CHAPTER IV

DISCUSSION AND CONCLUSION

4.1 Genomic DNA isolation from whole blood.

The method described by Steger and co-workers (1994) seems to be appropriate for routine genomic DNA isolation from whole blood. Less than 70 µl, freshly taken or stored for a long time at -70°C, is required. The method was practical and yielded enough genomic DNA for PCR. However, because of the small amount of blood required, the cell pellet containing nucleic acid may get lost in any washing step, and genomic DNA normally obtained was less than 50 ng/µl. Although the author suggested that washing 1-2 times with TE buffer was enough, it was not appropriate for all of the whole blood samples. Some were still red after washing 2 time with TE buffer due to too much heme molecules contaminating the pellet and this might be the inhibitor for PCR. The KOH they used to denatured the DNA and destroyed all proteins and also haemoglobin molecules and HCl which had been used to neutralize the pH of the solution, however, the final pH of the solution in each sample could not be meassured in an appropriate fashion because of the inadiquate volume. The inappropriate pH was then caused the degradation of the DNA after long time storage. EDTA,

which is normally used to protect the DNA in the solution was also not included. Moreover, when these genomic templates failed in some PCR, especially for the PCR experiments that test the deletion of some sequences in specific regions of the gene, α-thalassemia-2 (-3.7 and -4.2 kb) for example, it could not be concluded whether or not the failure of the PCR was from the template not occupying the specific deletional region or from the template itself, until it was checked with another pair of primers that could be amplify the undeletion region or checked in other PCR experiments.

Phenol-Chloroform extraction for genomic DNA described by Sambrook and co-workers (1989) requires too much volume of whole blood, 5-10 ml indeed. Phenol and chloroform are also harmful. The DNA obtained was too purified for PCR and the procedure was too complicated.

The application method for the isolation of genomic DNA from whole blood without preisolating of white blood cell described by McIndoe and co-workers (1995) was quite complicated due to the special equipment (13 ml Vacutainer™, Becton Dickinson), as much as 5 ml of whole blood and toxic chemicals such as phenol-chloroform were required.

Proteinase-K treatment after simple cell lysis with TE buffer is an interesting choice for genomic DNA isolation from whole blood.

Proteinase-K, the proteolytic enzyme isolated from *Tritirachium album* Limber, has been shown to inactivate nucleases rapidly and is widely used for the isolation of high molecular weight DNA and RNA from mammalian cells. As described by Chih-Chia and co-workers (1993), some components in K-buffer were modified as follows : Tris buffer with pH of 8.0 (at 25°C) and the stock (10%) sodium dodecyl sulfate (SDS) solution adjusted the pH to 7.0 to ensure the pH of above 7.5 when they were incubated with the homogenate at 45°C. Detergent such as SDS that included in K-buffer was the inhibitor of DNase and RNase. $MgCl_2$, as usually included in the K-buffer described by Badly and co-workers (1988), was also omitted from the buffer since it might enhance aggregration among nucleic acids and between proteins and nucleic acids. The buffer was preheated to 45°C, and then higher concentrations of proteinase-K than previously reported (Badly *et al.*, 1988) were added to the mixture to allow the rapid inactivation of endogenous nucleases that might contaminate the buffer during the preparation. By combining the two methods described above, 50-200 µl of whole blood was required for each preparation, washed 1 time with 0.5% Triton X100/TE and 1-4 times with TE buffer pH 8.4 or until a white cell pellet was observed, and then treated with proteinase-K in K-buffer. Approximately 40 µg/50 µl with $OD_{260/280} > 1.2$ was normally obtained. That high DNA concentration and purity was appropriate for

the PCR optimization experiments that the concentration of template required was still not indicated. This modified method was practical to use, took 6 hours to process, and was a good choice for genomic DNA isolation from whole blood for PCR.

4.2 High GC-content DNA template amplification by "Semi-touchdown" PCR

According to Molchanova and co-workers (1994), α_2- and α_1-globin genes were ampliflied by asymmetric PCR. The single-stranded PCR products, according to 25 folds of the reverse primer concentration over the forward primer used, were appropriate as templates for manual sequencing with Sequenase Version 2.0 (United State Biochemical, Cleveland, OH, USA). However, their PCR systems were not repeatable. In order to improve their PCR system, the titration of some important parameters in the reaction mixture such as formamide, $MgCl_2$, and DNA template concentrations and the annealing temperature used in the native-three step PCR protocol were tested but the system could still not be improved.

By using the two-step "Hot" PCR reported by Schuchard and co-workers (1993), the native PCR reaction mixtures still failed when 72, 74 or 76°C annealing/extension temperatures were used. Even with the "Touchdown" PCR protocol, the result were still the same. It seemed to

be that the native reaction mixtures as reported were not appropriate for the α_2- and α_1-globin genes amplification.

Specific oligonucleotide primers, according to Molchanova and co-workers (1994) were used in the modified α_2- and α_1-globin genes PCR. Although some were shown to be occupied the unusual sequences (stretch of Gs and Cs) which possibly formed the secondary structure and primer dimer, they were the best primer sets for α_2- and α_1-globin genes specific amplification according to the parameters used for the consideration in primer selection step.

By replacing the native PCR reaction buffer (20.0 mM MOPS, 40.0 mM KCl, 0.6 % Tween-20, and 7.5% Freshly-deionized formamide) with the *Taq* 2Δ PCR buffer, which has been modified from McKeown (1994), (45.0 mM Tris-HCl (pH 8.4, at 25°C), 11.0 mM $(NH_4)_2SO_4$, 6.7 mM 2-Mercaptoethanol, 4.4 mM EDTA, 11.3 mg/ml heat-inactivated BSA and 7.5% DMSO), α_2- and α_1-globin genes were successfully amplification, in the symmetric PCR condition, by using "Touchdown" PCR protocol as indicated. Moreover, these specific amplified products were obtained either with two-step PCR protocol (70°C annealing/extension temperature) or native-three steps PCR protocol (68°C annealing temperature). The intensity and specificity of the PCR products observed on the gel were further improved by using a

modified reaction mixture with "Semi-touchdown" PCR protocol as indicated.

The pH of the modified reaction buffer was adjusted to alkaline according to the stock Tris (pH 8.9 at 25°C) and EDTA pH 8.9 which was appropriate for the amplification of longer DNA fragments and also for high GC-content DNA templates. By using alkaline pH's PCR reaction buffer, the longer genomic DNA would be protected from damage when the reaction was heated up in PCR cycle and denatured as a single strand along the sequence which was amplified. The longer genomic DNA template would also be protected with EDTA, which was not included in the native reaction mixture. 2-Mercaptoethanol was used for retarding the oxidation of biological compound, for example acetylated BSA, in the reaction mixture. Nuclease which commonly contaminates in BSA was inactivated when BSA was in the acetylated form. Heat-inactivated acetylated BSA was reported to enhance the minisatellite variant repeat PCR (MVR-PCR) as reported by McKeown (1994). Formamide, according to Molchanova and co-workers (1994), was freshly deionized before combining with the PCR reaction mixture and was reported to enhanced the specific amplification of α_2- and α_1-globin genes. However, the titration of formamide (from 0-10% final conentration) showed no improvement of those PCR systems. As reported by Sarkar and co-workers (1990), more than 5% of

formamide inhibited the PCR of dopamine D2 receptor gene. On the other hand, 7.5% of DMSO, directly used in the modified reaction mixture, could enhance the α_2- and α_1-globin genes amplification as indicated. Further titration experiment showed the necessity of 7.5% DMSO for those specific amplification with the modified PCR reaction mixture. Without DMSO, the specific products were not enhanced.

"Semi-touchdown" PCR protocol is the PCR protocol that is modified from theoretical cycling parameters appearing in the "Touchdown" PCR. The exact annealing temperature was optimized to 67°C in which the highest intensity of the PCR products were obtained. The Tm calculated from the two widely used equations showed up to 21°C difference, depending on which factors were considered in each equation. By less optimization of the PCR system, the exact annealing temperature was fixed somewhere around the lowest Tm value from those two equations (67°C in this case, after optimization). For the stages that the non-specific products generated, it can be possible to use the average Tm value of 2 primers calculated from each equation. In this case, 80°C was from the average Tm value of (72+82+86) (from equation 1) and 73.5°C was from the middle value between 80°C and 67°C. After some optimization experiments, it could be concluded that, the first 10 cycles (with higher annealing temperature) were enough to generate a pool of specific and non-specific products in which the

specific one would be generated in the next 20 cycles when exact annealing temperature was used.

From the PCR results, it can be concluded that specific amplification of α_2- and α_1-globin genes are enhanced by modified reaction mixtures and "Semi-touchdown" PCR protocol as follows :

1) 785 and 795 bp DNA fragments, as expected for specific α_2- and α_1-globin genes PCR products respectively, were obtained (from gel electrophoresis along with 100 bp DNA ladder).

2) 7 bp deletion occurring in exon 3 of α_2-globin gene was observed from the sequencing data.

3) 684, 391, 293 and 111 bp *Alw44* I digestion products of Hb Chiang Mai's α_1-globin gene PCR product were detected in gel electrophoresis, while the same digested pattern was not observed in the *Alw44* I digested's α_2-globin PCR product. And also, 677 and 108 bp of *Stu* I digestion products of Hb Chiang Mai's α_2-globin gene PCR product were detected in gel electrophoresis, while the same digested pattern was not observed in the *Stu* I digested's α_1-globin PCR product.

4.3 Vertical Agarose Gel Electrophoresis (VAGE).

According to a new type of gel electrophoresis, VAGE combines the advantage of the horizontal agarose gel electrophoresis and PAGE

altogether. Agarose gel is non toxic, easy to prepare, and has no influence on the migration of DNA caused by their base sequences. Native 3 mm thick-horizontal agarose gel always takes a long time (normally more than 1 hr) for running electrophoresis due to the low electricity applied to the gel system, otherwise the gel would be heated up and lead to the poor resolution of DNA fragments separated on the gel. Vertical agarose gel takes only half the time required for electrophoresis compared with horizontal agarose gel at the same concentration. Also, there is no clinging as reported earlier (Badly *et al.*, 1988). This was very helpful for PCR optimization experiments because the results from gel electrophoresis could be obtained within half an hour after PCR had finished making it possible to perform 3-4 PCRs per day.

The 1.0 mm-thick mini-slab vertical gel requires less than a half of the agarose gel volume per 1 gel compared to 3 mm-thick horizontal agarose gel with 5.0 x 8.0 x 0.3 cm^3. It should be noted that, 100 ml of agarose gel solution was enough for 10-12 mini-slab vertical gel (maximum sample/gel = 9, total = 108 samples) compared with 3-4 of 3 mm-thick horizontal gel (maximum sample/gel = 7, totally = 28 samples)

Furthermore, VAGE could be used as the gel matrix for the purpose of PCR products purification. PCR products could also be

purified by using polyacrylamide as a gel matrix ; however, the recovering yield was low due to its smaller pore size. For the purpose of DNA purification by using agarose gel as the gel matrix, the electrophoresis buffer for this situation was Tris-Acetic acid-EDTA (TAE) (Sambrook *et al.*, 1989). Tris-Boric Acid-EDTA (TBE) buffer's interaction with agarose results in a smaller apparent pore size of agarose gel. This tighter gel reduces the broadering of DNA bands due to dispersion and diffusion and reduces the recovered yield after gel extraction.

There are many possible methods to extract DNA fragments from agarose gel. By using low-melting point agarose as the gel matrix, DNA can be recovered after low-melting point agarose gel is melted and digested with b-agarase. However, low melting point agarose and β-agarase are very expensive. Phenol-chloroform can be used to extract DNA from low-melting point agarose gel, however, the methods was so complicated and also phenol and chloroform were harmful.

Wizard™ PCR Preps DNA Purification system provides a simple procedure for DNA purification from high-melting agarose gel, or directly from PCR products. Typical yields of 70-90% for 500 bp DNA fragments can be obtained by this system. After gel electrophoresis and staining with ethidium bromide staining solution, vertical agarose gel was visualized under long wavelength UV transluminater and the

specific band was excised by using a sterile blade and collected into a microfuge tube. In the staining step, however, only 1 min staining, without destaining step, was recomended. The longer staining time may cause a loss of PCR products from the gel by diffusion due to its low thickness. The recovered yield from VAGE (1 min staining) was nearly the same as that obtained from native 3 mm-thick agarose gel.

The excised gels containing the specific PCR products were mixed with resin supplied with a purification system and heated at 70°C for 10 min or until the gel was completely melted. The DNA were released from the melted agarose gel and bound to the resin and then collected on a filter in the minicolumn. After washing with 80% isopropanol, any salt remaining was removed by simple drawing out the isopropanol and passing it through a syringe barrel attached to the minicolumn. The DNA was eluted from PCR Preps Resin in water or TE buffer pH 8.4 and collected into a microfuge tube with 50 µl final volume by simple centrifuging the minicolumn at 14,000 rpm for 1 min. PCR products purified in this manner was simple and required less than 15 min for processing.

4.4 Chain-termination cycle sequencing of Hb Chiang Mai's α_2- and α_1-globin genes.

Thermostable DNA polymerase enzymes incorporate different bases and base analogues with different efficiencies. For this reason, the relative concentration of each constituent of the termination mix needs to be optimized to facilitate the most efficient sequencing reaction. Although most commercially available kits supply termination mixtures in proportions already optimized for the particular enzyme and base/base analogue components, it may be possible to alter read lengths/signal intensities by changing the relative amounts of dNTPs/ddNTPs, or the concentration of the co-solvent may be added in each termination mixture in any specific case.

However, "Chain-termination Cycle Sequencing" ready reaction mixture supplied by Perkin Elmer (Cetus, USA.) did not facillitate the optimization of any constituents. For 20 µl cycle sequencing reaction mixture, 8 µl of sequencing ready-reaction mixture (5% DMSO, water, sequencing primer, DNA template and water not included) was already fixed. Therefore, this may have reduced the sequencing efficiency for those high GC-content DNA templates.

According to the cycle sequencing reaction mixture described by the instruction manual from the company, 5% DMSO may be added to the reaction mixture to improve the reading length of the products. The

maximum readable length of each template was approximately 600-700 bp. However, by including 5% DMSO into the cycle sequencing reaction mixture as reported in this thesis, the maximum readable length were increased only to 400-500 bp.

From the sequencing data, the two single bases substitution were detected from the electropherogram, one as the equally high peaks and the another as the single peak. The first single base substitution, detected as the equally peak-heigth of G and C nearly at the same position occurred at the nucleotide number 10853 (position was listed according to the sequence from GeneBank, HUMHBA4). This G-->C substitution was located in the exon 2 of Hb Chiang Mai's α_1-globin gene which corresponded to the amino acid substitution of Asp-->His at the residue number 74 ($\alpha_1{}^{74}$ (EF3) $^{Asp\text{-}\text{-}>His}$). This agrees with the study of Sithipreechacharn (1994) that the amino acid substitution which occurred in α-chain of Hb Chiang Mai was an acidic to basidic and caused the separation of the abnormal haemoglobin from the normal Hb A in cellulose acetate gel electrophoresis technique. This finding agrees with the one of haemoglobin variant, called Hb Mahidol (Hb Q-Thailand, ($\alpha_1{}^{74}$(EF3)$^{Asp\text{-}\text{-}>\ His}$), Zeng *et al.*, 1992).

Furthermore, another single base substitution, C-->G, was detected at the nucleotide number 7330 (position was listed according to the sequence from GeneBank, HUMHBA4), located in the exon 3 of Hb

Chiang Mai's α_2-globin gene, which corresponded to the amino acid substitution of His-->Gln at the reisdue number 122 (α_2^{122} (H5) His-->Gln). This was nealy the same charge of amino acid substitution (basidic to basidic) and should cause no change in total negative charge of α-globin chain and therefore could not be detected by using cellulose acetate gel electrophoresis (Fleming *et al.*, 1980). This type of Hemoglobin variant, as reported by Fleming and co-workers (1980), was called Hb Westmead.

4.5 Confirmation of two single base substitution in Hb Chiang Mai's α_2- and α_1-globin genes by using *Alw44* I and *Stu* I.

To confirm these two single base substitutions which occurred in Hb Chiang Mai's α_2- and α_1-globin genes, the specific restriction enzyme, *Stu* I (for Hb Chiang Mai's α_2-globin gene) and *Alw44* I (for Hb Chiang Mai's α_1-globin gene), were used in the restriction enzyme digestion experiments. From the *Alw44* I digestion experiment, which agrees with the sequencing data of Hb Chiang Mai's α_1-globin gene, it was be stated that the single base substitution, G-->C, occurred in exon 2 at the nucleotide number 10853 (position was listed according to the data derived from GeneBank HUMHBA4) which corresponded to the

first nucleotide of the amino acid residue 74 (GAC-->CAC). This caused the amino acid substitution of Asp-->His ($\alpha_1$74(EF3)$^{Asp-->His}$) which was equal to the haemoglobin variant firstly called Hb Q-Thailand (Zeng *et al.*, 1992) or Hb Mahidol (Pootrakul *et al.*, 1972).

Moreover, 785 bp of Hb Chiang Mai's α_2-globin gene that was not digested to 681/104 bp fragments with *Alw44* I, agreed with the sequencing data of Hb Chiang Mai's α_2-globin gene, indicating a second single base substitution. According to the sequencing data of Hb chaing Mai's α_2-globin gene, *Stu* I was allowed to confirm this second single base substitution. It can be stated that, this single base substitution, was occurred at the nucleotide number 7330 (position was listed according to the data derived from GeneBank, HUMHBA4) which corresponded to the third nucleotide of the amino acid residue 122 (CAC-->CAG). This caused the amino acid substitution of His-->Gln ($\alpha_2$122(H5)$^{His-->Gln}$) which was equal to haemoglobin variant initially called Hb Westmead (Fleming *et al.*, 1980). It can be concluded that this study subject occupied the two haemoglobin variants, Hb Mahidol and Hb Westmead.

Hb Mahidol (Q) was reported to localize at a chromosome having a leftward α-globin gene deletion (α-thalassemia-2 leftward deletion - 4.2 kb) (Pagnier *et al.*, 1982 : Pootrakul *et al.*, 1972). As a result of the linkage arrangement between α-chain structural variant alleles which

are localized at chromosomes that also contain an α-thalassemia determinant, individuals expressing these abnormal haemglobins also exhibit an α-thalassemia-2 phenotype. Hb Mahidol, also known as Hb Q-Thailand [$\alpha_1$74(EF3)$^{Asp-->His}$], was also reported by Zeng and co-workers (1992). The Thai patient with Hb Q-Thailand/Hb H disease and his mother were studied at the DNA level, and the gene organization of Hb Q-Thailand in the Thai patient was found to be located on the α_1-gene of chromosome 16 while the -4.2 kb or leftward deletion involving the α_2-gene. According to the sequencing data and the results from restriction enzyme digestions reported in this study, a single point mutation in α_1-globin gene caused the synthesis of the α-globin chain variant of Hb Mahidol in the heterozygous form and the single point mutation in α_2-globin gene caused the synthesis of the α-globin chain variant of Hb Westmead in the homozygous form. It can be hypothesized that α_1-globin gene containing a point mutation caused Hb Mahidol and α_2-globin gene containing a point mutation caused Hb Westmead were localized on the different chromatids of chromosome 16. Then, the genotype of this study subject was [-$_{(-4.2)\alpha}$(Hb Chiang Mai)/$_{\alpha}$(Hb Westmead)$_{\alpha}$].

However, there is no data on the heredity of these two haemoglobin variants from the father to the β-thalassemia-major child,

it can not be concluded that Hb Chiang Mai is actually Hb Mahidol or the new haemoglobin variant caused by two haemoglobin variants, Hb Mahidol and Hb Westmead.

According to the structural variant affected on α_1 and α_2-globin gene, there is no hypermutable "hot spot" that favors an unusual mutation rate. The data from clinical haematologic study was firstly used for further detection by using cycle sequencing and finally confirmed by using specific restriction enzyme digestion. However, according to the higher transcribtion rate of α_2-globin gene is approximately 26% higher than α_1-globin gene, the non-deletion mutation (point mutation) affecting the α_2-globin gene should result in more severe in total α-globin synthesis. Thereby the detection of any point mutations expected from the abnormal haemoglobin recovered from preliminary clinical study such as cellulose acetate gel electrophoresis should first concentrated on α_2-globin gene. By using PCR of specific α-globin gene and then sequencing, the point mutation was easily observed from sequencing data. The specific restriction enzyme digestion was then the confirmation technique that was appropriate for the further study of the frequency of the abnormal haemoglobin in a large population.

Hb Mahidol and Hb Westmead have been studied by many scientific groups (Lorkin and co-workers., 1970 ; Pootrakul and co-

workers., 1972 ; Lie-Injo and co-workers., 1979 ; Zeng and co-workers., 1992 for Hb Mahidol and Jiang and co-workers., 1991 ; Gu and co-workers., 1991 for Hb Westmead). Hb Mahidol is rare in Thais while Hb Westmead is very rare in Chinese (Honig, 1986). However, the data about the frequencies of those two haemoglobin variants in the Thai population was limited. The restriction enzyme digestion of the DNA fragments amplified from specific α-globin gene as reported in this study was easy to perform, the results could be obtained within 1 day, and could be an appropriate technique for the screening of the Thai population.

The inheritance of the mutant α-globin gene and the mutant β-globin gene are independent. However, the severity of β-thalassemia can be significantly modified by the coinheritance of α-thalassemia since the latter will decrease the level of excess α-globin chains. The more markedly α-globin synthesis is decreased by the loss of 1, 2 or 3 α-globin genes, the milder the β-thalassemia became.(Liebhaber, 1989). However, there is no data about the coinheritance of $\alpha_1^{\text{Hb Mahidol}}$ and $\alpha^{-4.2}$ from the study subject to his β-thalassemia-major child. The less clinical severity of β-thalassemia major found in his child according to his heterozygous α-thalassemia-2 (-4.2 kb) carrier was not documented.

A further study to complete the knowledge about Hb Mahidol and Hb Westmead found in this family should concentrate on 1)

confirmation of the link between the deletional α_2-globin gene to the $\alpha_1^{Mahidol}$ in this β-thassemia-major child's father. The detection of this deletional α_2-globin gene by using PCR should be based on the method reported by Baysal and co-workers., 1994. 2) The detection of the coinheritance of $\alpha_1^{Hb\ Mahidol}$ and $\alpha^{-4.2}$ genes from the father to his son. *Alw44* I digestion of α1-globin gene PCR product should be used for the detection of Hb Mahidol and PCR method according to Basal and co-workers., 1994 should be used for detection of the deletional α_2-globin in the β-thalassemia-major child. 3) A study of the frequency of Hb Mahidol and Hb Westmead in the Thai population by using *Alw44* I and *Stu* I digestion of specific α_1- and α_2-globin PCR products (the methods as reported in this study), respectively. And 4) The interaction of α-thalassemia with β-globin structural mutation in the β-thalassemia-major child. If this β-thalassemia-major child carried the $\alpha^{-4.2}$ gene, a study about the level of severity of β-thalassemia compared with other subjects carrying only homozygous β-thalassemia genotype should be done.