CHAPTER 5

DISCUSSION AND CONCLUSION

In this study, the whole-genome amplification by PEP with random 15-mer primers, a PCR amplification with specific primers for detection of the α-thalassemia-1 (SEA) haplotype, and analysis by 3% agarose gel electrophoresis were chosen for diagnosis of homozygous α-thalassemia in preimplantation embryos. Therefore the α-globin genes are contained of %GC rich, then PCR for detection of the α-thalassemia-1 (SEA) haplotype was difficult to performed. The PCR was optimized in a way that all three primers were used in one amplification reaction. The optimization of PCR components : glycerol concentration, annealing temperature, $MgCl_2$ concentration, primer concentration, *Taq* DNA polymerase, and cycle numbers were necessary to produce the specific bands, 314 bp and 188 bp fragments. The standard amounts of DNA template extracted from blood were used for optimization. In amplification of the heterozygote of the α-thalassemia-1 (SEA) haplotype, glycerol help in production of the 314 bp fragment, expected that glycerol improved the separation of double-strand DNA molecules. And also glycerol was increased the intensity of specific bands and reduced the unspecific bands (Cheng and Mitchelson, 1994 ; Nagai *et al.*, 1998), the result was shown in Figure 10. For cheaper testing, in house *Taq* DNA polymerase was used in this experiment and the activity of in house *Taq* DNA polymerase was as good as *Taq* DNA polymerase of another supplier.

The optimal conditions for detection of the α-thalassemia-1 (SEA) haplotype was developed to a ready-to-use PCR Kit and applied to analysis in the prospective screening of expected couples and fetuses (Table 5 and 6). A ready-to-use Kit can save time in preparation of reagents and can be kept for long time. For detection in the prospective screening of expecting couples, out of 3,347 cases 1,064 which had EOF values <60% were amplified. The heterozygotes of the α-thalassemia-1 (SEA) haplotype were found in 260 cases. The percentage is 7.8% (260/3,347) when calculated according to Hardy-Weinberg equilibrium, predicted that there are approximately 2 Hb Bart's hydrops fetalis cases in 1,000 births similar to Kitsirisakul, 1997. All samples should be successfully amplified but 3 of 29 fetuses PCR gave unambiguous results because of these samples were contaminated from maternal DNA which confirmed the results with high-performance liquid chromatography from cordocentesis. The results indicated that these PCR conditions were suitable to detection of the α-thalassemia-1 (SEA) haplotype with high efficiency.

Amplification of the small amount DNA was started in testing the sensitivity of PCR system amplifications which were performed with the serial dilutions of DNA extracts in PCR buffer (Figure 17). PCR of diluted DNAs about 1 cell can find the expected bands, indicated that the PCR was possible to amplify from the little amount of DNA. Although even PCR was optimized extensively a high cycle number because was necessary to guaranty the successful amplification.

Since embryos were available in a very limited amount, amniocytes counted under a microscope were used for finer adjustments of PCR, assuming that the knowledge gained from this 'model' cells can then be applied for experiments with real embryos. Amniocytes were used to optimization of cell lysis conditions and amplification conditions. The thermal, chemical, and proteinase K lysis protocol were tested. DNA extraction by chemical lysis protocol was sensitive to acidity/alkalinity, and difficult to neutralization. While PCR yields of amplification from DNA extracted by proteinase K and Chelex-100 method were not difference. Thus amniocytes were lysis by proteinase K and amplified with PEP method then PCR for detection of the α-thalassemia-1 (SEA) haplotype. This experiment was avoided contamination, PEP and PCR amplifications were carried out in separate laboratories. Cotton filter tips were used and pipetted in hood equipped with an uv-light (Paunio *et al.*, 1996). PCR of the α-thalassemia-1 (SEA) haplotype was failure amplification, the result disagreed to the positive control (Figure 19). For checking efficiency of PEP method, PEP product was analyzed in the STR locus. The result showed the successful amplification. Occurrence of failure amplification may be caused with PEP method did not involved extensive manipulations of the samples. During experiment the amount of DNA is too low to know the size distribution of the PEP products. The molecules must be greater than 314 bp in length, which is the size of the longest PCR product from detection of the α-thalassemia-1 (SEA) haplotype. Increasing the denaturation to 96°C during the first few cycles of amplification may be

solved occurrence of failure amplification because of the most likely cause for failure of a PCR is incomplete denaturation of the target template. Higher temperatures may be appropriate, especially for %GC rich targets (Innis et al., 1990 ; Ray and Handyside, 1996).

Although amplification of amniocytes were not successful. But this experiment tries to detect the α-thalassemia-1 (SEA) haplotype from embryos. These embryos were not investigated the molecular genetic background before, thence identification of PCR result can not be done. Various numbers of embryos were performed with the same method for PEP and PCR amplification. The results were not still successful, no PCR signals. Unless in above-mentioned, the nested PCR was tested to amplify the α-thalassemia-1 (SEA) haplotype. Primer A, B, and C were used as the outer primers, while the inner primers were newly designed. All samples were positive to heterozygotes, indicating that was contaminating DNA and failure amplification.

Inconclusion, the method for detection of the α-thalassemia-1 (SEA) haplotype from embryos in this study is too difficult because much more step (PEP method and PCR amplification) in the reaction and have to be careful about DNA contamination, allele dropout, and failure amplification. In the further study, adjustment of the condition for diagnosis of homozygous α-thalassemia should be done, and increasing denaturation temperature to 96°C may be avoided these problem. Although the optimized PCR conditions for detection of the α-thalassemia-1 (SEA) haplotype can

not use to analysis of PEP product but it can be applied to analyze in the prospective screening of expecting couples and for prenatal diagnosis.