Development of new technological applications for post- and prenatal diagnosis of the hemoglobinopathies

Phylipsen, M.

Chapter 2

Improving postnatal diagnostics for hemoglobinopathies

Chapter 2.1

A single-tube multiplex gap-PCR for the detection of eight β-globin gene cluster deletions common in Southeast Asia

Tritipsombut J, Phylipsen M, Viprakasit V, Chalaow N, Sanchaisuriya K, Giordano PC, Fucharoen S, Harteveld CL

Hemoglobin. 2012;36(6):571-80

Abstract

Up to now, more than 200 different β-thalassemia (β-thal) mutations have been characterized. The majority are point mutations causing expression defects. Only approximately 10.0% of the defects are caused by large deletions involving the β-globin gene cluster causing β^0-thal, $(\delta\beta)^0$-thal, $^G\gamma(^A\gamma\delta\beta)^0$-thal and other conditions with or without persistence of fetal hemoglobin (Hb). For the prevention of severe forms of β-thal intermedia and β-thal major, it is important to identify carriers of point mutations as well as carriers of deletions.

β-Thalassemia and related disorders are most commonly present among populations from all Mediterranean countries as well as Southeast Asia, India, Africa, Central America and the Middle East. Twelve relatively frequently occurring deletion types have been described involving the β-globin gene cluster. These include the 105 bp β^0-thal deletion, the 619 bp deletion, the 3.5 kb deletion, the Southeast Asian (SEA) deletion, the Filipino deletion, Hb Lepore, the Thai $(\delta\beta)^0$-thal, the Siriraj I $^G\gamma(^A\gamma\delta\beta)^0$-thal, the Chinese $^G\gamma(^A\gamma\delta\beta)^0$-thal, the Asian Indian deletion-inversion $^G\gamma(^A\gamma\delta\beta)^0$-thal as well as the (hereditary persistence of fetal hemoglobin) HPFH-6 and HPFH-7 deletions.

To improve the rapid detection of the eight common β-globin cluster deletions in Southeast Asian countries, a simple molecular technique based on a single-tube multiplex gap-polymerase chain reaction (PCR) has been developed in this study. This technique provides a fast, simple and cost effective diagnostic test for deletion-types of β-thal that can be applied in every molecular diagnostic laboratory having standard PCR equipment.

Introduction

β-Thalassemia (β-thal) is a group of disorders resulting from a reduced or absent synthesis of β-globin chains [1]. In human, the β-globin gene cluster is approximately 50,000 base pairs long and consists of five functional genes (ε, $^A\gamma$, $^G\gamma$, δ and β) and one pseudogene (ψβ) on the short arm of chromosome 11 (11p15.5). The expression of these genes is regulated by the locus control region (LCR) that lies 20 kb upstream of the ε-globin gene. As normal individuals have two allelic β-globin genes, β-thal may exist in a heterozygous or homozygous state [2].

The World Health Organization has estimated that almost 70,000 infants with β-thal are born worldwide each year and 270 million people are carriers [3]. β-Thalassemia and related disorders are commonly present among populations in all Mediterranean countries as well as in Southeast Asia, India, Africa, Central America and the Middle East [1]. However, because of migration, the carrier rate of β-thal is increasing in countries that previously had low prevalence.

For the prevention of β-thal intermedia and β-thal major in a population, carriers of β-globin gene defects and couples at risk should be identified and referred to a genetic center for counseling, preferably before the first pregnancy. The most common reason for referral for couples at risk in Thailand is if both parents are carrier of Hb E [β26(B8)Glu>Lys, GAG>AAG] and β-thal or, less frequently, both are carrier of β-thal.

Up to the present time, more than 200 different β-thal [4] mutations have been characterized. Most of them result from point mutations causing a reduced expression of the β-globin gene [5]. Alternatively, deletions may involve the β-globin gene or regulatory elements, preventing the allelic expression of β-globin. Only approximately 10.0% are large deletions involving the β-globin gene cluster causing β^0-thal, $(\delta\beta)^0$-thal, $^G\gamma(^A\gamma\delta\beta)^0$-thal and other forms of hereditary persistence of fetal hemoglobin (Hb) (HPFH). Since these deletions can lead to mild,

intermediate or severe diseases in combination with a β-thal point mutation, it is important to include deletional types of β-thal in the molecular screening for couples at risk.

In Southeast Asian countries, approximately 12 types of deletions in the β-globin gene cluster have been described. They include the β^0-thal 105 bp deletion, the 619 bp deletion, the 3.5 kb deletion, the novel $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal; Siriraj I deletion (~118 kb) (Nipon Chalaow & Vip Viprakasit; unpublished data 2012), the Southeast Asian (SEA) deletion (~27 kb), the Filipino deletion (~45 kb), Hb Lepore (~7.4 kb deletion), the Thai $(\delta\beta)^0$-thal deletion (~12.5 kb), the Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal deletion (~100 kb) and the Asian Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^0$-thal, as well as HPFH-6 and Vietnamese HPFH (known as HPFH-7 in HbVar) deletions. Their deletion breakpoints have been presented in Table 1 [3,4-24].

A number of polymerase chain reaction (PCR)-based techniques such as restriction endonuclease (RE), Southern blotting and multiplex ligation-dependent probe amplification (MLPA) have been applied for characterization of rare and unknown β-globin gene cluster deletions. However, the most widely technique used is gap-PCR that uses only two specific primers complementary to the sense and antisense strand in the DNA regions flanking the deletion. The importance of enzymatic amplification of deletions causing $\delta\beta)^0$-thal or HPFH was already demonstrated back in 1994 by Craig et al. [25] to facilitate the molecular diagnosis. Nine different deletions including a subset of Mediterranean, African and Asian deletions were performed in single-plex. To facilitate the use of gap-PCR in a diagnostic setting, multiplex-PCR assays containing a mixture of primers on each side of the breakpoints were designed for the detection of common thalassemia deletions in the α- and β-globin gene clusters [26-28].

To improve rapid diagnostics for detection of eight β-globin cluster deletions in Southeast Asian countries, a simple molecular technique based on a single-tube multiplex gap-PCR assay has been developed in this study. This technique provides a fast, simple and cost effective diagnostic test for β-thal and deletional types of HPFH which can be applied in every molecular diagnostic laboratory equipped with standard PCR apparatus.

Materials and methods

Details of all primers in the single-tube multiplex gap-PCR for the detection of eight β-globin gene cluster deletions in Southeast Asian countries are shown in Table 2 and Figure 1. The PCR reaction was performed in a total volume of 50 microliters (μL) and this multiplex PC reaction took about 2 hours 30 min. Each PC reaction contained 0.2 mM of each dNTPs, 1.2 mM $MgCl_2$, 1 unit of GoTaq DNA polymerase in supplied reaction buffer (Promega, Madison, WI, US), 100 ng of genomic DNA and 0.08-1 pmole of 15 different primers (Table 2).

The PCRs were performed on a Biometra TProfessional Thermocycler (Biometra, Göttingen, Germany). After an initial denaturation at 94°C for 1 min., 27 cycles with template denaturation at 94°C for 30 seconds, primer annealing at 62°C for 2 min. and 59°C for 1 min., DNA extension at 72°C for 1 min. and a final extension at 72°C for 10 min., were performed. Eight microliters of each amplified product was analyzed by electrophoresis on 1.2% agarose gel in 1X Tris Borate-EDTA (TBE) buffer at 120 volts for 3 hours After staining with ethidium bromide, the agarose gel was visualized under UV-light.

Among the 12 deletions described in Southeast Asian countries, eight deletions with available genomic DNA were selected for the development of the gap-PCR technology. Genomic DNA of carriers for the eight different β-thal deletions previously characterized by MLPA, by

Disorders	Types [References]	Locations on NCBI_ AC104389.8		Distribution
		5' Deletion breakpoint	3' Deletion breakpoint	
β^0-thalassemia	105 bp deletion [6]	29,436	29,331	Thailand
	619 bp deletion [7]	28,214	27,595	India Pakistan Thailand
	3.5 kb deletion [8,9]	29,539-30,221	26,128-26,881	Thailand
	SEA deletion (~27 kb deletion)	31,394	3,982	Cambodia Vietnam China
	Filipino deletion [11,12]	33,691-33,699	184,733-184,743[a]	Philippines Malaysia Indonesia
$\delta\beta$ hybrid	Hb Lepore [13-17]	36,324-36,666	28,910-29,254	Mediterranean Brazil Thailand
$(\delta\beta)^0$-thalassemia	Thai (~12.5 kb deletion) [18]	35,432-35,620	23,090	Thailand Laos PDR [21]
$^G\gamma(^A\gamma\delta\beta)^0$-thalassemia	Asian Indian deletion-inversion [20,21]	51,802	27,826	India Bangladesh Kuwait Thailand
	Chinese [22]	51,161	127,698[b]	Southern China
	Siriraj I (~118 kb deletion)	52,507	165,744[c]	Thailand
HPFH	HPFH-6 [23]	54,361	124,872[d]	Thailand
	HPFH-7 [24]	33,468	4,799	Vietnam

Table 1 Deletion breakpoints of β-globin gene cluster found in Southeast Asian countries [4-24]. [a]Data from HbVar ID 989 (HGVS name NG_000007.3), [b]Data from HbVar ID 1046 (HGVS name NG_000007.3), [c]Characterized by MLPA and direct sequence analysis, [d]Data from HbVar ID 1048 (HGVS name NG_000007.3).

Southern blotting or direct sequence analysis of breakpoint fragments were used as positive controls for optimizing the conditions of multiplex gap-PCR. In first instance, due to a limited availability of DNA of patients to be used as positive control for the detection of the Siriraj I $^G\gamma(^A\gamma\delta\beta)^0$-thal and the Southeast Asian β^0-thal deletions, reamplified PCR products were used to set up conditions together with genomic DNA of the other six rearrangements. To reduce the presence of non-specific background and primer dimers, the first PCR amplification was used at

a dilution of 1:1,000,000. To rule out amplification artifacts or failure as a consequence of PCR products used as positive controls, the experiment was repeated on genomic DNA for all eight deletions as positive controls, which confirmed the previously obtained results.

Results

A single-tube multiplex gap-PCR for detection of eight β-globin gene cluster deletions in Southeast Asian countries has been successfully optimized in this study. In this multiplex PCR system, the 304 and 1,889 bp fragments serve as an internal control of the PCR reaction. The 620, 688, 743, 836, 974, 1159, 1270 and 1447 bp fragments indicate the presence of the $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal Siriraj I deletion, β^{0}-thal 3.5 kb deletion, Filipino β^{0}-thal, Southeast Asia (SEA) β^{0}-thal, HPFH-6, Hb Lepore, β^{0}-thal 619 bp deletion and Thai $(\delta\beta)^{0}$-thal, respectively (Table 2). The first and last lanes in Figure 2 contain a 100 bp ladder as a size standard.

Discussion

Literature for Thai $(\delta\beta)^{0}$-thal and Laotian $(\delta\beta)^{0}$-thal has been reviewed in this study [21, 22]. The results confirm that both deletional types of $(\delta\beta)^{0}$-thal are most likely the same as the deletion size and breakpoints are similar. The 5' breakpoint is located in the second intervening sequence (IVS-II) of the δ-globin gene and the 3' breakpoint lies within the L1 repetitive sequences at 4.7 kb 3'of the β-globin gene. As shown in Table 1, the deletion is approximately 12.5 kb in length.

In general, the profile of the PCR has three steps including template denaturation, primer annealing and DNA extension steps. In order to get a clearly positive band for Hb Lepore, the extra annealing step at 62^{0}C for 2 min. was added to the PCR program. This step is to support the binding of the Hb Lepore primers before the 619 bp primers, because of the higher melting temperature (Tm) of the Lepore primer pair as shown in Table 2. The double-step annealing multiplex PCR has been shown to work well with the high Tm primers [29].

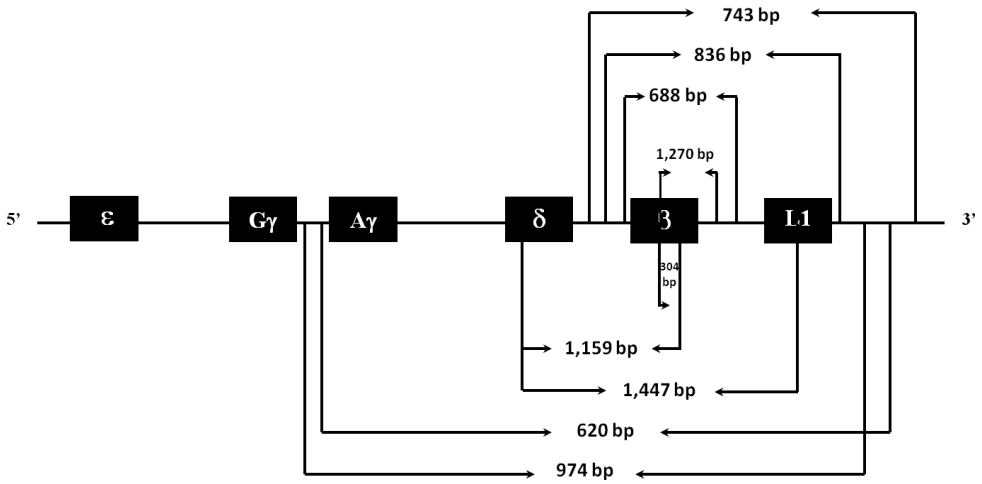

Figure 1 Schematic representation of the β-globin gene cluster, indicating the extent of the eight deletions and relative positions of the primer pairs.

Disorders	Primer name	Primer sequence (5'→3')	Primer concentration (μM)	Tm (°C)	Product size (bp)
$^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal (Siriraj I deletion)	Siriraj-Fw Siriraj-Rev	GGTCACGAATTTGCTTGGTC GCGCTGAAACTGTGGCTTTA	0.08 0.08	60.50 61.46	620
β^{0}-thal (3.5 kb deletion)	3.5 kb-Fw 3.5 kb-Rev	CAGTCACGATGCTGTACATTAGA TGCAGATTAGTCCAGGCAGA	0.16 0.08	58.00 59.55	688
Filipino β^{0}-thal (~45 kb deletion)	Filipino-Fw Filipino-Rev	CCTTGAAGCTGGGTAGTGTGA GCAGAGCTACTCAGGGCATT	0.16 0.16	60.30 59.60	743
SEA β^{0}-thal (~27 kb deletion)	SEA-Fw SEA-Rev	TGCTTAGACATTTTCCCAAGG GGTGACAATTTCTGCCAATCA	0.4 0.4	59.21 60.89	836
HPFH-6	HPFH 6-Fw HPFH 6-Rev	CAGGATGGGGCTCAGAAATA AAGGAAATGAGCCAGCAGAA	1.0 1.0	60.03 59.96	974
Hb Lepore	Lepore-Fw Lepore-Rev	TGGTGCAAAGAGGCATGATA GGAGGACAGGACCAGCATAA	0.2 0.8	60.22 60.07	1,159
β^{0}-thal (619 bp deletion)	619 bp-Fw 619 bp-Rev	TTATGGTGTAAGACAAGGGTCTGA CACAGTCTGCCTAGTACAT	0.8 0.4	59.93 48.03	1,270
Thai $(\delta\beta)^{0}$- thal (~12.5 kb deletion)	Thai-Fw Lepore-Rev	TTCTCCCCATCACTTTCAGC GGAGGACAGGACCAGCATAA	0.16 0.8	60.20 60.07	1,447
Internal control	Lepore-Fw 619 bp-Rev	TGGTGCAAAGAGGCATGATA CACAGTCTGCCTAGTACAT	0.2 0.4	60.22 48.03	304
	619 bp-Fw 619 bp-Rev	TTATGGTGTAAGACAAGGGTCTGA CACAGTCTGCCTAGTACAT	0.8 0.4	59.93 48.03	1,889

Table 2 Primer details in a single-tube multiplex gap-PCR for detection of the eight β-globin gene cluster deletions in Southeast Asian countries (Tm is the melting temperature of each primer)

Several types of Hb Lepore have been described based on their deletion breakpoints. They include Hb Lepore-Hollandia (δ22Ala/β50Thr), Hb Lepore-Baltimore (δ50Ser/β86Ala or δ68Leu/β84Thr or δ59Lys/β86Ala) and Hb Lepore-Boston-Washington (δ87GIn/βVSII-8 or δ87GIn/β116His) [32-34]. The primer pair selected to amplify a fragment of 1159 bp suits all types of Hb Lepore. As three types are not clinically different, further direct genomic sequencing of the Hb Lepore deletion breakpoint will only be necessary for further anthropological studies to identify the origin of different subtypes of Hb Lepore. Practically, our simple multiplex-PCR should be sufficient for routine laboratory diagnosis for thalassaemia and Hb variants.

Even though PCR amplified DNA was used as positive control for the Siriraj I $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal and the Southeast Asia β^{0}-thal deletions for the optimization of PCR conditions, the validation of the multiplex PCR had been successfully performed using genomic DNA as positive control for all 8 mutations. No DNA samples were available to be used as positive controls for the rare 105 bp β^{0}-thal deletion, the Vietnamese HPFH-7 deletion, the Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal and Asian

Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal. By adding four more set of primers to the mix (primer sequences and primer concentrations available on request), no interference of amplified PCR fragments from other known deletions was observed. However, the robustness and efficiency of these added primers on detecting four rare deletions remain elusive and warranted further analysis when these rare deletional control DNA samples become available.

So far, the actual prevalence of these mutations in the population is unknown. However, the 105 bp β^{0}-thal deletion, the 3.5 kb deletion, the Filipino deletion, the Thai $(\delta\beta)^{0}$-thal, HPFH-6 and Hb Lepore have been reported occasionally in Thailand and other Southeast Asian populations [4,6,9,30-34]. Siriraj I $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thal is a novel mutation. It has been found in two unrelated families from the southern part of Thailand, one from Hat Yai and another from Phuket (unpublished data). It can be assumed that there may be more if the detection method is available. The multiplex gap-PCR developed in this study is simple enough for any molecular laboratory to apply in routine practice.

In conclusion, a simple molecular technique based on a single-tube multiplex gap-PCR for the detection of eight β-globin cluster deletions in Southeast Asian countries has been successfully developed in this study. A single-tube multiplex gap-PCR will help diagnostic laboratories to perform diagnostics on β-thal quickly and accurately, using the presently described multiplex PCR assay. This screening assay is widely applicable as it only requires a PCR machine, which is available in most clinical molecular laboratories screening for hemoglobinopathies in Asia where the incidence of β-thal is high.

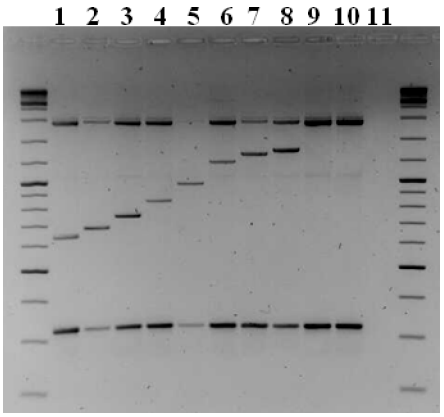

Figure 2 Representative 1.2% agarose gel electrophoresis of the PCR product. Lane 1: positive for $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia (Siriraj I deletion); lane 2: positive for β^{0}-thalassemia (3.5 kb deletion); lane 3: positive forβ^{0}-thalassemia (Filipino deletion); lane 4: positive for β^{0}-thalassemia (SEA deletion); lane 5: positive for HPFH-6;lane 6: positive for Hb Lepore; lane 7: positive for β^{0}-thalassemia (619 bp deletion); lane 8: positive for Thai $(\delta\beta)^{0}$-thalassemia; lanes 9 and 10: negative control; lane 11: blank.

Acknowledgements

We thank all the staff at the Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands for participation in this study.

Declaration of Interest

This work was supported by grants from the Office of the Higher Education Commission [the National Research University (NRU) Project, Khon Kaen University, Khon Kaen, Thailand]. J. Tritipsombut was supported by the CHE-PHD-Scholarship program of the Office of the Higher Education Commission, Ministry of Education, Thailand. V. Viprakasit and N. Chalaow were supported by the NRU Grant to Mahidol University, Mahidol, Thailand. The authors alone are responsible for the content and writing of this article.

References

1. Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th ed. Oxford, England: Blackwell Science, 2001.

2. Higgs DR. Gene regulation in hemopoiesis: New lessons from thalassemia. Hematology Am Soc Hematol Educ Program 2004; 1: 1-13.

3. Modell B, Khan M, Darlison M. A national register for surveillance of inherited disorders: beta thalassemia in the United Kingdom. Bull World Health Organ 2001; 79: 1006-13.

4. Patrinos GP, Giardine B, Riemer C, Miller W, Chui DH, Anagnou NP, et al. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies. Nucleic Acids Res 2004; 32: D537-41.

5. Old JM. Screening and genetic diagnosis of haemoglobin disorders. Blood 2003; 17: 43-55.

6. Nopparatana C, Panich V, Saechan V, Sriroongrueng V, Nopparatana C, Rungjeadpha J, et al. The spectrum of beta-thalassemia mutations in southern Thailand. Southeast Asian J Trop Med Public Health 1995; 26: 229-34.

7. Pritchard CC, Tait JF, Buller-Burckle AM, Mikula M. Annotation error of a common β^0-thalassemia mutation (619 bp-deletion) has implications for molecular diagnosis. Am J Hematol 2010; 85: 978.

8. Lynch JR, Brown JM, Best S, Jennings MW, Weatherall DJ. Characterization of the breakpoint of a 3.5 kb deletion of the beta globin gene. Genomics 1991; 10:509-11.

9. Sanguansermsri T, Pape M, Laig M, Hundrieser J, Flatz G. Beta zero-thalassemia in a Thai family is caused by a 3.4 kb deletion including the entire beta-globin gene. Hemoglobin 1990; 14: 157-68.

10. Dimovski AJ, Divoky V, Adekile AD, Baysal E, Wilson JB, Prior JF, et al. A novel deletion of approximately 27 kb including the beta-globin gene and the locus control region 3'HS-1 regulatory sequence: beta zero- thalassemia or hereditary persistence of fetal hemoglobin? Blood 1994; 83: 822-7.

11. Waye JS, Eng B, Hunt JA, Chui DHK. Filipino β-thalassemia due to a large deletion: identification of the deletion endpoints and polymerase chain reaction (PCR)-based diagnosis. Hum Genet 1994; 94: 530-2.

12. Motum PI, Kearney A, Hamilton TJ, Trent RJ. Filipino beta zero thalassaemia: a high Hb A2 beta zero thalassaemia resulting from a large deletion of the 5' beta globin gene region. J Med Genet 1993; 30: 240-4.

13. Waye JS, Eng B, Patterson M, Chui DH, Chang LS, Cogionis B, et al. Hb E/Hb Lepore-Hollandia in a family from Bangladesh. Am J Hematol 1994; 47: 262-5.

14. Villegas A, Espinós D, Alvarez-Sala JL, Calero F, Valverde F, Robb L, et al. Haemoglobin Lepore-Baltimore in a Spanish family. Acta Haematol 1983; 69: 192-4.

15. Ribeiro ML, Cunha E, Gonçalves P, Martin Núñez G, Fernandez Galan MA, Tamagnini GP, et al. Hb Lepore-Baltimore (delta 68Leu-beta 84Thr) and Hb Lepore-Washington-Boston (delta 87Gln-beta IVS-II-8) in central Portugal and Spanish Alta Extremadura. Hum Genet 1997; 99: 669-73.

16. Viprakasit V, Pung-Amritt P, Suwanthon L, Clark K and Tanphaichitr VS. Complex interactions of $\delta\beta$ hybrid haemoglobin (Hb Lepore-Hollandia), HbE (β26 G>A) and β+ thalassaemia in a Thai family. European Journal of Haematology 2002; 68: 107-11.

17. Lanclos KD, Patterson J, Efremov GD. Characterization of chromosomes with hybrid genes for Hb Lepore-Washington, Hb Lepore-Baltimore, Hb P-Nilotic, Hb Kenya. Hum Genet 1987; 77: 40-5.

18. Trent RJ, Svirklys L, Jones P. Thai (delta beta)0-thalassemia and its interaction with gamma-thalassemia. Hemoglobin 1988; 12: 101-4.

19. Zhang JW, Stamatoyannopoulos G, Anagnou NP. Laotian $(\delta\beta)^0$-thalassemia: Molecular characterization of a novel deletion associated with increased production of fetal hemoglobin. Blood 1988; 72: 983-8.

20. Mishima N, Landman H, Huisman TH, Gilman JG. The DNA deletion in an Indian delta beta-thalassaemia begins one kilobase from the A gamma globin gene and ends in an L1 repetitive sequence. Br J Haematol 1989; 73: 375-9.

21. Gilman JG, Brinson EC, Mishima N. The 32.6 kb Indian delta beta-thalassaemia deletion ends in a 3.4 kb L1 element downstream of the beta-globin gene. Br J Haematol 1992; 82: 417-21.

22. Mager DL, Henthorn PS, and Smithies O. A Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$ thalassemia deletion: comparison to other deletions in the human beta-globin gene cluster and sequence analysis of the breakpoints. Nucleic Acids Res 1985; 13: 6559-75.

23. Kosteas T, Palena A, Anagnou NP. Molecular cloning of the breakpoints of the hereditary persistence of fetal hemoglobin type-6 (HPFH-6) deletion and sequence analysis of the novel juxtaposed region from the 3' end of the beta-globin gene cluster. Hum Genet 1997; 100: 441-5.

24. Motum PI, Hamilton TJ, Lindeman R, Le H, Trent RJ. Molecular Characterisation of Vietnamese HPFH. Human Mutation 1993; 2: 179-184.

25. Craig JE, Barnetson RA, Prior J, Thein SL. Rapid detection of deletions causing $\delta\beta$ thalassemia and hereditary persistence of fetal hemoglobin by enzymatic amplification. Blood 1994; 83: 1673-82.

26. Liu YT, Old JM, Miles K, Fisher CA, Weatherall DJ, Clegg JB. Rapid detection of alpha-thalassaemia deletions and alpha-globin gene triplication by multiplex polymerase chain reactions. Br J Haematol 2000; 108: 295-9.

27. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. Blood 2000; 95: 360-2.

28. Bhardwaj U and McCabe ER Multiplex-PCR assay for the deletions causing hereditary persistence of fetal hemoglobin. Mol Diagn 2005; 9: 151-6.

29. Biotools Hotsplit DNA polymerase. Manual guide Biotools Biotechnological & Medical Laboratories. [n.p.]; 2008.

30. Chaibunruang A, Srivorakun H, Fucharoen S, Fucharoen G, Sae-ung N, Sanchaisuriya K. Intteractions of Hb Lepore ($\delta\beta$ hybrid hemoglobin) with various hemoglobinopathies: A molecular and hematological characteristics and differential diagnosis. Blood Cells Mol Dis 2010; 44: 140-5.

31. Fucharoen S, Pengjam Y, Surapot S, Fucharoen G, Sanchaisuriya K. Molecular and hematological characterization of HPFH-6/Indian deletion-inversion $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$-thalassemia and $^{G}\gamma(^{A}\gamma\delta\beta)^{0}$- thalassemia/Hb E in Thai patients. Am J Hematol 2002; 71: 109-13.

32. Fucharoen S, Pengjam Y, Surapot S, Fucharoen G, Sanchaisuriya K. Molecular characterization of $(\delta\beta)^{0}/\beta^{0}$-thalassemia and $(\delta\beta)^{0}$-thalassemia/hemoglobin E in Thai patients. Eur J Haematol 2001; 67: 258-62.

33. Yamsri S, Sanchaisuriya K, Fucharoen G, Sae-ung N, Ratanasiri T, Fucharoen S. Prevention of severe thalassemia in northeast Thailand: 16 years of experience at a single university center Prenat Diagn 2010; 30: 540-6.

34. Yamsri S, Sanchaisuriya K, Fucharoen G, Fucharoen S. Genetic origin and interaction of the Filipino β^{0}-thlassemia with Hb E and α-thalassemia in a Thai family. Translational Research 2012; 159: 473-6.