# CHAPTER 4
# DISCUSSION

Thalassemia and hemoglobinopathies are the life-threatening globin gene disorders highly endemic throughout Thailand. To avoid emergence of new patients, prevention and control are required. One important strategy in this approach is an identification of the carriers. The carrier is, however, one who carries abnormal globin gene in heterozygous state without clinical manifestation. To identify the carriers, 2 major consecutive steps comprising an initial screen followed by the diagnostic confirmation have been routinely employed in Thailand and most developing countries.

The initial screening methodologies including OFT or MCV combined with the HbE screening tests such as DCIP tests or the AMS-HbE Tube test provide only preliminary information (Fucharoen and Winichagoon 2011, Tatu and Kasinrerk 2011, Wasi 1983, Weatherall and Clegg 2001). Those positive for the initial tests are usually subjected for Hb identification; i.e. to determine $HbA_2$/E levels for identifying the β-thalassemia and HbE carriers. Employing these two laboratory categories, one cannot determine types of α- and β-globin gene mutations which absolutely require the DNA analysis for identification.

Although several multiplex PCR protocols for α- and β-thalassemia diagnosis were introduced (Chong, *et al* 2000, Eng, *et al* 2000, Liu, *et al* 2000, Tan, *et al* 2001, Wang, *et al* 2003), this thesis established the in-house multiplex allele-specific PCR protocols with the ultimate aim to simplify the diagnostic strategy of thalassemia carriers that are common in Thailand. This development began using the PCR systems that were previously set up in Dr. Thanusak's laboratory (Sritong, *et al* 2004, Suwannasin 2009, Tatu, *et al* 2012). Those established PCR techniques were however mostly of singleplex forms and did not cover the common types of thalassemia/hemoglobinopathies in Thailand. It hence would be worthwhile for multiplexing those PCR primers in order to simultaneously identify either α- or β-globin gene defects. Based on this notion, this thesis re-designed the PCR protocols by multiplexing the α-globin specific primers together and also by multiplexing the β-globin specific primers together. Then, two in-house multiplex allele-specific PCR protocols were proposed to be developed in this thesis; i.e. that for the α-thalassemia/hemoglobinopathies and that for the β-thalassemia/hemoglobinopathies.

In case of the multiplex allele-specific PCR for α-thalassemia/hemoglobinopahies, the singleplex PCR for SEA-α thalassemia 1, α-thalassemia 2 (3.7-kb deletion) and HbCS routinely employed in Dr. Thanusak's laboratory were proposed to be multiplexed. The singleplex PCR for SEA-α thalassemia was previously developed in this laboratory (Suwannasin 2009, Tatu, *et al* 2012). The singleplex PCR protocol for α-thalassemia 2 (3.7-kb deletion) and HbCS was described elsewhere (Fucharoen, *et al* 2002, Fucharoen, *et al* 2003). These PCR protocols were, however, modified and re-optimized in this

thesis. One modification was the addition of internal control primers. Since only mutant primers were utilized in these modified singleplex PCR reactions, the additional internal control primers were required. The primers that were designed to generate the internal control products were αG-17 and C3. The αG-17, C3 primers were initially designed for using in PCR protocol to identify the HbCS allele (Fucharoen, *et al* 2003). Addition of the αG-17 and C3 primers in the singleplex PCR reactions created 391-bp amplified products spanning the α2-globjn gene to promoter region of the α1-globin gene (nt 163367-163757 of AE006462) (Figure 8A). Thus, presence of this 391-bp fragment would indicate success of the PCR reaction as well as the heterozygous state of the deletional α-thalassemia being tested. Having been optimized, the αG-17 and C3 primers were then added into the singleplex PCR reaction for either SEA-α thalassemia 1 or α-thalassemia 2 (3.7-kb deletion) or HbCS, the PCR conditions of which were also subjected for re-optimization.

As expected, the efficiencies of the established singleplex PCR were reduced after fortification of the internal control primers. Thus, annealing temperature, $MgCl_2$ concentration were re-determined with the major criteria that the 391-bp control and α-globin mutation specific products must be efficiently produced. The results of re-optimzation was finally promising and the development of multiplex PCR was encouraged. It was concluded in this stage that before combining the primer, efficiencies should be tested and re-optimization should be carried out as the original singleplex PCR condition certainly altered. This conclusion was in the same way as those described by

other investigators (Dieffenbach and Dveksler 1995, Edwards and Gibbs 1994, Elnifro, *et al* 2000, Radstrom, *et al* 2004, Shuber, *et al* 1995).

Development of the multiplex allele-specific PCR for α-thalassemia/hemoglobinopathies started in the singleplex PCR condition optimized for detecting the α-thalassemia 2 (3.7 kb deletion) and gradually optimized after additions of other primers. As also expected, addition of other primers led to declined amplification efficiencies of this multiplex PCR. Thus, concentration of α-thalassema specific primers as well as numbers of thermal cycles were re-adjusted while $MgCl_2$ concentration remained the same as that used in the singleplex PCR. The re-adjustment of the PCR condition provided satisfactory outcomes. This prompted concluding that re-optimization was always needed in establishment of multiplex PCR and that conditions to be optimized were obviously variable for different protocols. This notion was also true in other platforms described by other workers (Chong, *et al* 2000, Fucharoen, *et al* 2003, Fucharoen, *et al* 2007, Lin, *et al* 1991, Liu, *et al* 2000, Panyasai, *et al* 2004, Siriratmanawong, *et al* 2001, Suwannasin 2009, Wang, *et al* 2003). Interestingly, a 280-bp amplified product was always produced in this multiplex allele-specific PCR. In fact, αG-17 primer has another binding site within α1 globin gene. Thus, it should be able to generate amplified product with the Alpha 3.7B shown in Figure 7(A). Having aligned these 2 primers in AE006462 reference sequence, the 280-bp fragment was revealed and it should then be considered another internal control.

For β-thalassemia/hemoglobinopathies, the multiplex allele-specific PCR was modified from that routinely used in Dr. Thanusak's laboratory. The modifications included addition of M-28M1 and internal control primers (αG-17 and C3) followed by re-optimization of the β-globin gene specific primers as well as re-adjustment of numbers of thermal cycles. Since the multiplex allele-specific PCR for β-globin gene defects previously established in this laboratory was intended to detect only $\beta^{41/42(-TTCT)}$, $\beta^{17(A-T)}$ and $\beta^{E}$ in the carriers and only mutant primers were used, it was then hard to determine success or failure of the reaction in those having no mutations being tested. Fortification of the αG-17 and C3 primers which are specific for α-globin cluter overcame this problem. Moreover, M-28M1 was also added in this multiplex allele-specific PCR in order to extent ability of the developed multiplex allele-specific PCR to detect the A-G substitution in nucleotide -28 relative to Cap site on β-globin promoter region ($\beta^{-28(A-G)}$). The $\beta^{-28(A-G)}$ mutation is fairly common in Thailand, accounting for 5.0% of the population (Fucharoen and Winichagoon 1997, Nopparatana, *et al* 1995). It affects transcriptional efficiency and leads to only mild phenotype of the β-thalassemia. Compound heterozygote of this mutation and other β-globin gene defects such as $\beta^{E}$ could lead to mild clinical manifestation (Cao and Galanello 2010, Galanello and Origa 2010, Lebnak, *et al* 2005, Luawsombut 1998, Steinberg, *et al* 2001, Weatherall and Clegg 2001). Thus, identifying this mild mutation should be done in all diagnosed as being carrier of the β-thalassemia/hemoglobinopathies. This was particular important in married couples at high risk for HbE/β-thalassemia since it has been shown that individual compound heterozygous of HbE and $\beta^{-28(A-G)}$ always presented with a mild

clinical phenotype. Thus, those couples possessing these particular β-globin mutations have no risk of terminating pregmancies.

Addition of the αG-17, C3 and M-28M1 altered the conditions of the multiplex allele-specific PCR previously optimized and in routine use in this laboratory. This prompted re-titration of the "beta 17 multiplex" and "beta 41/42 multiplex" primers. However, after optimal primer concentrations were met, non-specific amplified fragments were still evident and mis-interpretation was likely. Numbers of thermal cycles were thought to be the cause as it has been shown that numbers of thermal cycles could lead to non-specific amplification (Dieffenbach and Dveksler 1995, Edwards and Gibbs 1994, Elnifro, *et al* 2000, Radstrom, *et al* 2004, Shuber, *et al* 1995). Fortunately, reduction of the numbers of thermal cycles greatly reduced non-specific bands and no further optimizations were required. It was also concluded in this stage that re-optimization was needed in establishment of the multiplex allele-specific PCR with no straightforward optimization criterions.

Efficiencies of the developed in-house α- and β-multiplex allele-specific PCR were evaluated in unknown blood samples following the main rationale whether this PCR technique could be performed directly after the initial carrier screen. Based on this concept, only blood samples with high probability of being the thalassemia carriers (i.e. MCV < 80 fL and MCH < 27 pg) were recruited. The results clearly showed that these PCR protocols could accurately identify the α- and β-globin mutations and common globin gene mutations (in both single and double heterozygous states) were identified in

majority of the samples. This indicated that these developed in-house multiplex allele-specific PCR was effective in genotyping the common thalassemia/hemoglobinopathies in Thailand. This also indicated that it was possible to carry out this PCR analysis directly after the MCV or MCH determinations without the need for Hb identification. This finding confirmed the notions raised previously by other investigators (Clarke and Higgins 2000, Flint, *et al* 1998, Fucharoen, *et al* 2004, Pornphannukool, *et al* 2008, Sanguansermsri, *et al* 1998, Sirinawin, *et al* 2004, Tujinda, *et al* 2010, Weatherall and Clegg 2001). This finding also highlighted the possibility to establish this strategy in other populations in which specific globin gene mutations are well characterized.

This thesis also developed the whole blood PCR protocols for identifying the globin gene mutations. The aim of this development was to simplify the PCR protocol by skipping the DNA purification step prior to the PCR reaction. However, several aspects have to be considered such as means of releasing genomic DNA from leukocytes and means of removing the PCR inhibitor that might exist in blood. Mercier, B. *et al* and Burckhard, J. *et al* were successful in releasing the genomic DNA from the leukocytes using high temperature (Burckhardt 1994). Thus, the so-called "heat-cool" step was introduced in this developed whole blood PCR protocol. However, prolonged exposure to high temperature inserted fatal effect on DNA polymerase; i.e. DNA polymerase might loss its activity quickly (Dieffenbach and Dveksler 1995, Radstrom, *et al* 2004). This was the reason while the "heat-cool" technique was employed. Repeated heat-cool procedure was finally found to be sufficient for liberating the DNA from nucleus of white blood cells. Interestingly, it was noticed that only 3 repeated "heat-cool" steps were enough for

aged blood lysate, whereas 10 were required for the fresh and intact blood. It was postulated that leukocytes existing in the aged blood lysate were already or almost dead as a result of nutrient shortage. These leukocytes were then easily disrupted by high temperature. In fresh blood, however, the nutrient was still sufficient to maintain the energy within the cells and cellular integrity then stabilizes. More heat-cool cycles were thus needed to destroy leukocytes in fresh blood.

Heme liberated from hemoglobin has been proven to be potent PCR inhibitor. Its inhibitory action was direct inactivation of the thermostable DNA polymerase (Al-Soud and Radstrom 2000, Al-Soud and Radstrom 2001). This is the reason why the traditional PCR reaction was not possible using whole blood directly added into the reaction. Kreader, C.A. *et al* and Abu Al-Sold, W.A. *et al* demonstrated that addition of BSA or betaine to reaction mixtures significantly reduced the inhibitory effect of blood (Al-Soud and Radstrom 2001, Kreader 1996). Thus, BSA and betaine were tested in this developed protocol and, finally, betaine was shown to be the best PCR facilitator in the presence of whole blood. Betaine structurally is a polyglycine compound (N, N, N-trimethylglycine) (Frackman, *et al* 1998, Rees, *et al* 1993). It has been utilized as the enhancer in traditional purified DNA-based PCR reaction due to its ability to reduce formation of secondary structure in GC-rich regions (Frackman, *et al* 1998, Musso, *et al* 2006, Radstrom, *et al* 2004, Santoro, *et al* 1992). In whole blood PCR, however, betaine may absorp heme, hence, preventing its interaction with DNA polymerase (Al-Soud and Radstrom 2001). This mechanism was similar to that of BSA and other proteins such as gp32 (Al-Soud and Radstrom 2000, Al-Soud and Radstrom 2001, Kreader 1996, Radstrom, *et al* 2004).

The power of betaine was in part dependent on quantity of whole blood added into the reaction. Large amount of blood would limit the facilitating effect of the betaine. Thus, volume of the whole blood used in the reaction must be optimized and 0.5-2 μl whole blood was found to be optimal. However 2-μl blood was used in this developed whole blood PCR protocol. This was based on the fact that this amount of blood ensured enough DNA for amplification and contained heme group that was completely removed by the betaine.

The developed in-house whole blood PCR was applied for identifying the globin gene mutations in the singeplex Gap-PCR for SEA α-thalassemia 1, singleplex allele-specific PCR for HbCS and developed multiplex allele-specific PCR for β-thalassemia described recently in the Chapter II. After slight protocol adjustments, the globin gene specific PCR reactions were able to identify globing gene defects in whole blood samples without the need for prior DNA isolation. Although whole blood PCR has been applied in several other fields (Al-Soud and Radstrom 2000, Al-Soud and Radstrom 2001, Burckhardt 1994, Kreader 1996, McCusker, *et al* 1992, Mercier, *et al* 1990, Yang, *et al* 2007), this was the first whole blood PCR for identifying human globin gene mutations utilizing both aged blood lysate and fresh whole blood with no prior whole blood treatment at all. Thus, due to its simplicity, this newly developed PCR procedure was then suitable for detecting the globin gene mutations in large sample numbers where between-sample contamination is prone if DNA purification is still performed. Moreover, owing to its ease and swiftness, this whole blood PCR would proudly be served as the front-line test in thalassemia/hemoglobinopathy diagnosis.