA3	AE006462	175268-175290
Alpha 3.7A	AE006462	161976-161999
Alpha 3.7B	AE006462	167429-167451
CS-2	AE006462	163578-163599
αG-17	AE006462	163367-163387
C3	AE006462	163738-163757
beta common multiplex	HUMHBB	61993-62017
beta 17 multiplex	HUMHBB	62238-62260
beta E multiplex	HUMHBB	62265-62283
beta 41/42 multiplex	HUMHBB	62437-62470
M-28M1	HUMHBB	62090-62129

Table 3.2 Sizes of amplified products deduced from position of each primer in the AE006462 and HUMHBB GenBank databases

Primer pairs	Size of amplified product (bp)
SEA1 + SEA3	762
SEA1 + SEA2	652
Alpha 3.7A + Alpha 3.7B	1,671
CS-2 + C3	180
αG-17 + C3	391
αG-17 + Alpha 3.7B	280
Alpha 3.7A + C3	1,782
beta common multiplex + beta 17 multiplex	267
beta common multiplex + beta E multiplex	290
beta common multiplex + beta 41/42 multiplex	469
beta common multiplex + M-28M1	156

3.1.2 Optimization of the singleplex allele-specific PCR

A. Singleplex allele-specific PCR for internal control

Efficiency of the singleplex allele-specific PCR of internal control primers was initially tested as described in the section 2.2.1.3. It was found that this primer pair was in fact working well as the expected 391-bp amplified products were generated (Figure 3.3).

It was also found that this PCR was in a good condition as non-specific amplified products were almost absent as seen in the Figure 3.3.

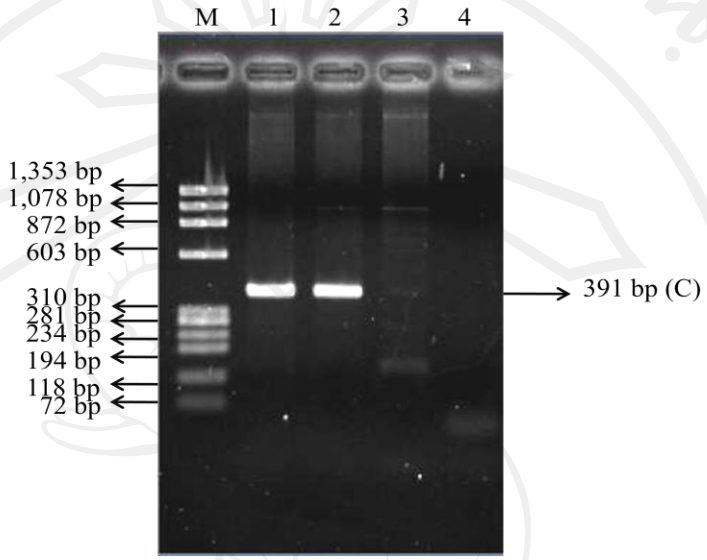

Figure 3.3 PCR products (391-bp) of internal control primers; αG-17 + C3. Lane 1 is amplified fragments of normal individual. Lane 2 is amplified fragments of SEA-α thalassemia 1 heterozygote. Lane 3 is amplified fragments of HbH disease sample. "M" is the ϕX174 *Hae*III DNA ladder. "C" is internal control. Note that presence of the 391-bp in lanes 1, 2 and absence of this band in lane 3 indicates success of the amplification.

B. Singleplex Gap-PCR for SEA-α thalassemia 1

Optimization of the singleplex Gap-PCR for detecting SEA-α thalassemia 1 was performed in the Section 2.2.1.3. It was found that the optimal annealing temperature was 60°C and that the optimal $MgCl_2$ concentration was 2.5 mM (Figures 3.4 and 3.5). It was also found that the optimal final concentrations of SEA1 and SEA3 primers were 0.175 μM and 0.105 μM, respectively. (Figures 3.6-3.11).

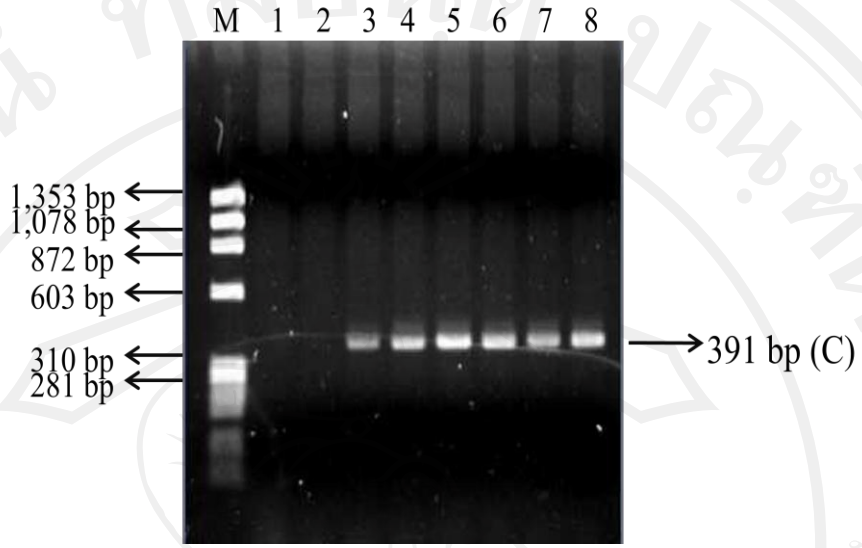

Figure 3.4 The optimization of annealing temperature for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. DNA sample from the carrier of SEA-α thalassemia 1 was utilized. Lanes 1-8 are the amplified products generated at 65, 64.5, 63.4, 62, 60.2, 58.7, 57.6 and 57 ^{O}C respectively. "M" is ϕX174 *Hae*III DNA ladder. "C" is internal control. The annealing temperature at 60.2 ^{O}C was selected

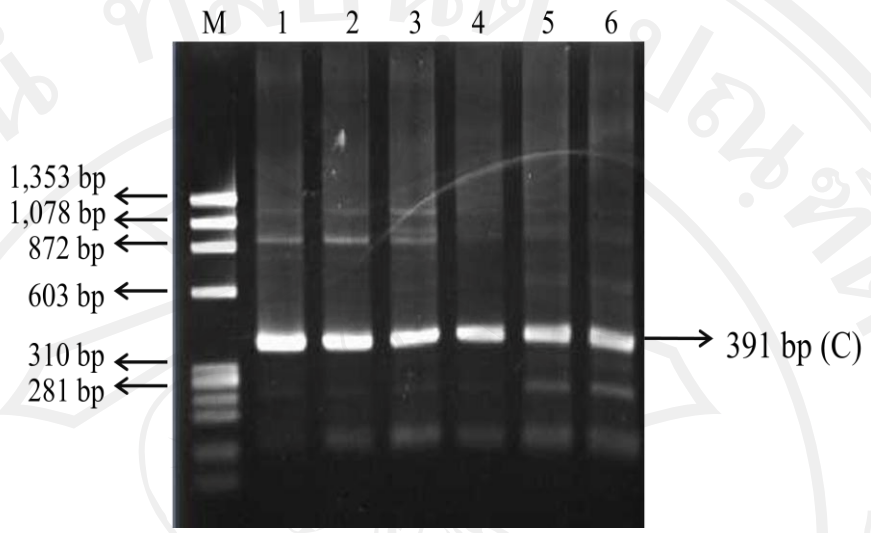

Figure 3.5 The optimization of concentration of $MgCl_2$ for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. DNA sample from the carrier of SEA-α thalassemia 1 was utilized. Lanes 1-6 are amplified products generated using final concentration of $MgCl_2$ at 1, 1.5, 2, 2.5, 3 and 3.5, respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. The concentration of 2.5 mM was chosen.

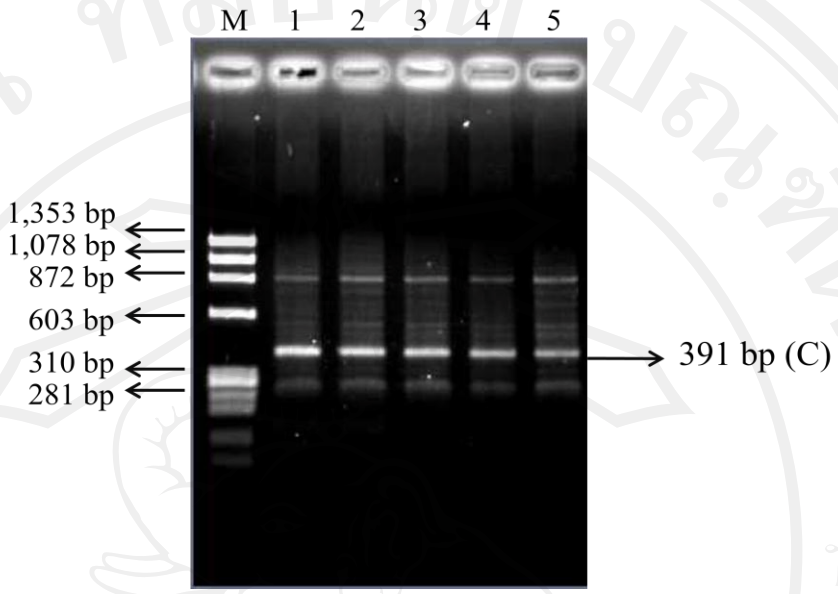

Figure 3.6 Titration of amount of SEA primers for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. In this step, SEA3 concentration was fixed at 0.035 μM and that of SEA1 varied. Lanes 1-5 are amplified products generated by SEA1 at the final concentration of 0.035, 0.07, 0.105, 0.14 and 0.175 μM, respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that no optimal concentrations of SEA1 and SEA3 primers were met in this experiment.

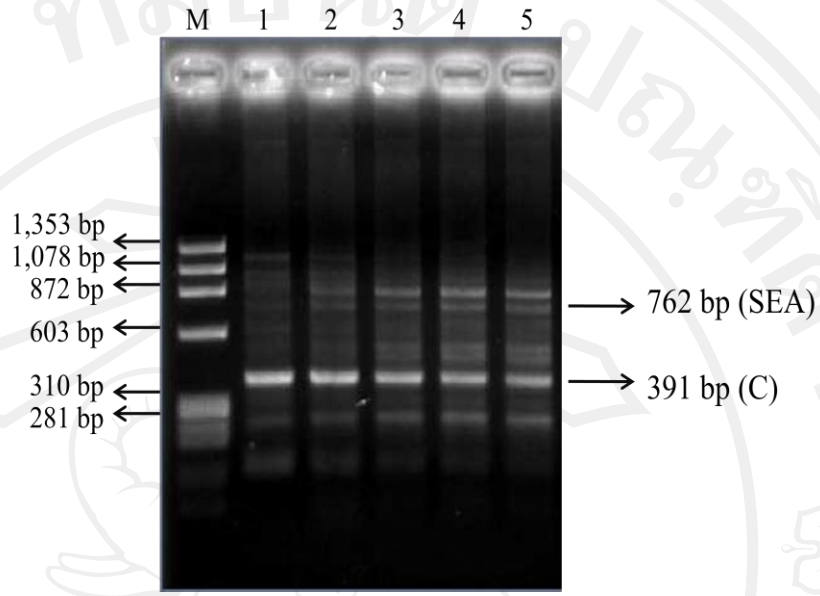

Figure 3.7 The titration of amount of SEA primers for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. In this step, SEA3 concentration was fixed at 0.07 μM and that of SEA1 varied. Lanes 1-5 are the amplified products of SEA1 at the final concentration of 0.035, 0.07, 0.105, 0.14 and 0.175 μM respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that lanes 3-5 represent the optimal concentrations of SEA1 and SEA3 primers in this experiment.

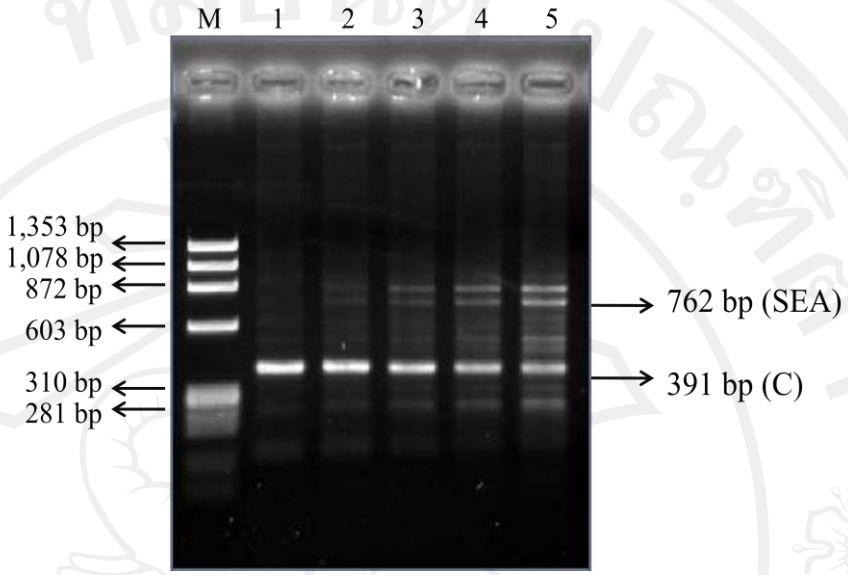

Figure 3.8 The titration of amount of SEA primers for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. In this step, SEA3 concentration was fixed at 0.105 μM and that of SEA1 varied. Lanes 1-5 are the amplified products of SEA1 at the final concentration of 0.035, 0.07, 0.105, 0.14 and 0.175 μM respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that lanes 3-5 represent the optimal concentrations of SEA1 and SEA3 primers in this experiment.

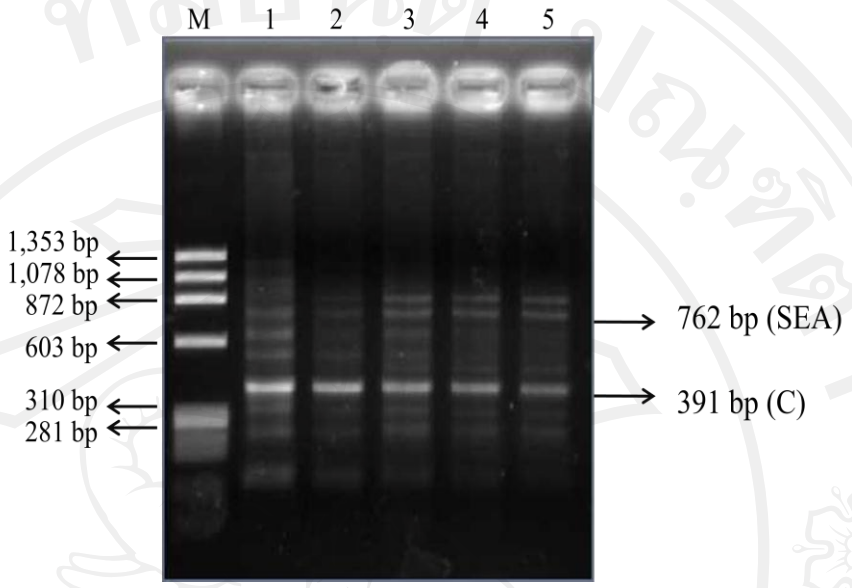

Figure 3.9 The titration of amount of SEA primers for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. In this step, SEA3 concentration was fixed at 0.14 μM and that of SEA1 varied. Lanes 1-5 are the amplified products of SEA1 at the final concentration of 0.035, 0.07, 0.105, 0.14 and 0.175 μM respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that lanes 3-5 represent the optimal concentrations of SEA1 and SEA3 primers in this experiment.

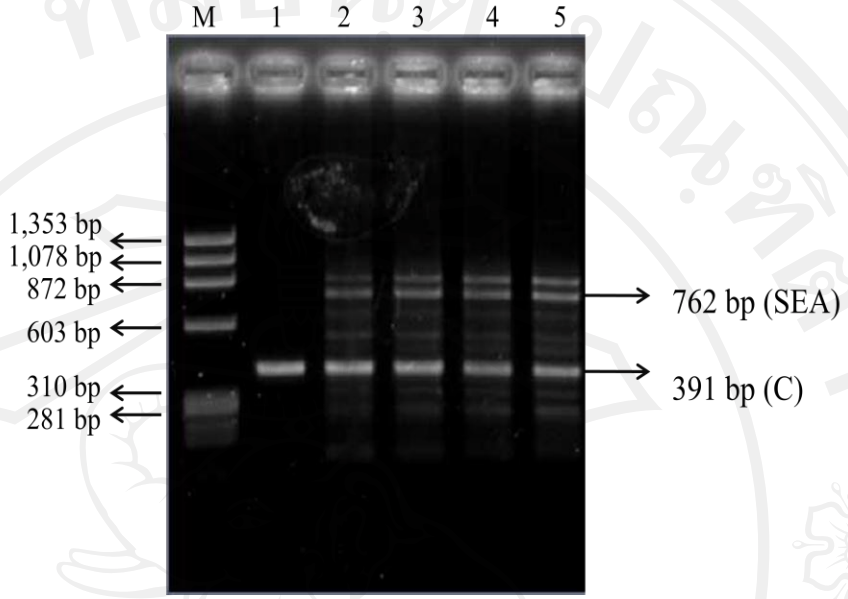

Figure 3.10 The titration of amount of SEA primers for the singleplex Gap-PCR for detection of the SEA-α thalassemia 1. In this step, SEA3 concentration was fixed at 0.175 μM and that of SEA1 varied. Lanes 1-5 are the amplified products of SEA1 at the final concentration of 0.035, 0.07, 0.105, 0.14 and 0.175 μM respectively. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that lanes 2-5 represent the optimal concentrations of SEA1 and SEA3 primers in this experiment.

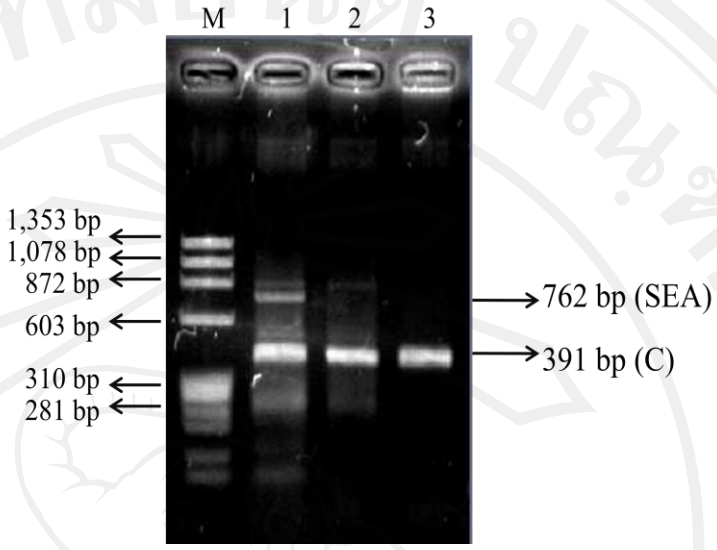

Figure 3.11 Agarose electrophoretic pattern of the amplified fragments of the singleplex Gap-PCR for detection of the SEA-α thalassemia 1 utilizing 0.175 μM SEA1 and 0.105 μM SEA3. Lane 1 is the amplified products of DNA from the SEA-α thalassemia 1 carrier. Lanes 2-3 are DNA from non SEA-α thalassemia 1 carrier. "C" is internal control. "SEA" is SEA-α thalassemia 1. "M" is ϕX174 *Hae*III DNA ladder. Note that the 762-bp fragments are seen in only lane 1.

C. Singleplex allele-specific PCR for Hb Constant Spring

The CS-2 primer was allele specific-primer of HbCS that was designed with 3'-end mismatch. It was found that introducing this mismatch was able to generate specific 180-bp amplified products in case bearing the HbCS allele. However, non-specific amplified fragments were still seen in this protocol. Thus, to reduce these non-specific bands, titration of amount of αG-17 primer was carried out and 0.025 μM final concentration was found to be optimal (Figure 3.12). The PCR reaction was repeated

utilizing this optimized amount of the αG-17 primer and clear 180-bp HbCS specific products as well as the 391-bp internal control products were observed on the agarose gel (Figure 3.13).

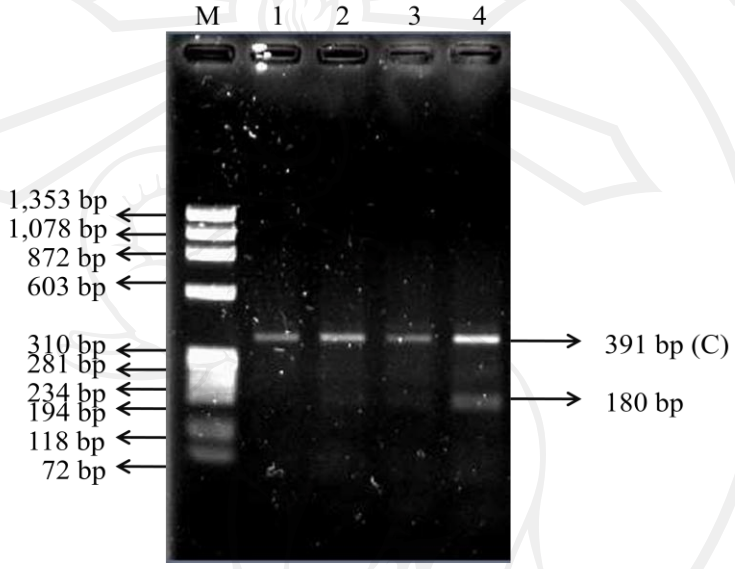

Figure 3.12 Titration of amount of αG-17 primer (0.016, 0.025, 0.034 and 0.04 μM final concentration) in the singleplex allele-specific PCR for HbCS. DNA sample was prepared from non HbCS carrier. Lanes 1-4 are the amplified products generated using 0.016, 0.025, 0.034 and 0.04 μM of αG-17. "C" is internal control. "M" is ϕX174 *Hae*III DNA ladder. Note that lane 2 is the best and 0.025 μM final concentration of αG-17 primer chosen.

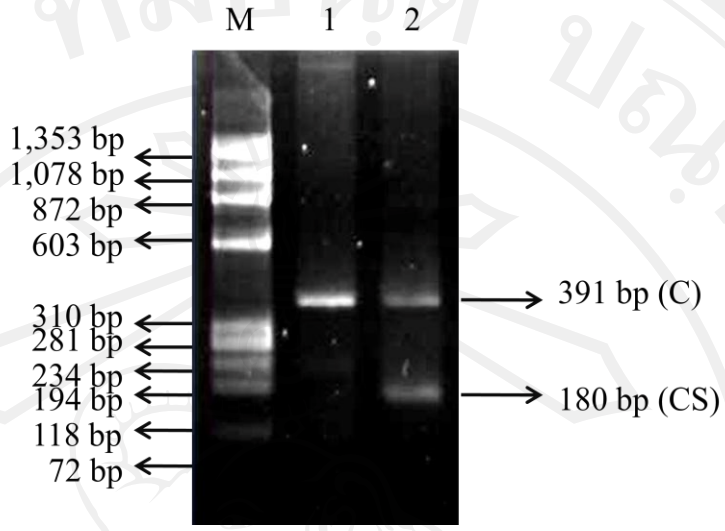

Figure 3.13 Agarose electrophoresis pattern of the amplified fragment of the singleplex allele-specific PCR for detection of the HbCS employing the optimized conditions. Lane 1 is amplified products of DNA sample from non-HbCS. Lane 2 is amplified product of DNA sample from HbCS carrier. "C" is internal control. "CS" is HbCS. "M" is ϕX174 *Hae*III DNA ladder. Note that the 180-bp band is only seen in lane 2.

D. Singleplex allele-specific PCR for α-thalassemia 2 (3.7-kb deletion)

As stated, 2-step optimization of PCR for this type of α-thalassemia was performed. In the first step, the final concentrations of $MgCl_2$ were titrated and 1 mM was found to be optimal (Figure 3.14). The betaine concentration was also optimized in this step and finally found that the best concentration was 2 M (Figure 3.15). In the second step, αG-17 and C3 primers were added into the optimized PCR reaction. The specific 1,671-bp product was weakly amplified as shown in Figure 3.16. The thermal

cycles were then increased from 22 to 32 and stronger signals of amplified fragments were observed as shown in Figures 3.15, 3.16.

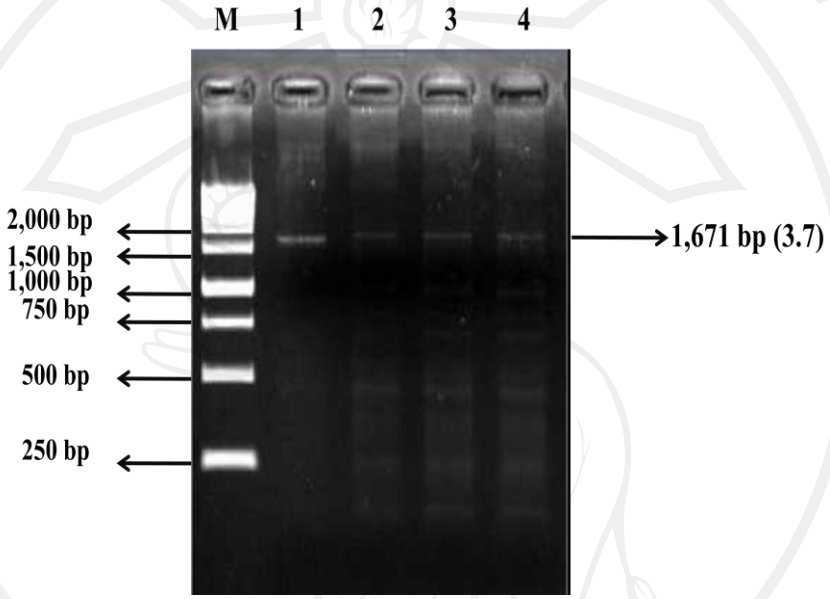

Figure 3.14 Titration of concentration of $MgCl_2$ in the singleplex allele-specific PCR for α-thalasseia 2 (3.7-kb deletion). DNA samples from HbH disease was utilized. Lanes 1-4 are PCR products generated from 1, 2, 3, 4 mM final concentration of $MgCl_2$. "3.7" is α-thalassemia 2 (3.7 kb deletion). "M" is 1 kb DNA ladder marker. Note that lane 1 (1 mM $MgCl_2$) has the strongest intensity of specific 1,671-bp product.

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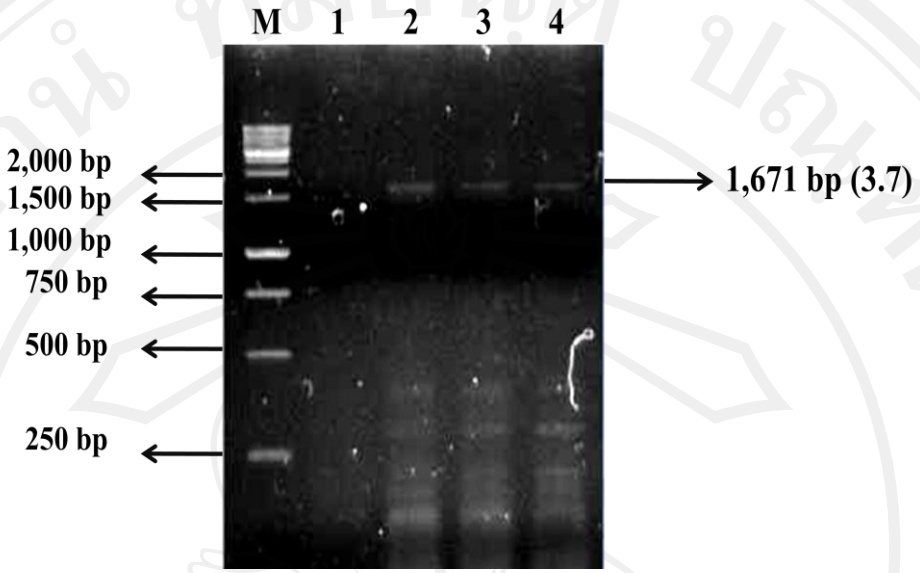

Figure 3.15 Titration of concentration of betaine in the singleplex allele-specific PCR for α-thalasseia 2 (3.7-kb deletion). DNA samples from HbH disease was utilized. Lanes 1-4 are PCR products generated from 0.5, 1, 2, 3 mM final concentration of betaine. "3.7" is α-thalassemia 2 (3.7 kb deletion). "M" is 1 kb DNA ladder marker. Note that lanes 2-4 show identical 1,671-bp product and lane 3 (2 M betaine) was selected.

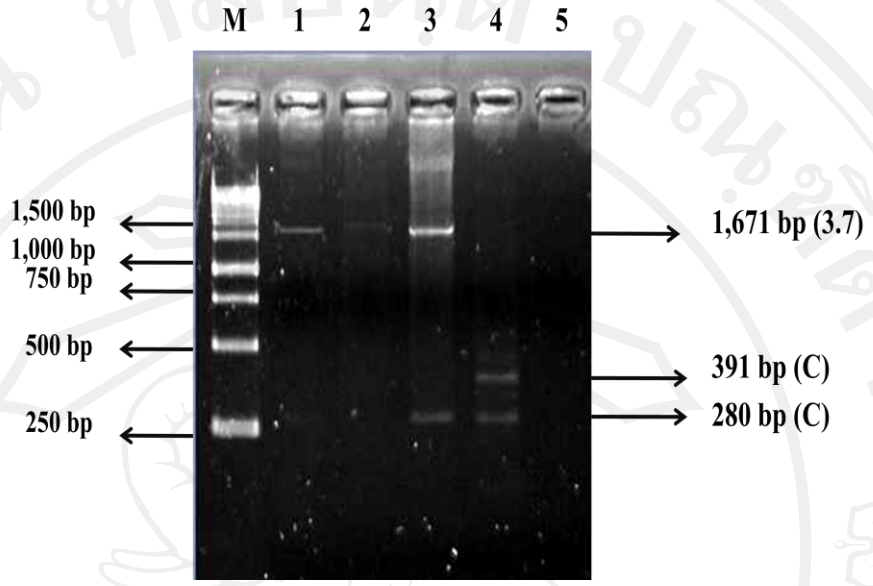

Figure 3.16 Agarose electrophoresis pattern of the amplified fragment of the singleplex allele-specific PCR for detection of the α-thalassemia 2 (3.7 kb deletion) after addition of αG-17 and C3 primers and 22-round thermal cycle performed. Lane 1 is PCR product using DNA sample from HbH disease. Lane 2 is PCR product using DNA sample from carrier of SEA-α thalassemia 1. Lane 3 is PCR product using DNA sample from HbH disease. Lane 4 is PCR product using DNA sample from normal individual. "C" is internal control. "3.7" is α-thalassemia 2 (3.7 kb deletion). "M" is 1 kb DNA ladder.

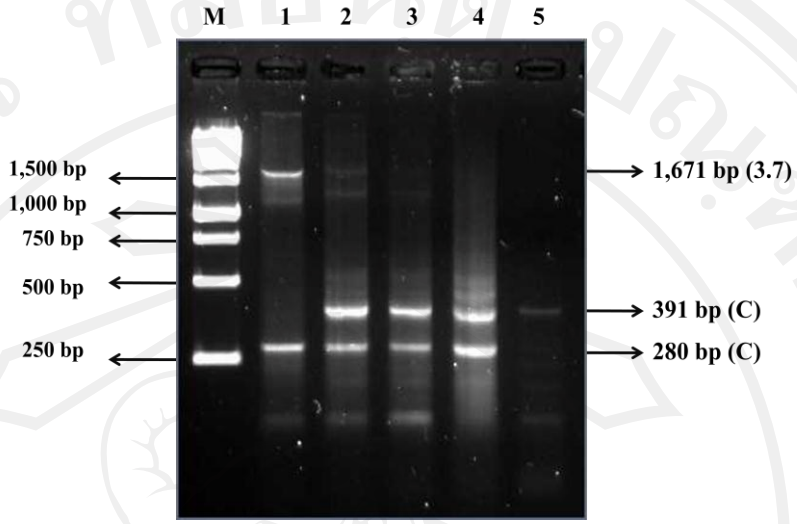

Figure 3.17 Agarose electrophoresis pattern of the amplified fragment of the singleplex allele-specific PCR for detection of the α-thalassemia 2 (3.7 kb deletion). The PCR reaction completed in 32 thermal cycles. Lane 1 is PCR product using DNA from α-thalassemia 2 (3.7 kb deletion). Lane 2-4 are PCR products using DNA samples from normal individuals. Lane 5 is the blank lane (no DNA added into the reaction). "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "M" is 1 kb DNA ladder marker. Note that signal intensities in all lanes are significantly increased.

3.1.3 Development and optimization of in-house multiplex allele-specific PCR

A. In-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy

This in-house multiplex allele-specific PCR protocol was proposed to detect SEA-α thalassemia 1, α-thalassemia 2 (3.7 kb deletion) and HbCS simultaneously. Firstly, 0.175 μM of SEA1 and 0.105 of SEA3 were added into the PCR reaction previously optimized for the 3.7-kb deletion of the α-thalassemia 2. However, the amplification was

not as good as expected (Figure 3.18). Then, concentrations of both SEA1 and SEA3 primers were increased to 0.4 μM and satisfactory amplification was obtained (Figure 3.19). Subsequently, 0.4 μM of CS-2 primer was added into the reaction and both specific and non-specific amplified products were generated as shall be seen in Figure 3.20. Thus, for the best condition, the concentrations of $MgCl_2$ were titrated and 1 mM was found to be suitable for this protocol (Figure 3.21). To further decrease the non-specific products, however, the thermal cycles were gradually decreased from 32 to 27 cycles. The best thermal cycles were finally shown to be 29 cycles (Figures 3.22-3.26). The optimized in-house multiplex allele-specific PCR was then tested against known samples again and the results were satisfactory as shown in Figure 3.27. The optimized condition is summarized in Table 3.3**.**

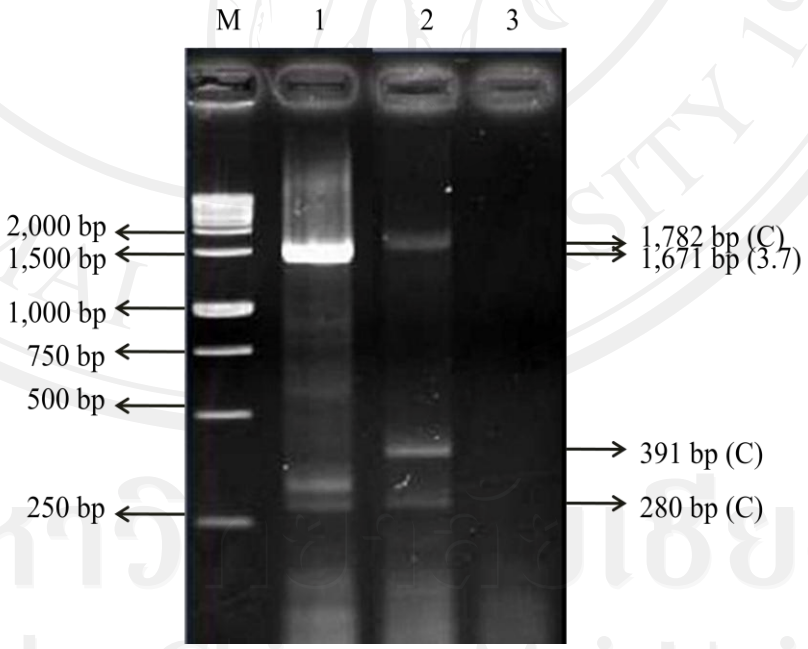

Figure 3.18 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy utilizing 0.175 μM

and 0.105 μM of SEA1 and SEA3, respectively, in the PCR protocol optimized for α-thalassemia 2 (3.7-kb deletion). Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from normal. Lane 3 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "M" is 1 kb DNA ladder marker. Note that no 762-bp SEA-specific products are seen.

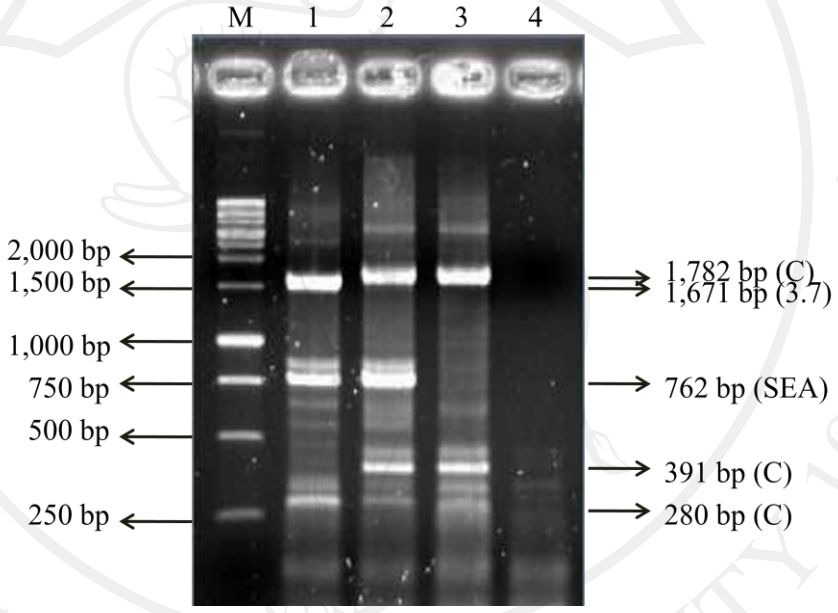

Figure 3.19 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy utilizing 0.4 μM of SEA1 and SEA3 primers in the PCR protocol in the PCR protocol optimized for α-thalassemia 2 (3.7-kb deletion). Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from normal. Lane 4 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "M" is 1 kb

DNA ladder marker. Note that specific PCR bands for SEA (762 bp) and 3.7-kb (1,671 bp) deletions are shown.

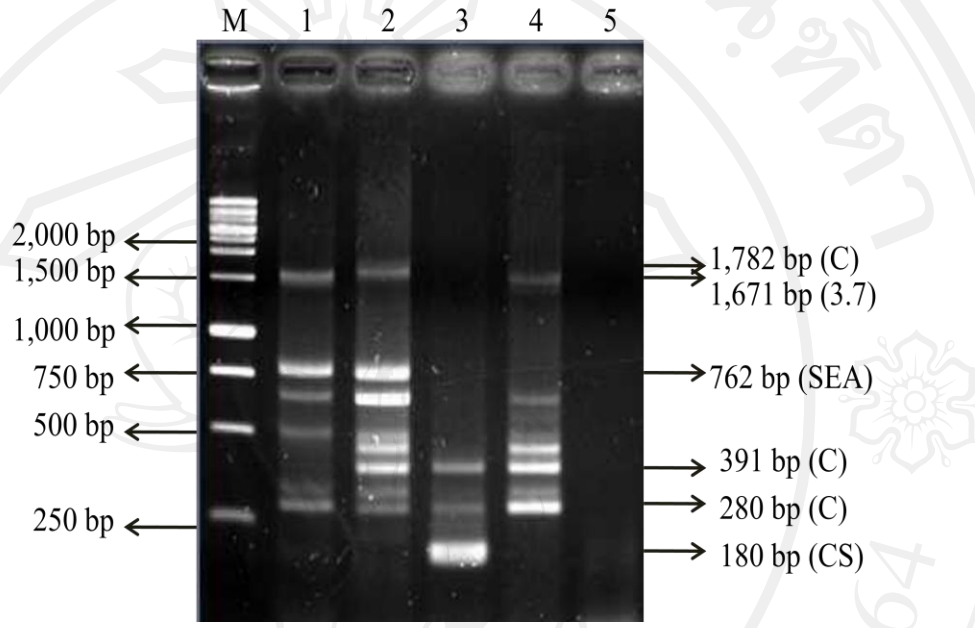

Figure 3.20 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy. The HbCS-specific primer; CS-2, was utilized in addition to the SEA and 3.7-kb specific primers. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of DNA from normal. Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker. Note that both specific and non-specific PCR products are seen.

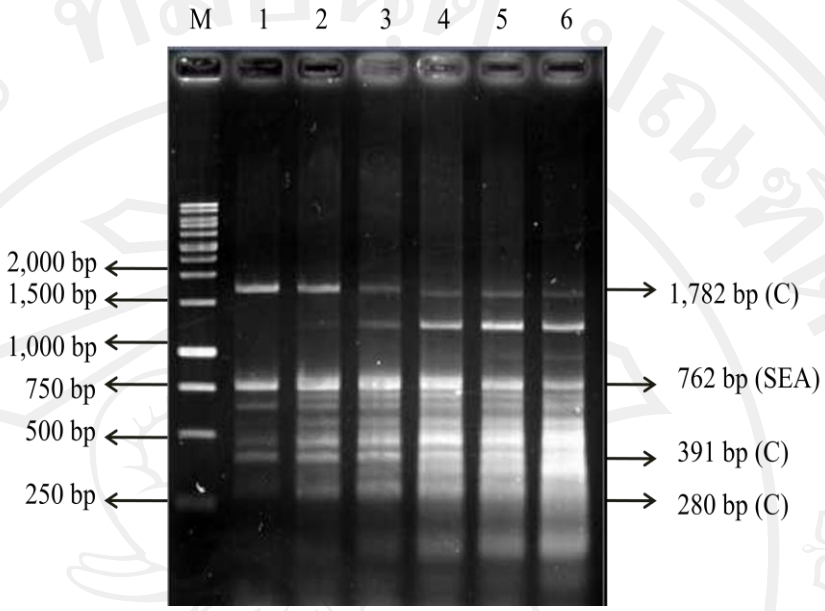

Figure 3.21 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy for the titration of $MgCl_2$ concentration. DNA sample used was from carrier of SEA-α thalassemia 1. Lane 1-6 are the amplified products resulted from $MgCl_2$ at concentrations 1, 1.5, 2, 2.5, 3 and 3.5 mM. "C" is internal control. "SEA" is SEA-α thalassemia 1. "M" is 1 kb DNA ladder marker. Note that lane 1 (1 mM) shows the best result.

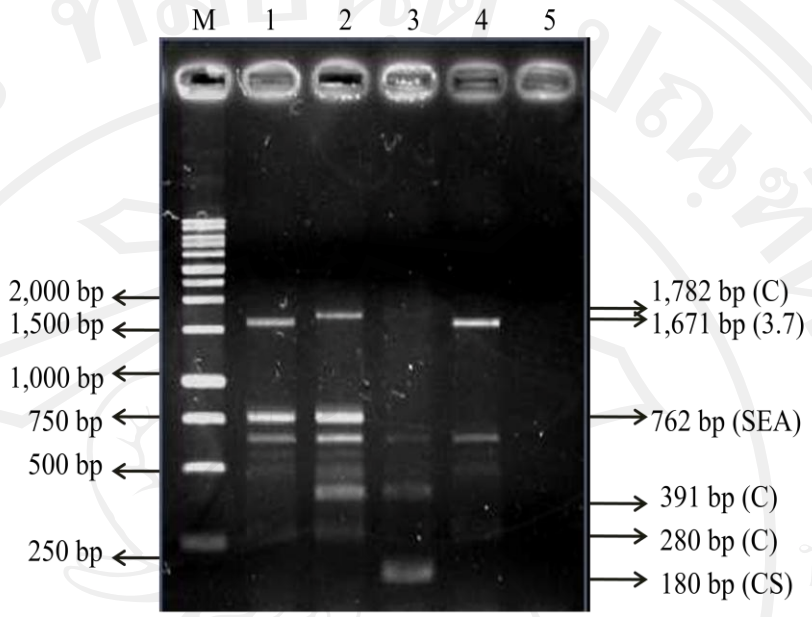

Figure 3.22 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy employing the condition just optimized with 31 the thermal cycles. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of DNA from α-thalassemia 2 (3.7 kb deletion). Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker.

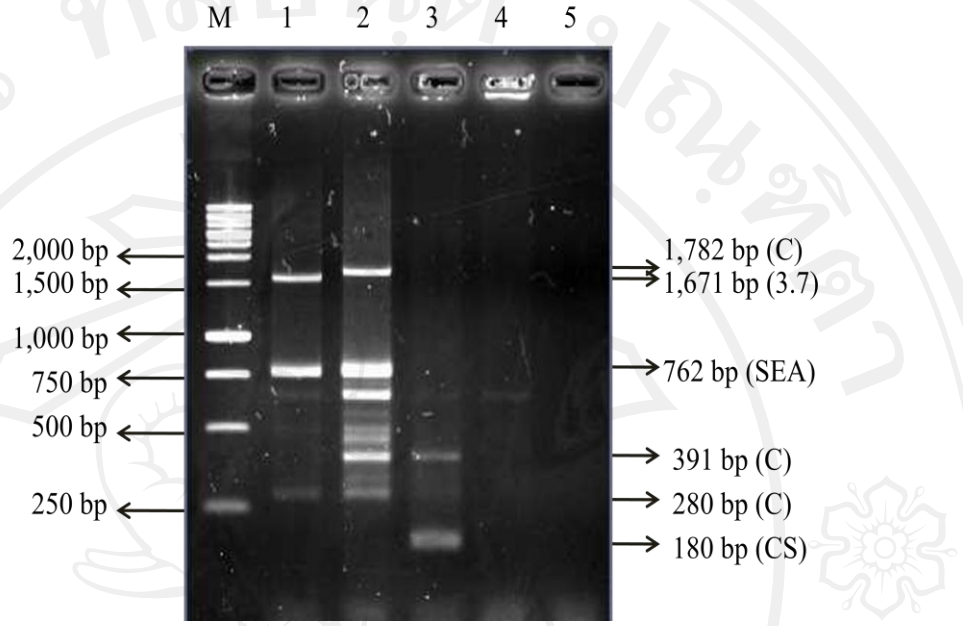

Figure 3.23 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy employing the condition just optimized with 30 the thermal cycles. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of normal. Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker.

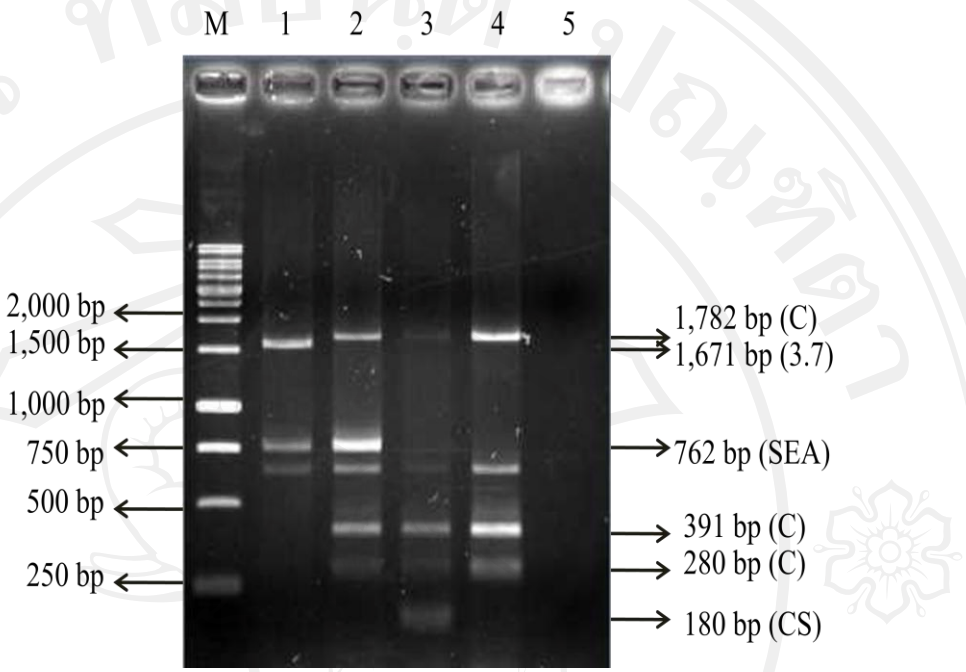

Figure 3.24 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy employing the condition just optimized with 29 the thermal cycles. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of normal. Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker.

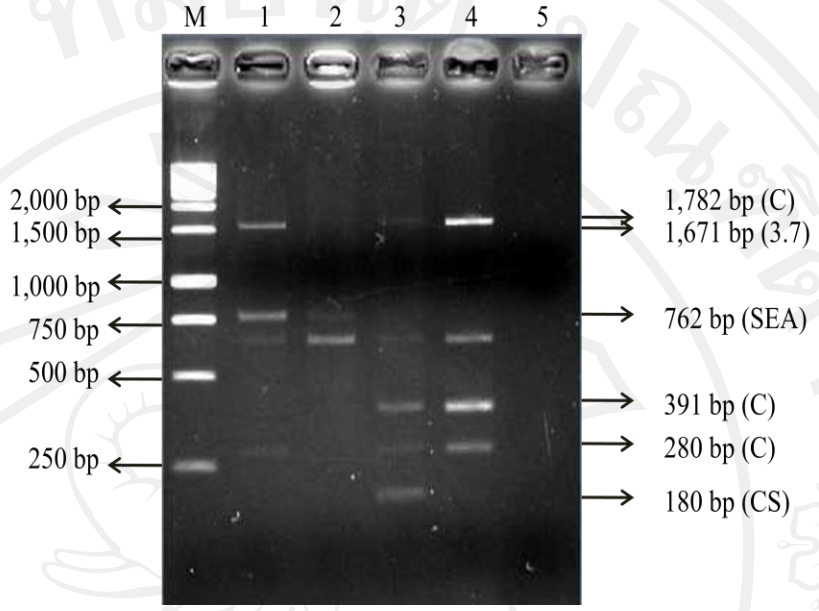

Figure 3.25 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy employing the condition just optimized with 28 the thermal cycles. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of normal. Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA α-thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker.

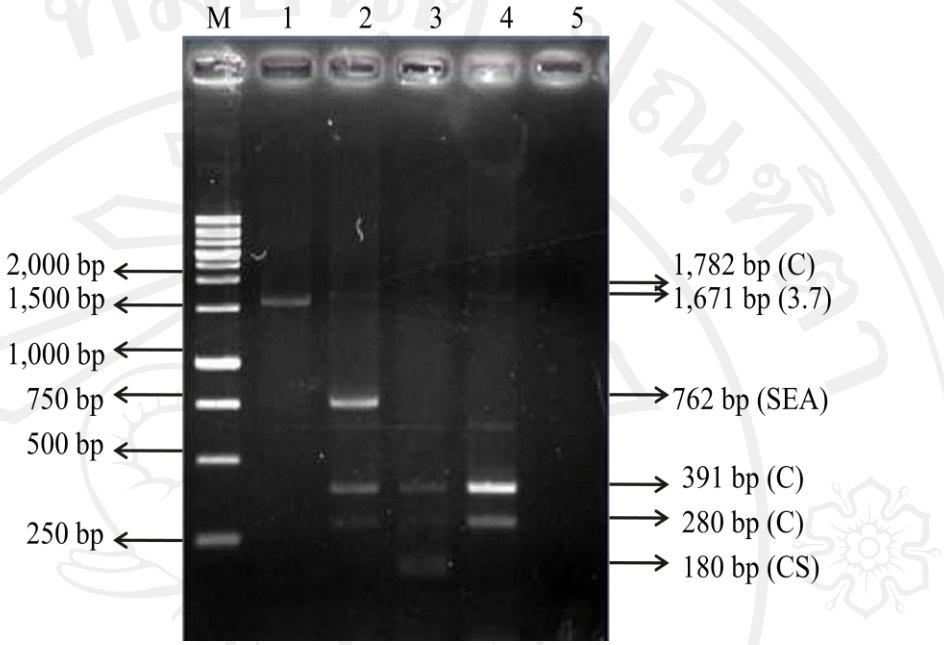

Figure 3.26 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for α-thalassemia/hemoglobinopathy employing the condition just optimized with 27 the thermal cycles. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from HbCS. Lane 4 is the amplified products of normal. Lane 5 is blank. "3.7" is α-thalassemia 2 (3.7 kb deletion). "C" is internal control. "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is 1 kb DNA ladder marker.

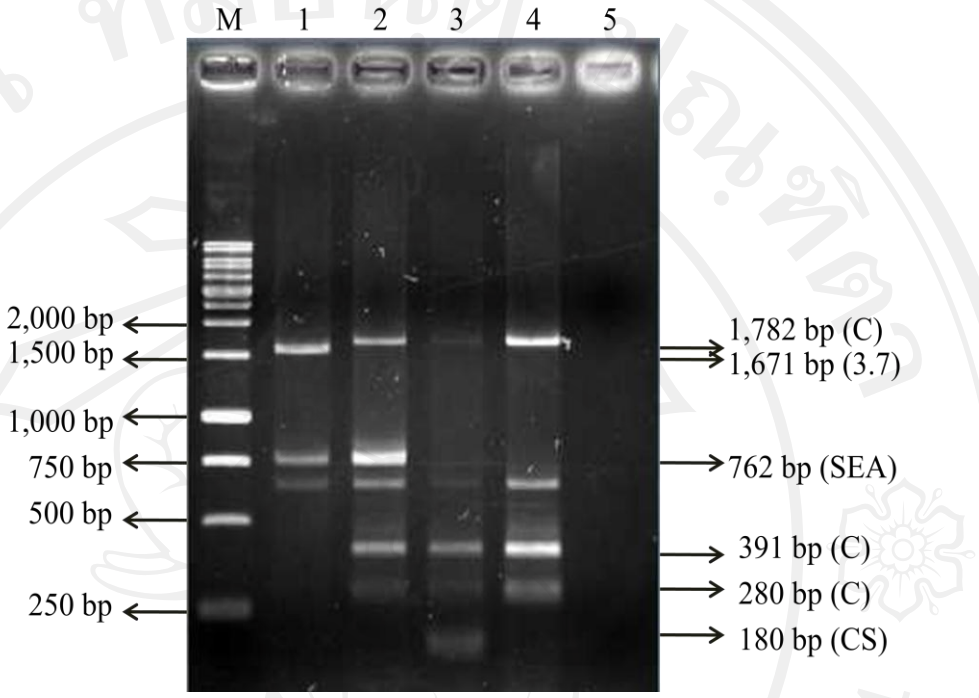

Figure 3.27 Agarose gel pattern of amplified products generated by the optimized in-house multiplex allele-specific PCR for α-thalassemia and hemoglobinopathy in which the specific amplified products were produced with less non-specific bands. Lane 1 is the amplified products of DNA from HbH disease. Lane 2 is the amplified products of DNA from carrier of SEA-α thalassemia 1. Lane 3 is the amplified products of DNA from carrier of HbCS. Lane 4 is the amplified products of DNA from normal. Lane 5 is a blank control. "C" is internal control. "3.7" is α-thalassemia 2 (3.7 kb deletion). "SEA" is SEA-α thalassemia 1. "CS" is HbCS. "M" is a 1-kb DNA size marker.

Table 3.3 The optimized condition of the in-house multiplex allele-specific PCR for α-thalassemia and hemoglobinopathy

Reagent	**Final conc.**
Alpha3.7A primer	1.2 μM
Alpha3.7B primer	1.2 μM
αG-17 primer	0.025 μM
C3 primer	0.4 μM
SEA1 primer	0.4 μM
SEA3 primer	0.4 μM
CS-2 primer	0.4 μM
Tris pH 8.5	10 mM
KCl	50 mM
$MgCl_2$	1 mM
dNTPs	140 μM
DMSO	2%
Betaine	2 M
DNA polymerase (iTaq;iNtRON Biotechnology, Inc.)	0.1 units
DNA template	250 ng

B. In-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy

The in-house multiplex allele-specific PCR protocol was proposed to simultaneously identify HbE, $\beta^{17(A-T)}$, $\beta^{41/42(-TTCT)}$ and $\beta^{-28(A-G)}$. As stated in the Materials and Methods (Section 2.2.1.4 (B)), the final concentration of "M-28M1" primer was firstly optimized and 0.14 µM was found to be optimal (Figure 3.28). The subsequent titrations of the "beta 41/42 multiplex" and "beta 17 multiplex" primers demonstrated that the best final concentration of these two primers in this PCR protocol were 0.54 µM (Figure 3.29, 3.30). The optimal concentration of $MgCl_2$ was also determined and found that 4 mM was the optimal concentration for this PCR protocol (Figure 3.31). Finally, the appropriate thermal cycles were evaluated (32, 27, 26, 25, 24, 23 and 22 cycles). It was found that the optimal thermal cycles were in fact 27 cycles (Figures 3.32-3.38). The optimized condition was then tested against the known DNA samples and the results were completely satisfactory as shown in Figure 3.39. The optimized condition is summarized in Table 3.4**.**

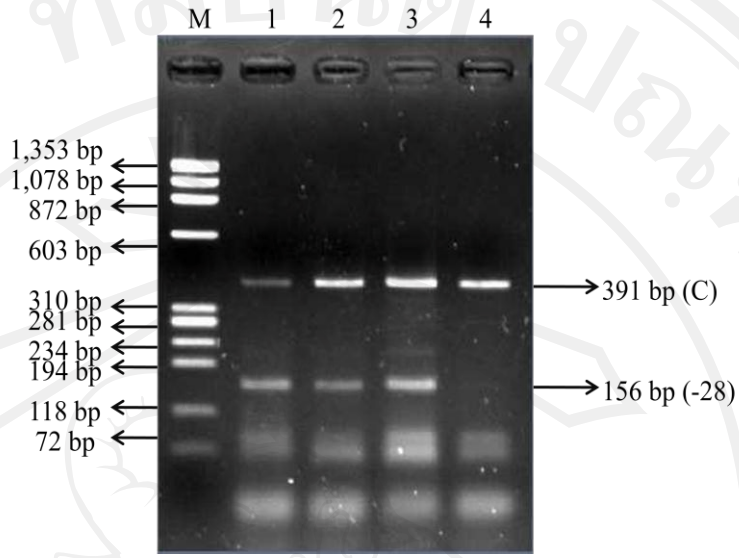

Figure 3.28 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy utilizing DNA sample from the carrier of the $\beta^{-28(A-G)}$thalassemia. Lanes 1-4 are the amplified products generated from 0.56, 0.28, 0.14 and 0.07 μM final concentration of M-28M1 primer. "391-bp (C)" is internal control. "156-bp (-28)" is specific product for $\beta^{-28(A-G)}$thalassemia. "M" is ϕX174 *Hae*III DNA ladder. Note lane 3 shows the best result.

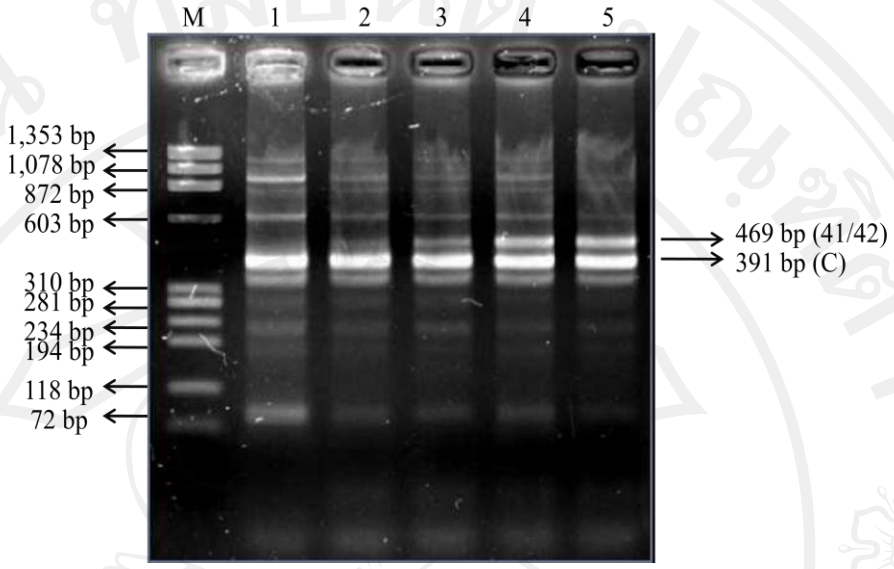

Figure 3.29 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy utilizing DNA sample from the carrier of the $\beta^{41/42(-TTCT)}$ thalassemia. Lanes 1-5 are the amplified products generated generated from 0.018, 0.027, 0.036, 0.045 and 0.054 μM final concentration of "beta 41/42 multiplex" primer. "391-bp (C)" is internal control. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "M" is ϕX174 *Hae*III DNA ladder. Note that lane 5 shows the best result.

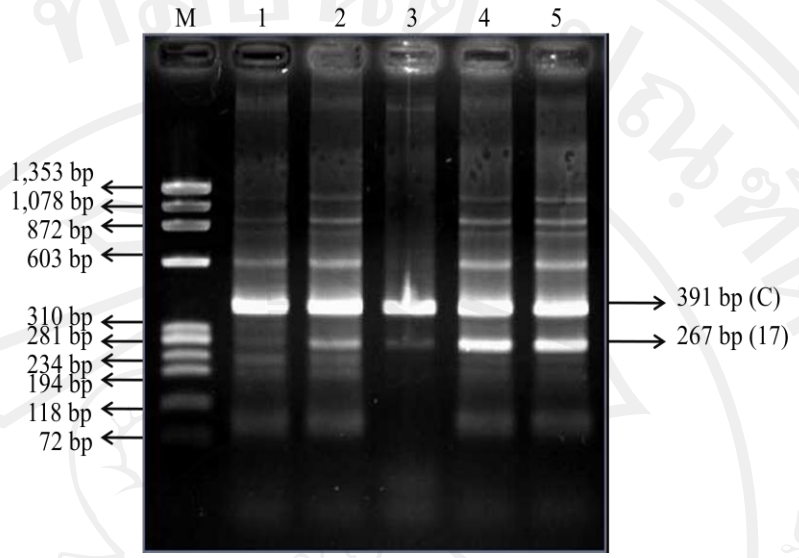

Figure 3.30 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy utilizing DNA sample from the carrier of the $\beta^{17(A\text{-}T)}$thalassemia. Lanes 1-5 are the amplified products generated from 0.018, 0.027, 0.036, 0.045 and 0.054 μM final concentration of "beta 17 multiplex" primer. "391-bp (C)" is internal control. "267-bp (17)" is specific for $\beta^{17(A\text{-}T)}$thalassemia. "M" is ϕX174 *Hae*III DNA ladder. Note that lane 5 shows the best result.

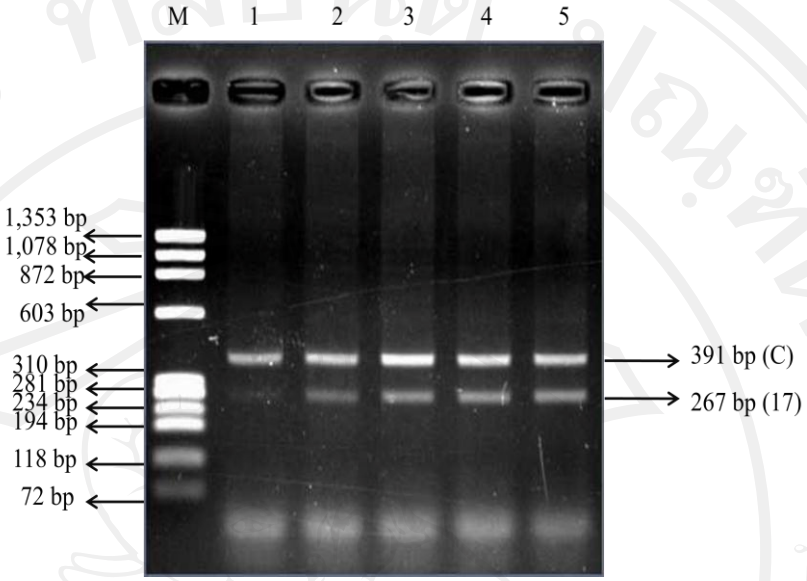

Figure 3.31 Titration of concentration of $MgCl_2$ (2, 2.5, 3, 3.5 and 4 mM final concentration) to be used in the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy using DNA sample from carrier of $\beta^{17(A-T)}$ thalassemia. Lanes 1-5 are amplified products generated from 2, 2.5, 3, 3.5 and 4 mM final concentration of $MgCl_2$. "391-bp (C)" is internal control. "267-bp (17)" is specific for $\beta^{17(A-T)}$ thalassemia. "M" is ϕX174 *Hae*III DNA ladder. Note that lane 5 shows the best result.

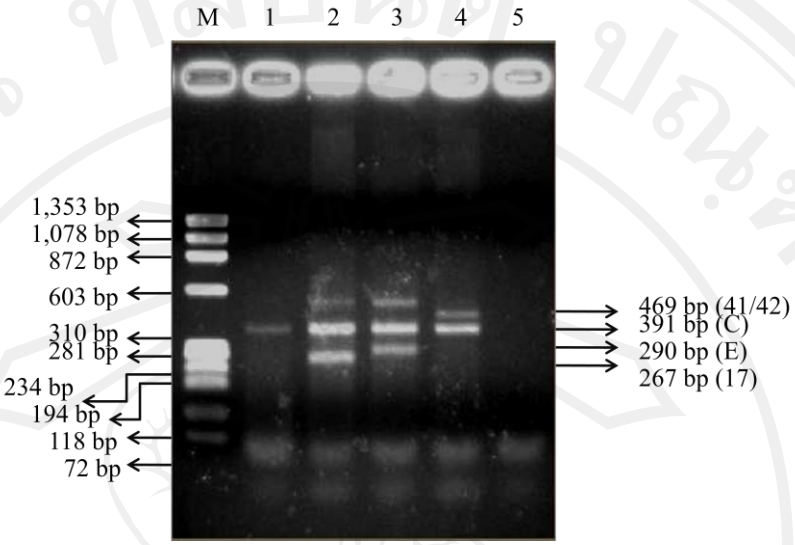

Figure 3.32 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 32 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from HbE. Lane 4 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$ thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

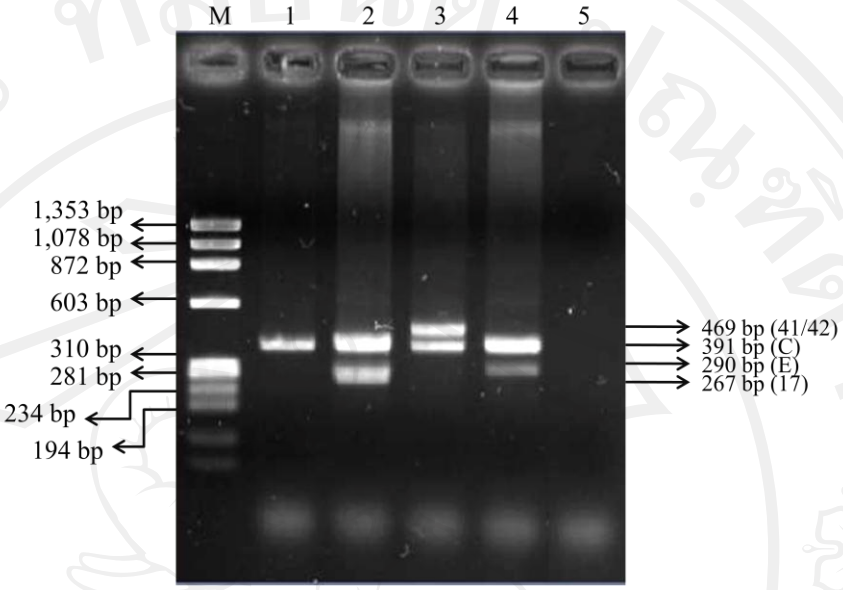

Figure 3.33 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 27 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. DNA of carrier of $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

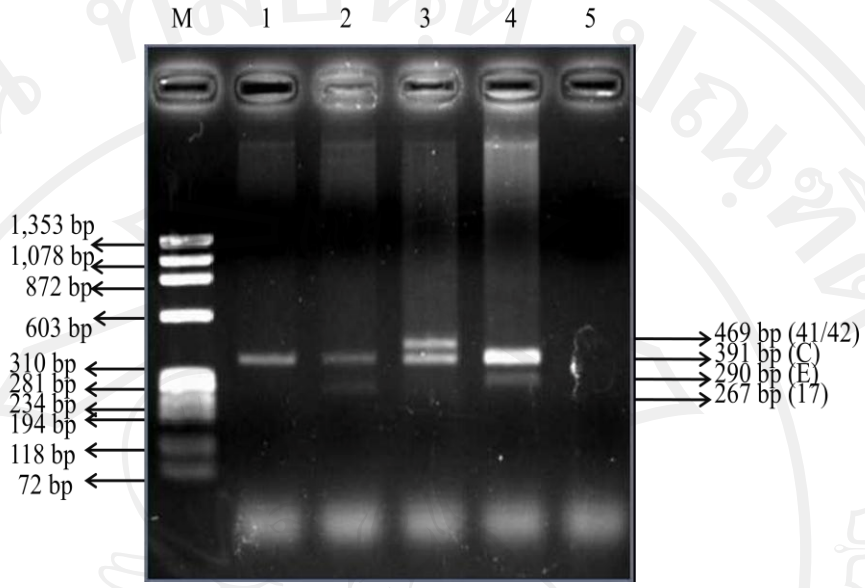

Figure 3.34 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 26 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. DNA of carrier of $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

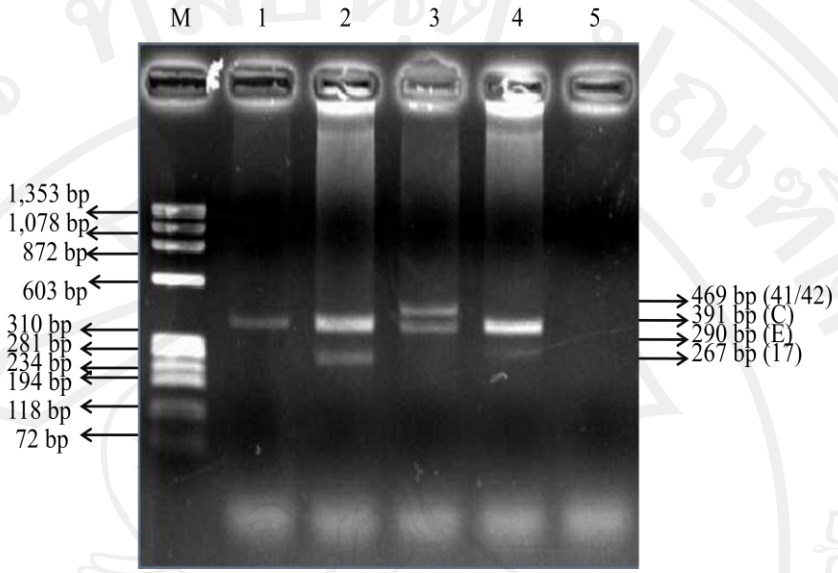

Figure 3.35 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 25 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. DNA of carrier of $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

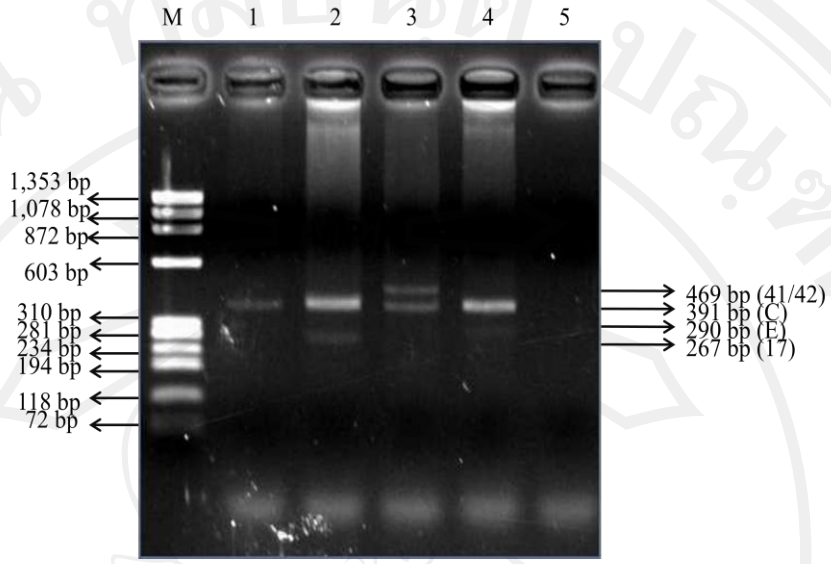

Figure 3.36 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 24 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. DNA of carrier of $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

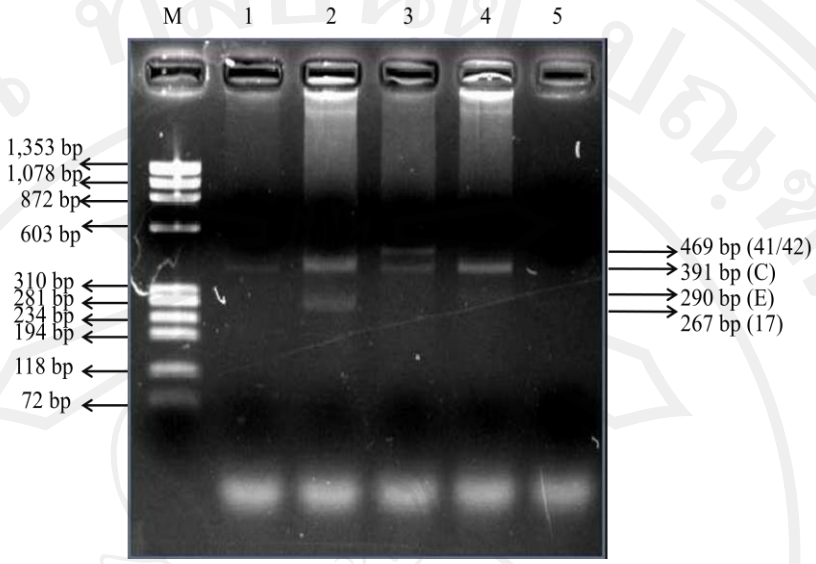

Figure 3.37 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 23 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. DNA of carrier of $\beta^{-28(A-G)}$thalassemia not done. "M" is ϕX174 *Hae*III DNA ladder.

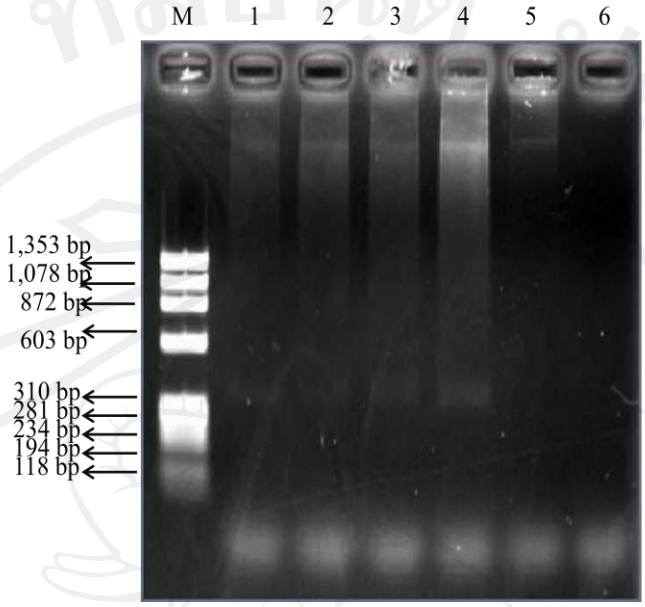

Figure 3.38 Agarose gel electrophoresis of amplified fragments for the in-house multiplex allele-specific PCR for β-thalassemia/hemoglobinopathy employing recently optimized PCR protocol with 22 thermal cycles. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$ thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is the amplified products of DNA from $\beta^{-28(A-G)}$ thalassemia. Lane 6 is blank. "M" is ϕX174 *Hae*III DNA ladder. Note that no amplified fragments were produced.

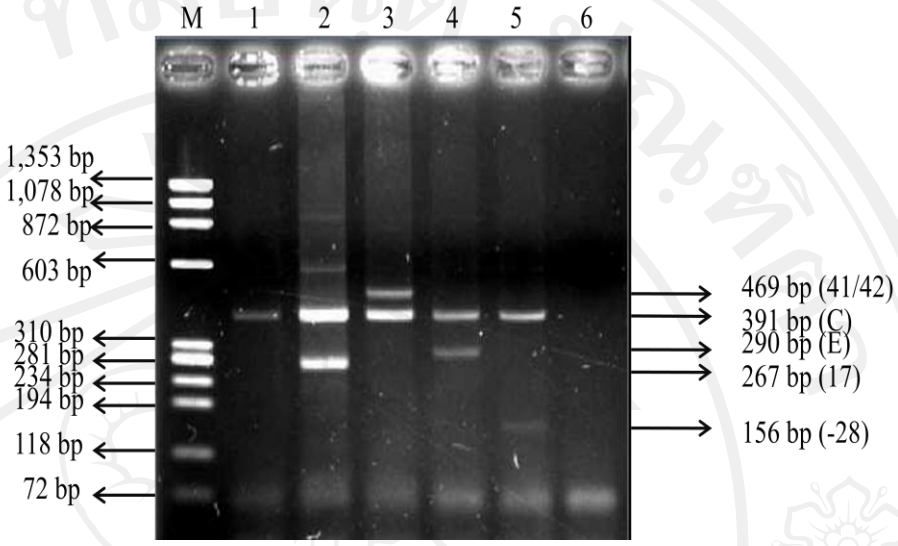

Figure 3.39 Agarose gel electrophoresis of amplified fragments genetared from the optimized in-house multiplex allele-specific PCR for β-thalassemia and hemoglobinopathy. Lane 1 is the amplified products of DNA from normal. Lane 2 is the amplified products of DNA from $\beta^{17(A-T)}$thalassemia. Lane 3 is the amplified products of DNA from $\beta^{41/42(-TTCT)}$thalassemia. Lane 4 is the amplified products of DNA from HbE. Lane 5 is the amplified products of DNA from $\beta^{-28(A-G)}$thalassemia. Lane 6 is blank. "469-bp (41/42)" is specific for $\beta^{41/42(-TTCT)}$thalassemia. "391-bp (C)" is internal control. "290-bp (E)" is specific for HbE. "267-bp (17)" is specific for $\beta^{17(A-T)}$thalassemia. "156-bp (-28)" is specific for $\beta^{-28(A-G)}$thalassemia. "M" is ϕX174 *Hae*III DNA ladder.

Table 3.4 The optimized condition of the in-house multiplex allele-specific PCR for β-thalassemia and hemoglobinopathy

Reagent	Final conc.
beta common multiplex primer	0.032 μM
beta E multiplex primer	0.018 μM
beta 17 multiplex primer	0.054 μM
beta 41/42 multiplex primer	0.054 μM
M-28M1 primer	0.14 μM
αG-17 primer	0.025 μM
C3 primer	0.4 μM
Tris pH 8.5	10 mM
KCl	50 mM
MgCl2	4 mM
dNTPs	140 μM
DMSO	2%
DNA polymerase (iTaq; iNtRON Biotechnology, Inc.)	0.1 units
DNA	250 ng

3.1.4 Evaluation of efficiency of the developed in-house multiplex allele-specific PCR

The newly developed in-house multiplex allele-specific PCR was employed in identifying common α- and β-globin genes in 70 anonymous blood samples whose screening results were indicative of being thalassemia carrier (Hb 13.1 ± 1.0 g/dl; mean ± SD), MCV 73.1 ± 5.6 fL; mean ± SD) and MCH (23.1 ± 2.2 pg; mean ± SD). Having genotyped all 70 samples, carriers of common α- and β-thalassemia/hemoglobinopathy were indentified in 57 samples accounting for 81.4% of the samples. These included single carriers of SEA-α thalassemia 1, HbE, HbCS, α-thalassemia 2 (3.7-kb deletion), $\beta^{41/42(-TTCT)}$thalassemia and $\beta^{17(A-T)}$thalassemia. These also included double carriers of α-thalassemia 2 (3.7-kb deletion) and $\beta^{17(A-T)}$thalassemia, HbCS and HbE, SEA-α thalassemia 1 and $\beta^{17(A-T)}$thalassemia and α-thalassemia 2 (3.7-kb deletion) and HbE. Table 3.5 summarizes the results. The nucleotide sequencing was performed to confirm the accuracy of mutation detection in these blood samples and the results generated from both techniques were completely comparable. Figures 3.40-3.45 shows the examples of nucleotide sequencing patterns in blood samples randomly selected for this confirmation.

Table 3.5 Frequencies of globin gene mutations in 70 thalassemia carriers as detected by the in-house multiplex allele-specific PCR developed in this thesis

	Number of samples	**Percentage**
SEA α-thalassemia-1	18	25.7
α-thalassemia-2 (3.7 kb deletion)	12	17.1
HbCS	1	1.4
$\beta^{17(A-T)}$-thalassemia	1	1.4
$\beta^{41/42(-TTCT)}$-thalassemia	2	2.8
HbE	15	21.4
HbCS/HbE	2	2.8
SEA α-thalassemia-1/$\beta^{17(A-T)}$-thalassemia	1	1.4
α-thalassemia-2 (3.7 kb deletion)/ $\beta^{17(A-T)}$-thalassemia	3	4.2
α-thalassemia-2 (3.7 kb deletion)/ HbE	2	2.8
Negative for PCR analysis	13	18.5
Total	70	

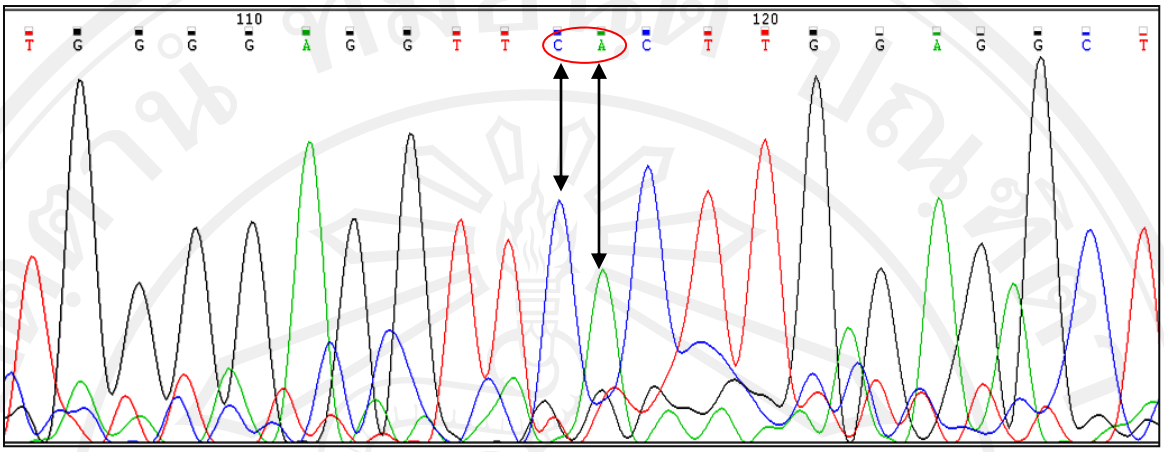

Figure 3.40 Nucleotide sequences of SEA-α thalassemia 1 breakpoint in blood sample number M149 who had been diagnosed as being the carrier by the developed in-house allele-specific PCR. The 5' breakpoint of SEA α-thalassemia 1 at nucleotides 116 and 3' breakpoint at nucleotide 117 are arrowed. Note that the nucleotide sequences are referred to the AE006462 GenBank access code.

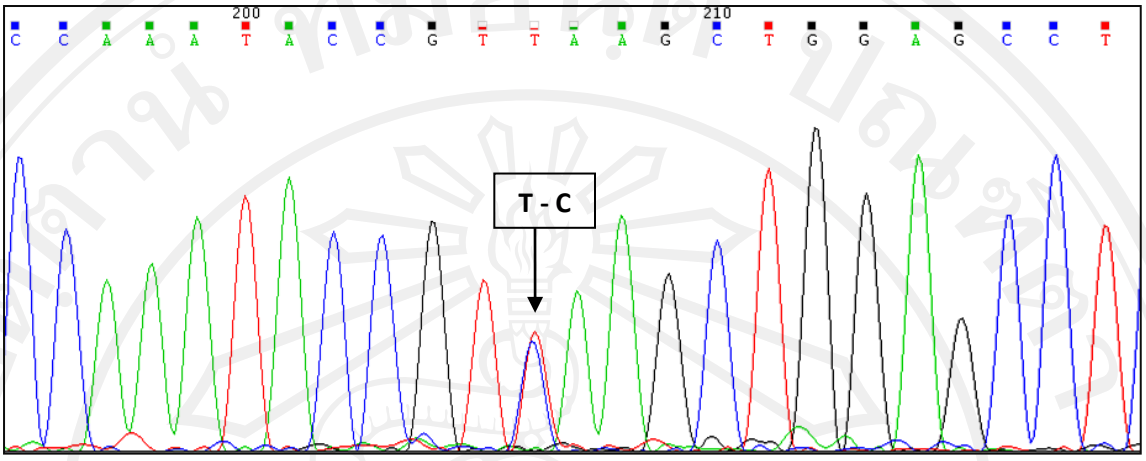

Figure 3.41 Nucleotide sequences of HbCS in blood sample number M96 who had been diagnosed as being carrier by the developed in-house allele-specific PCR. The HbCS is mutation at the terminal codon in the α2-globin gene (TAA>CAA) as shown by arrow. Note that the nucleotide sequences are referred to the AE006462 GenBank access code.

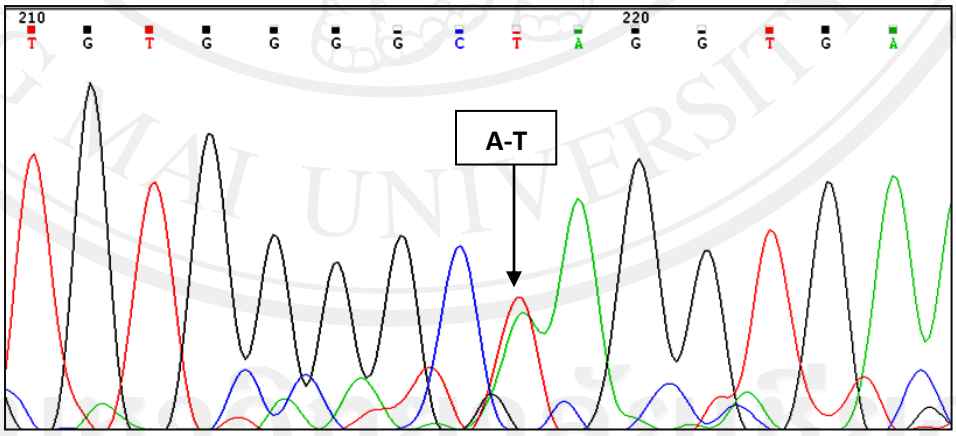

Figure 3.42 Nucleotide sequences of $\beta^{17(A-T)}$ in blood sample number LP109 who had been diagnosed as being carrier by the developed in-house allele-specific PCR. Arrowed is the mutation of $\beta^{17(A-T)}$thalassemia is AAG>TAG at codon 17 in β-globin

gene. Note that the nucleotide sequences are referred to the HUMHBB GenBank access code.

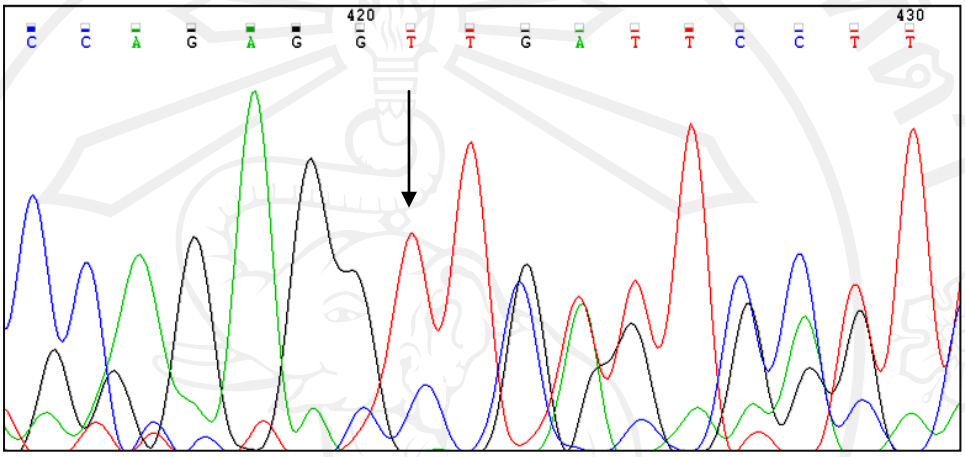

Figure 3.43 Nucleotide sequences of $\beta^{41/42(-TTCT)}$ in blood sample number M226 who had been diagnosed as being carrier by the developed in-house allele-specific PCR. The starting point of deletion of 4 nucleotides between codons 41 and 42 of β-globin gene is arrowed. This frameshift results in a new stop codon at codon 59. Note that the nucleotide sequences are referred to the HUMHBB GenBank access code.

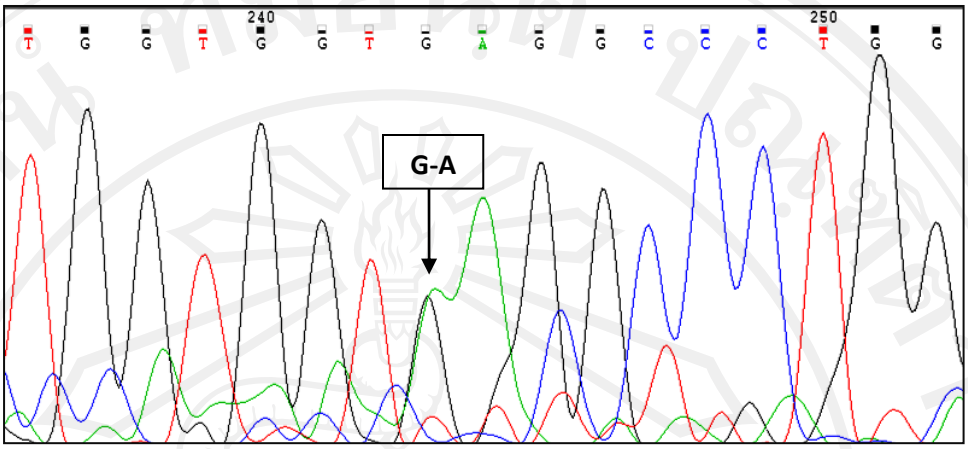

Figure 3.44 Nucleotide sequences of $\beta^{26(G-A)}$ or HbE in blood sample number LP224 who had been diagnosed as being carrier by the developed in-house allele-specific PCR. The G – A point at codon 26 in β-globin gene is arrowed. Note that the nucleotide sequences are referred to the HUMHBB GenBank access code.

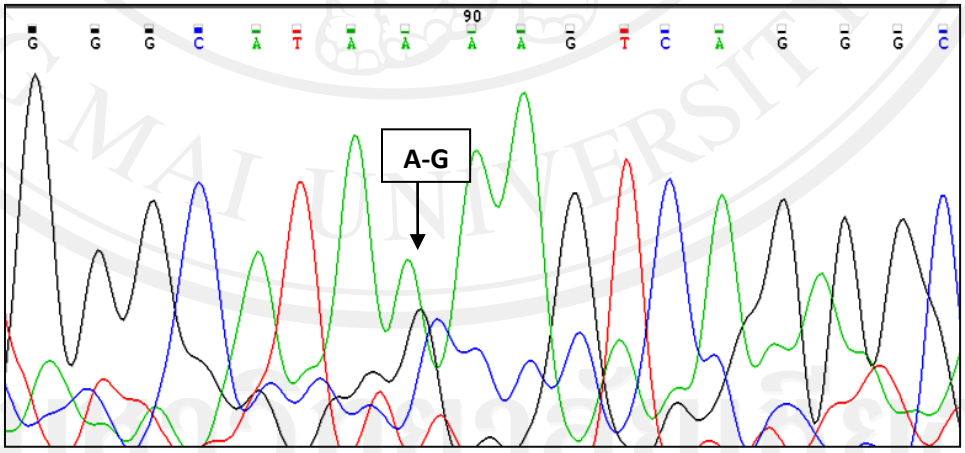

Figure 3.45 Nucleotide sequences of $\beta^{-28(A-G)}$ in known blood sample who had been diagnosed as being carrier by the developed in-house allele-specific PCR. The A-G

mutation at nucleotide -28 in β-globin gene is arrowed. Note that the nucleotide sequences are referred to the HUMHBB GenBank access code.

3.2 Development of whole blood PCR protocols for identifying globin gene mutations

3.2.1 Determining optimal thermal cycling pattern and suitable form of blood samples to be used in the PCR reaction

As stated, 2 forms of samples (lysate and fresh blood) may be encountered in the laboratory, thermal cycling patterns were initially tested for each type of samples to be used in the developed protocol. It was found that addition of three repeating extra heat-cool steps just prior to the typical thermal cycle was suitable for blood lysate (Figure 3.46). For fresh blood, however, these three repeating extra heat-cool steps were not enough to generate the 665-bp amplified fragments (Figure 3.47). Thus, five and ten repeating extra heat-cool steps were tested in this occasion. The result showed that ten repeating extra heat-cool steps were the best thermal cycles for this type of sample (Figure 3.48, 3.49). It was concluded, in this stage, that both blood lysate and fresh blood can be used in the developed whole blood PCR and adjustment of the thermal cycles must be undertaken for each type of samples used.

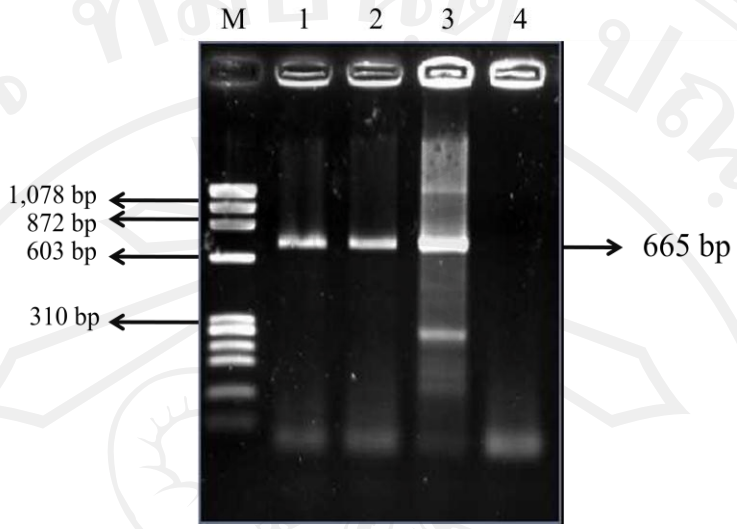

Figure 3.46 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using blood lysate with three repeating extra heat-cool steps prior to typical thermal cycles. Lanes 1-2 are the amplified products of blood lysate samples. Lane 3 is the amplified products of DNA sample. Lane 4 is blank. "665-bp" is specific amplified product. "M" is the ϕX174 *Hae*III digest as standard size marker. Note that the 665-bp products were successfully amplified.

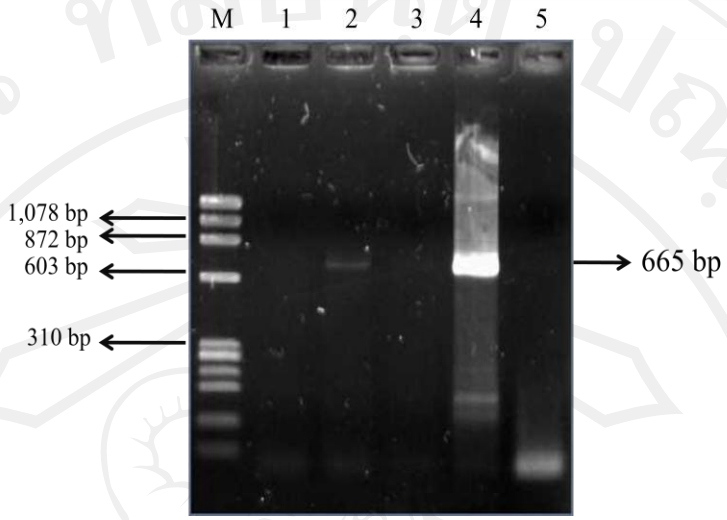

Figure 3.47 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using fresh blood with three repeating extra heat-cool steps prior to typical thermal cycles. Lanes 1-3 are the amplified products of fresh blood samples. Lane 4 is the amplified products of DNA sample. Lane 5 is blank. "665-bp" is specific amplified product. "M" is the ϕX174 *Hae*III digest as standard size marker. Note that weak amplified signals are seen in lanes 1-3.

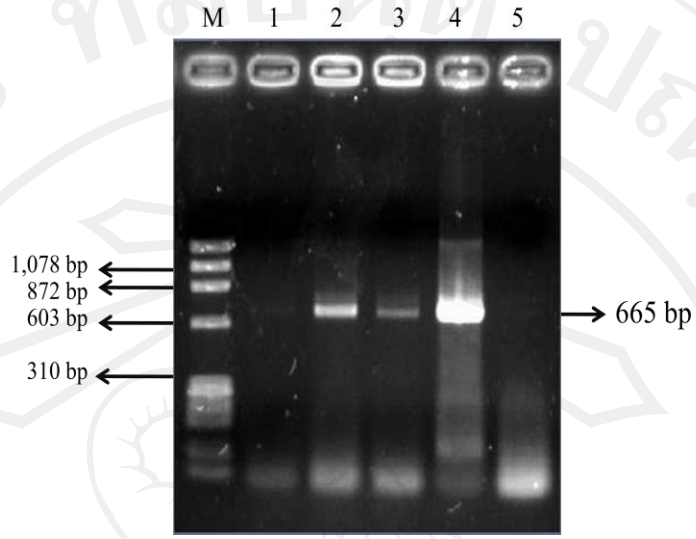

Figure 3.48 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using fresh blood with five repeating extra heat-cool steps prior to typical thermal cycles. Lanes 1-3 are the amplified products of fresh blood samples. Lane 4 is the amplified products of DNA sample. Lane 5 is blank. "665-bp" is specific amplified product. "M" is the ϕX174 *Hae*III digest as standard size marker. Note that increased amplified signals are seen in lanes 2-3.

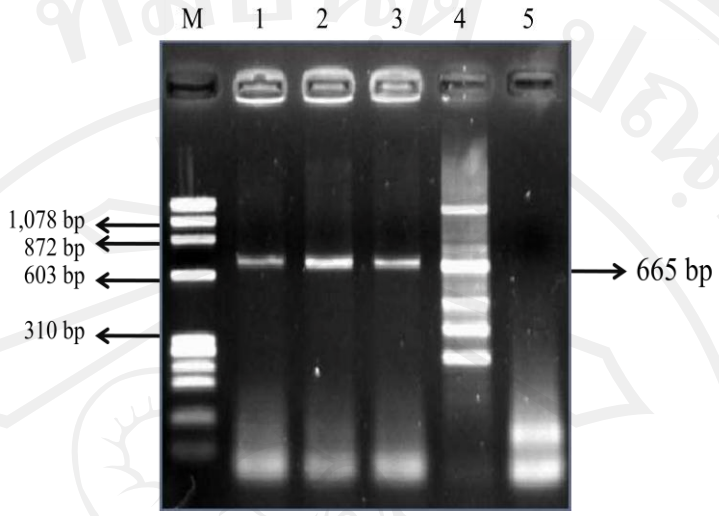

Figure 3.49 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using fresh blood with ten repeating extra heat-cool steps prior to typical thermal cycles. Lanes 1-3 are the amplified products of fresh blood samples. Lane 4 is the amplified products of DNA sample. Lane 5 is blank. "665-bp" is specific amplified product. "M" is the ϕX174 *Hae*III digest as standard size marker. Note that strong amplified signals are seen in lanes 1-3.

3.2.2 Search for the best PCR facilitators

Bovine serum albumin (BSA) and betaine were tested for being the facilitator in the developed whole blood PCR. The best concentration of BSA was found to be 0.6% (w/v) for blood lysate (Figure 3.50) and 0.2 % (w/v) for fresh blood (Figure 3.51). However, the PCR products generated in BSA as facilitator were not significantly increased. For betaine, the best concentration was found to be 7-15% (w/v) for blood lysate (Figure 3.52) and 5-13% (w/v) for fresh blood (Figure 3.53). Combinations of 9% (w/v) betaine with various concentrations of BSA showed that 0.2%, 0.4% and 0.6%

(w/v) BSA could be combined with betaine as shown in Figure 3.54. However, the combination seemed not to benefit as the PCR products were not increased significantly. Hence, the betaine alone at 9% (w/v) concentration was chosen to be the facilitator of this developed whole blood PCR.

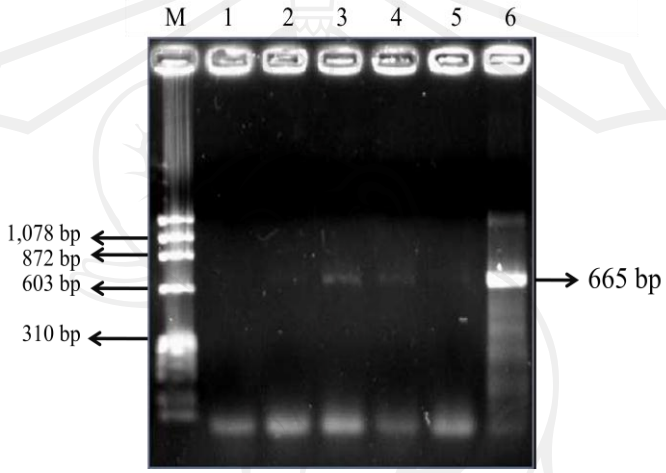

Figure 3.50 Amplification of 665-bp $^{G}\gamma$-globin specific fragments in presence of varying amount of BSA. Lane 1; 0.2% (w/v) BSA, Lane 2; 0.4% (w/v) BSA, Lane 3; 0.6% (w/v) BSA, Lane 4; 0.8% (w/v) BSA, Lane 5; 1.0% (w/v) BSA, Lane 6; no BSA. 2 μl of blood lysate were used in lanes 1-5 and 2 μl of DNA in lane 6. "M" is the ϕX174 *Hae*III digest standard size marker. Note lane 3 shows the strongest signal.

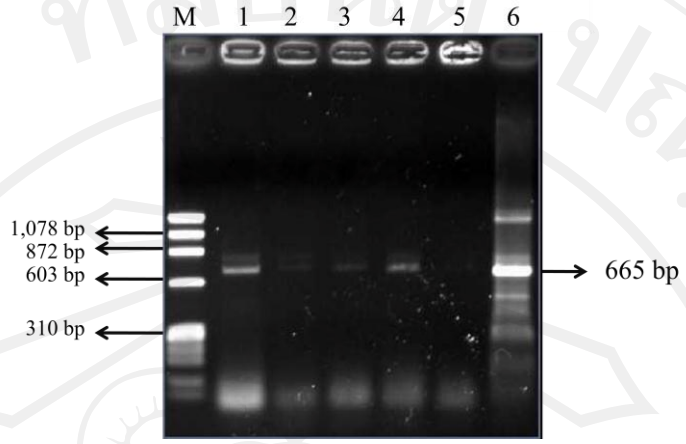

Figure 3.51 Amplification of 665-bp $^{G}\gamma$-globin specific fragments in presence of varying amount of BSA. Lane 1; 0.2% (w/v) BSA, Lane 2; 0.4% (w/v) BSA, Lane 3; 0.6% (w/v) BSA, Lane 4; 0.8% (w/v) BSA, Lane 5; 1.0% (w/v) BSA, Lane 6; no BSA. 2 μl of fresh blood were used in lanes 1-5 and 2 μl of DNA in lane 6. "M" is the ϕX174 *Hae*III digest standard size marker. Note lane 1 shows the strongest signal.

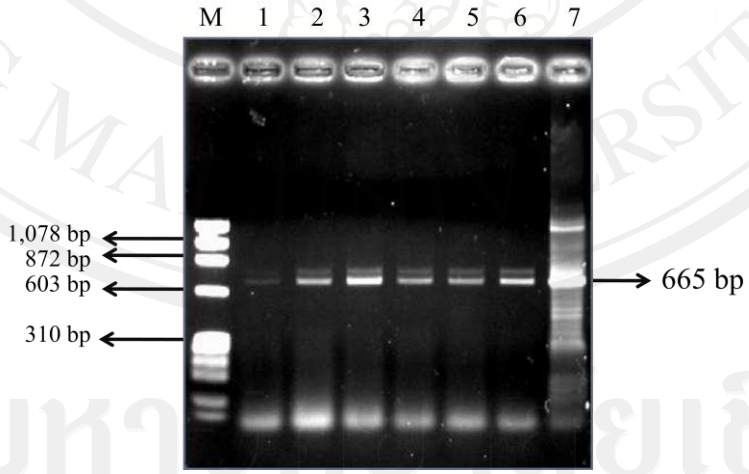

Figure 3.52 Amplification of 665-bp $^{G}\gamma$-globin specific fragments in presence of varying amount of betaine. Lane 1; 5% (w/v) betaine, Lane 2; 7% (w/v) betaine, Lane 3; 9% (w/v) betaine, Lane 4; 11% (w/v) betaine, Lane 5; 13% (w/v) betaine, Lane 6; 15%

(w/v) betaine, Lane 7; no betaine. 2 μl of blood lysate were used in lanes 1-6 and 2 μl of DNA in lane 7. "M" is the ϕX174 *Hae*III digest standard size marker. Note that lanes 2-5 show strong band signals.

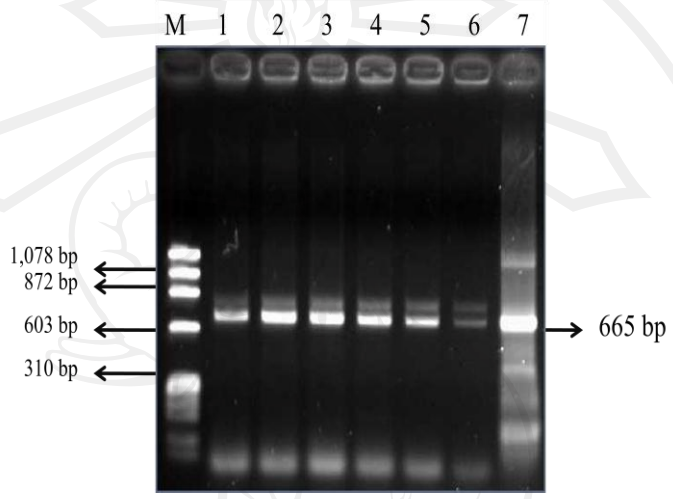

Figure 3.53 Amplification of 665-bp $^{G}\gamma$-globin specific fragments in presence of varying amount of betaine. Lane 1; 5% (w/v) betaine, Lane 2; 7% (w/v) betaine, Lane 3; 9% (w/v) betaine, Lane 4; 11% (w/v) betaine, Lane 5; 13% (w/v) betaine, Lane 6; 15% (w/v) betaine, Lane 7; no betaine. 2 μl of fresh blood were used in lanes 1-6 and 2 μl of DNA in lane 7. "M" is the ϕX174 *Hae*III digest standard size marker. Note that lanes 1-5 show strong band signals.

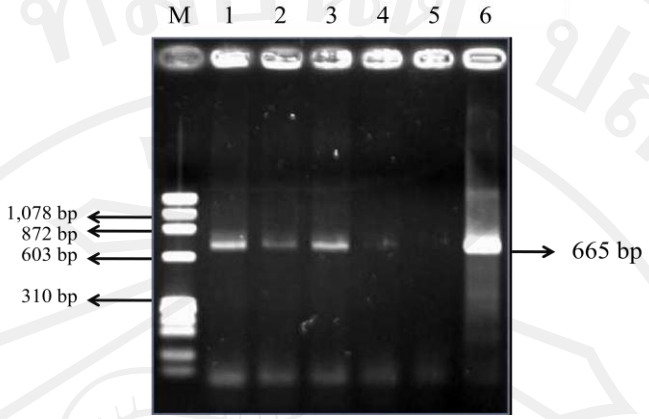

Figure 3.54 Amplification of 665-bp $^{G}\gamma$-globin specific fragments in presence of fixed amount of betaine at 9% and varying concentration of BSA. Lane 1; 9% (w/v) betaine + 0.2% (w/v) BSA, Lane 2; 9% (w/v) betaine + 0.4% (w/v) BSA, Lane 3; 9% (w/v) betaine + 0.6% (w/v) BSA, Lane 4; 9% (w/v) betaine + 0.8% (w/v) BSA, Lane 5; 9% (w/v) betaine + 1.0% (w/v) BSA, Lane 6; no facilitators. 2 μl of blood lysate was used in lanes 1-5 and 2 μl of DNA in lane 6. "M" is the ϕX174 *Hae*III digest standard size marker. Note that lanes 1-3 show strong signals.

3.2.3 Determining appropriate volume of blood samples used in the whole blood PCR

Varying amounts of either fresh blood or blood lysate were tested in the optimized whole blood PCR as described in Section 2.2.2.3. It was found that both blood lysate and fresh blood at the volume of 0.5μl, 1.0μl, 1.5μl and 2.0μl were suitable for this developed PCR protocol. However, 2 μl of either blood lysate or fresh blood was chosen for 50-μl PCR reaction (Figures 3.55, 3.56).

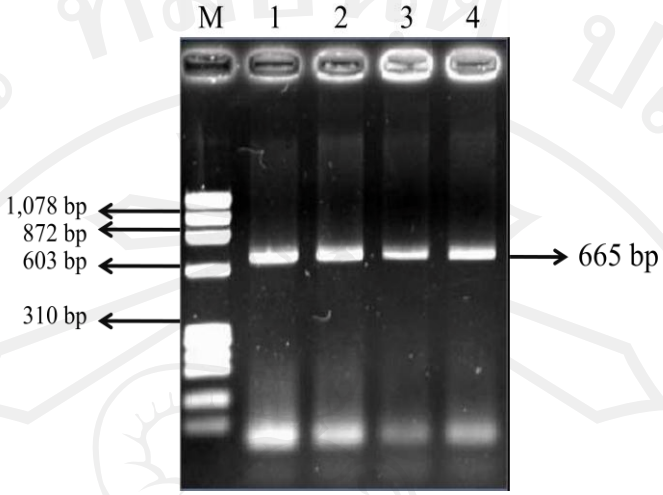

Figure 3.55 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using varying volume of blood lysate. Lane 1; 0.5 µl, Lane 2; 1 µl, Lane 3; 1.5 µl, Lane 4; 2 µl, Lane 5; 2 µl DNA. "M" is the ϕX174 *Hae*III digest standard size marker. Note that all lanes show strong signals.

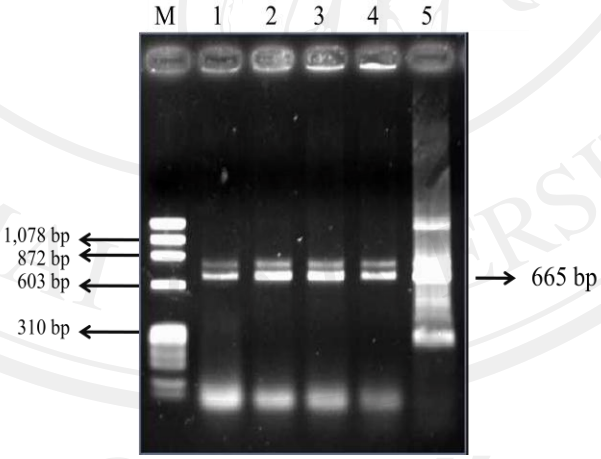

Figure 3.56 Amplification of 665-bp $^{G}\gamma$-globin specific fragments using varying volume of fresh blood. Lane 1; 0.5 µl, Lane 2; 1 µl, Lane 3; 1.5 µl, Lane 4; 2 µl, Lane 5; 2 µl DNA. "M" is the ϕX174 *Hae*III digest standard size marker. Note that all lanes show strong signals.

3.2.4 Applying the developed technique in detecting the carriers of α- and β-thalassemia and hemoglobinopathies common in Thailand

A. SEA-α thalassemia 1

The whole blood PCR was adapted to the singleplex Gap-PCR protocol for detection of SEA-α thalassemia 1 previously optimized. However, optimal concentrations of SEA1, SEA2 and SEA3 primers were re-adjusted in order to yield satisfactory results. It was finally found that 0.10 μM SEA1, 0.07 μM SEA2 and 0.04 μM SEA3 primers were sufficient for the whole blood Gap-PCR to identify the SEA-α thalassemia 1 (Figures 3.57-3.60). The optimized condition is summarized in Table 3.6.

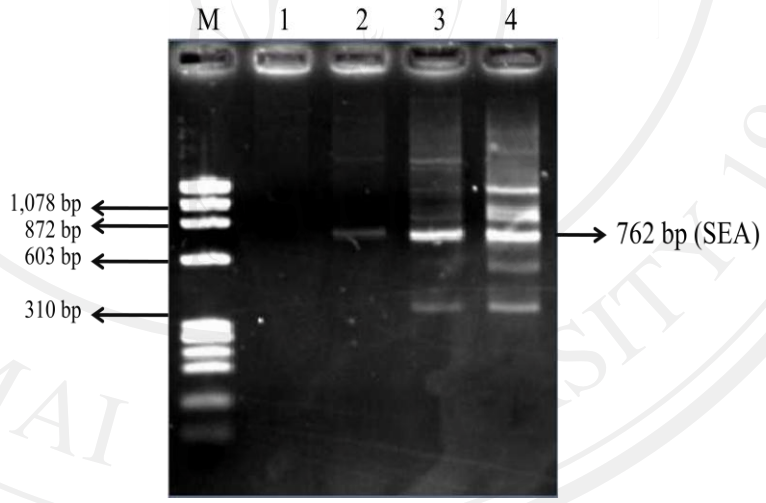

Figure 3.57 Titration of SEA1 primer concentration used in the whole blood Gap-PCR of SEA-α thalassemia 1. The concentration of SEA2 and SEA3 primers were fixed at 0.04 μM and 0.07 μM and that of SEA1 primer varied. Lane 1; 0.04 μM SEA1, Lane 2; 0.07 μM SEA1, Lane 3; 0.10 μM SEA1, Lane 4; 0.14 μM SEA1. "SEA" is the SEA-α

thalassemia 1. “C” is internal control. “M” is the ϕX174 *Hae*III digest standard size marker. Note that lane 3 shows the best results.

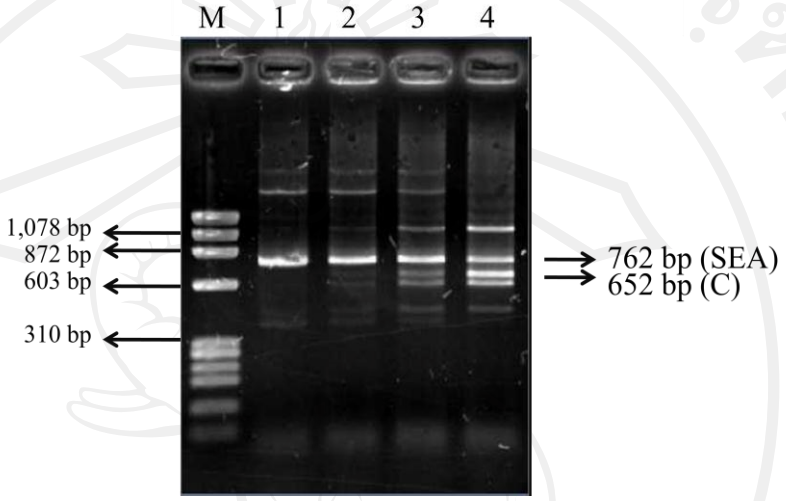

Figure 3.58 Titration of SEA2 primer concentration used in the whole blood Gap-PCR of SEA-α thalassemia 1. The concentration of SEA1 and SEA3 primers were fixed at 0.10 μM and 0.07 μM and that of SEA2 primer varied. Lane 1; 0.028 μM SEA2, Lane 2; 0.042 μM SEA2, Lane 3; 0.056 μM SEA2, Lane 4; 0.07 μM SEA2. “SEA” is the SEA-α thalassemia 1. “C” is internal control. “M” is the ϕX174 *Hae*III digest standard size marker. Note that lane 4 shows the best results.

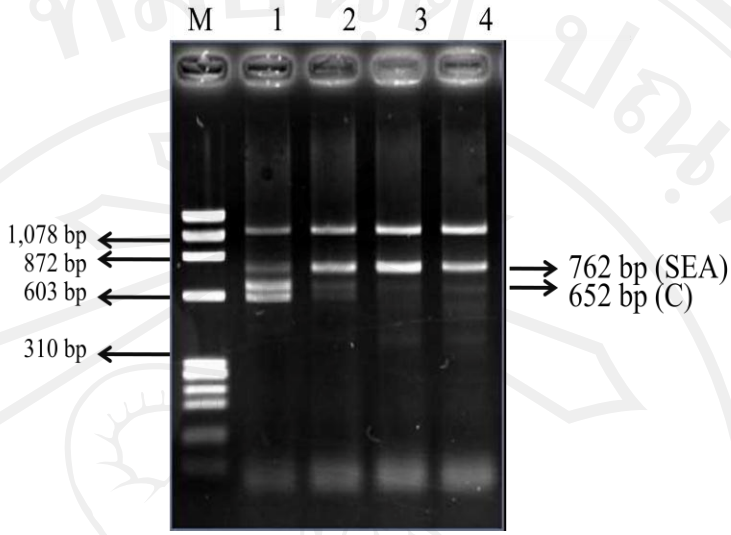

Figure 3.59 Titration of SEA3 primer concentration used in the whole blood Gap-PCR of SEA-α thalassemia 1. The concentration of SEA1 and SEA2 primers were fixed at 0.10 μM and 0.07 μM and that of SEA3 primer varied. Lane 1; 0.04 μM SEA3, Lane 2; 0.07 μM SEA3, Lane 3; 0.10 μM SEA3, Lane 4; 0.14 μM SEA3. "SEA" is SEA-α thalassemia 1. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker. Note that lane 1 shows the best result.

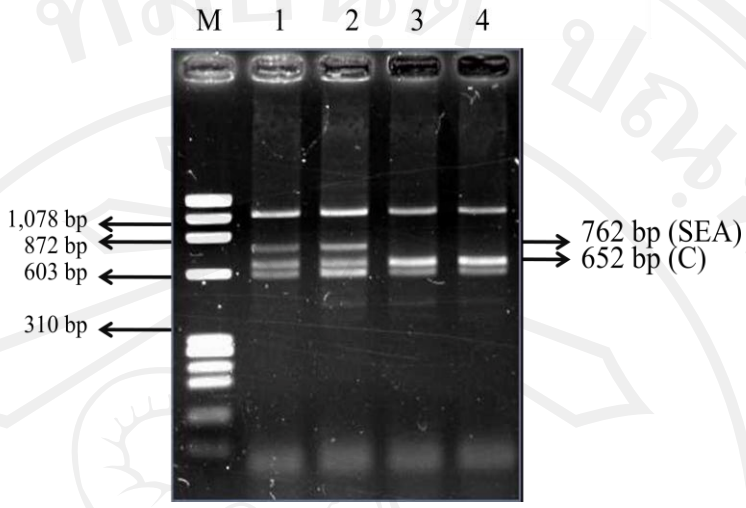

Figure 3.60 Detection of the SEA-α thalassemia 1 by the whole blood Gap PCR utilizing 0.10 μM SEA1, 0.07 μM SEA2 and 0.04 μM SEA3 primers. Lanes 1 and 2; whole blood from SEA-α thalassemia 1, Lanes 3 and 4 ; whole blood from non SEA-α thalassemia 1. "SEA" is SEA-α thalassemia 1. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

Table 3.6 The optimized condition of the whole blood Gap PCR for detecting SEA-α thalassemia 1

Reagent	Final conc.
i-Proof GC buffer	1X
$MgCl_2$	1.5 mM
dNTPs	140 μM
SEA1 primer	0.10 μM
SEA2 primer	0.07 μM
SEA3 primer	0.04 μM
DMSO	2%
betaine	9%
DNA polymerase (iTaq; iNtRON Biotechnology, Inc.)	0.02 units
Whole blood	1 μl

B. Hb Constant Spring

The whole blood PCR was adapted from the singleplex allele-specific PCR optimized previously for identifying HbCS. However, the concentrations of CS-2, αG-17 and C3 primers were also re-adjusted. Finally, CS-2 at 0.1 μM, αG-17 at 0.04 μM and C3 at 0.4 μM were found to be sufficient for this whole blood allele-specific PCR for detecting HbCS allele (Figures 3.61-3.63). The optimized condition is summarized in Table 3.7.

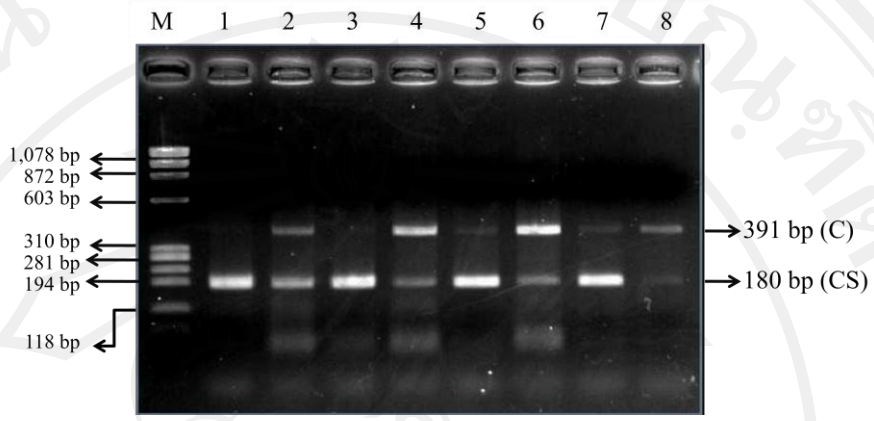

Figure 3.61 Titration of CS-2 primer concentration used in the whole blood allele-specific PCR for HbCS. The concentrations of αG-17 and C3 primers were fixed at 0.025 μM and 0.4 μM, respectively, and that of CS-2 varied. Lanes 1, 3, 5, 7 are the amplified products of carriers of HbCS and lane 2, 4, 6, 8 are the amplified products of non-HbCS carrier. The concentration of CS-2 primer was 0.4 μM in lane 1 and 2, 0.3 μM in lane 3 and 4, 0.2 μM in lane 5 and 6, 0.1 μM in lane 7 and 8. "CS" is HbCS. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

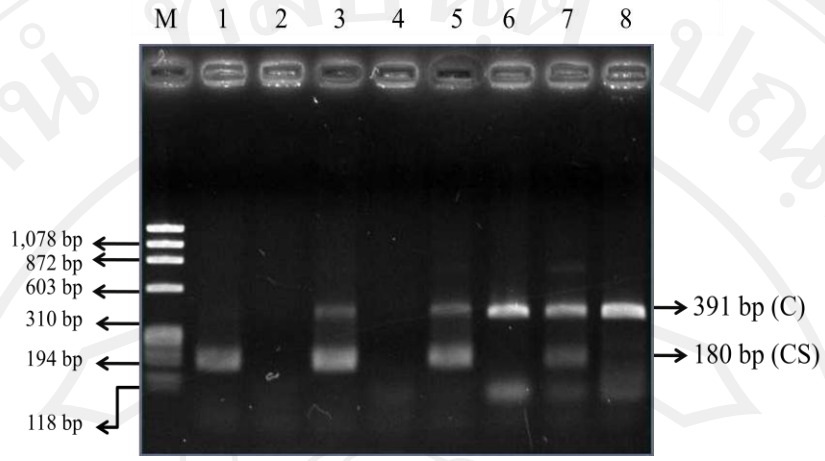

Figure 3.62 Titration of αG-17 primer concentration used in the whole blood allele-specific PCR for HbCS. The concentration of CS-2 and C3 primers were fixed at 0.1 μM and 0.4 μM, respectively, and that of αG-17 varied. Lanes 1, 3, 5, 7 are the amplified products of carriers of HbCS and lanes 2, 4, 6, 8 are the amplified products of non-HbCS carrier. The concentration of αG-17 primer was 0.025 μM in lane 1 and 2, 0.03 μM in lane 3 and 4, 0.04 μM in lane 5 and 6, 0.05 μM in lane 7 and 8. "CS" is HbCS. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

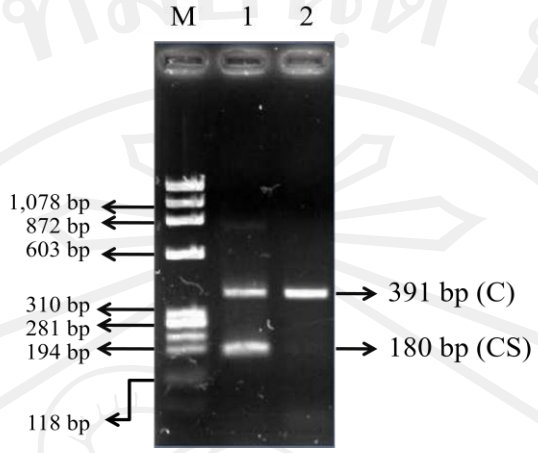

Figure 3.63 Detection of carrier of HbCS by the whole blood allele-specific PCR utilizing 0.1 μM CS-2, 0.04 μM αG-17 and 0.4 μM C3. Lane 1; whole blood from carrier of HbCS, Lane 2; whole blood from non-HbCS carrier. "CS" is HbCS. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

Table 3.7 The optimized condition of the whole blood PCR for detecting HbCS

Reagent	Final conc.
Tris pH 8.5	10 mM
KCl	50 mM
$MgCl_2$	2.5 mM
dNTPs	100 μM
CS-2 primer	0.1 μM
C3 primer	0.4 μM
αG-17 primer	0.04 μM
DMSO	2%
betaine	9%
DNA polymerase (iTaq; iNtRON Biotechnology, Inc.)	0.1 units
Whole blood	1 μl

C. β-thalassemia (β^{17}, $\beta^{41/42}$, β^{-28}, HbE)

The multiplex allele-specific PCR for detecting common β-thalassemia in Thailand previously optimized was adapted employing the conditions also previously optimized for the whole blood PCR. However, the HbE-specific primers and thermal cycles were re-adjusted. It was found that the optimal thermal cycles for this protocol was 24 cycles. It was also found that the optimal concentrations of "beta-common multiplex", "beta-E multiplex", "beta-17 multiplex", "beta-41/42 multiplex", αG-17, C3 and M-

28M1 were 0.032 μM, 0.0108 μM, 0.054 μM, 0.054 μM, 0.025 μM, 0.4 μM and 0.14 μM, respectively, (Figures 3.64-3.65). The optimized condition is summarized in Table 3.8.

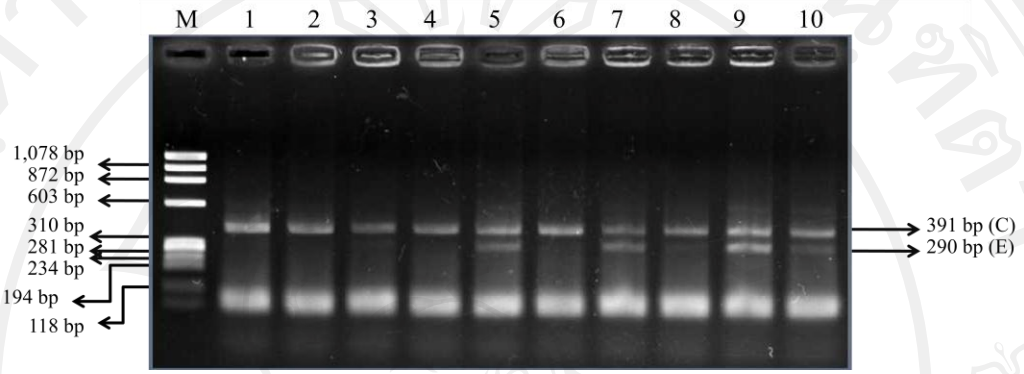

Figure 3.64 Titration of concentration of "beta-E multiplex" primer used in whole blood multiplex allele-specific PCR of common β-thalassemia in Thailand. Lanes 1, 3, 5, 7, 9 are the amplified products of HbE carriers and lanes 2, 4, 6, 8, 10 are the amplified products of non-HbE carrier. The concentration of "beta-E multiplex" primer was 0.0036 μM in lane 1 and 2, 0.0072 μM in lane 3 and 4, 0.0108 μM in lane 5 and 6, 0.0144 μM in lane 7 and 8, 0.018 μM in lane 9 and 10. "E" is HbE. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

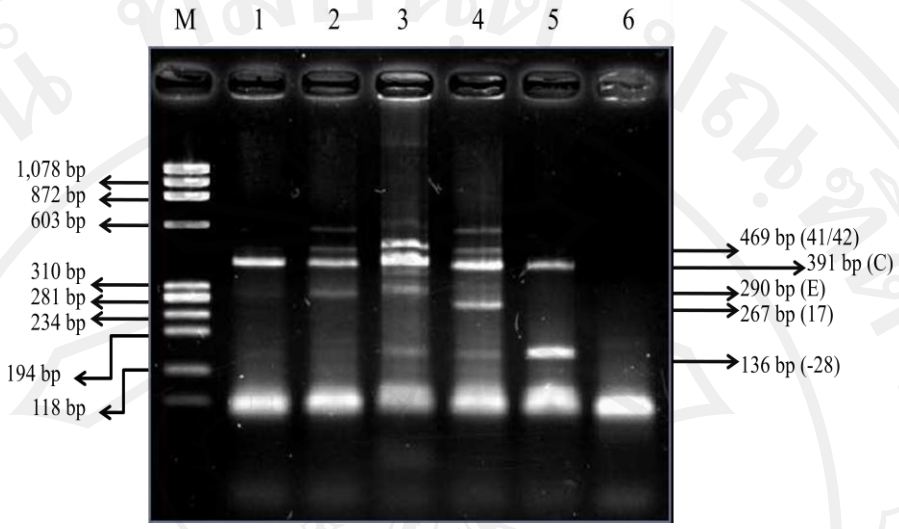

Figure 3.65 Whole blood multiplex allele-specific PCR for detecting the common β-thalassemia/hemoglobinopathy in Thailand. Lane 1 is the amplified products of normal, lane 2 is the amplified products of HbE carrier, lane 3 is the amplified products of HbE/$\beta^{41/42}$-thalassemia, lane 4 is the amplified products of $\beta^{17(A-T)}$-thalassemia carrier, lane 5 is the amplified products of $\beta^{-28(A-G)}$-thalassemia carrier, lane 6 is a blank control. "41/42" is $\beta^{41/42(-TTCT)}$-thalassemia. "17" is $\beta^{17(A-T)}$-thalassemia. "E" is HbE. "-28" is $\beta^{-28(A-G)}$-thalassemia. "C" is internal control. "M" is the ϕX174 *Hae*III digest standard size marker.

Table 3.8 The optimized condition of the whole blood multiplex allele-specific PCR for detecting the common β-thalassemia/hemoglobinopathy

Reagent	**Final conc.**
beta common multiplex primer	0.032 μM
beta E multiplex primer	0.0108 μM
beta 17 multiplex primer	0.054 μM
beta 41/42 multiplex primer	0.054 μM
M-28M1 primer	0.14 μM
αG-17 primer	0.025 μM
C3 primer	0.4 μM
Tris pH 8.5	10 mM
KCl	50 mM
$MgCl_2$	4 mM
dNTPs	140 μM
DMSO	2%
betaine	9%
DNA polymerase (iTaq; iNtRON Biotechnology, Inc.)	0.1 units
Whole blood	1 μl