CHAPTER II
RESEARCH DESIGNS AND METHODS

2.1 RESEARCH DESIGNS

The α-thalassemia-1 deletions have been reported in Thailand including ($--^{SEA}$), ($--^{THAI}$), and ($--^{FIL}$) deletions. Thus, in this study, the method of PCR was applied to detect the carriers of these deletions.

Firstly, using standard PCR systems to detect the ($--^{FIL}$), ($--^{THAI}$), and ($--^{SEA}$) α-thalassemia-1 deletions. Next, we were modified the standard PCR to multiplex PCR by choosing specific primers that used to amplify each deletion junction fragments, consisting of 7 primers: FIL-F, FIL-R, THAI-F, THAI-R, SEA-F, SEA-R, and α-R. Combining the primers in various mixtures and amplifying many targets DNA simultaneously, required alteration or optimization of some of the parameters of the reaction. After that, the optimized multiplex PCR was employed to detect the each of α-thalassemia-1 in 100 whole blood samples from patient with Hb H disease.

Whole blood samples were collected and kept in refrigerator during the experimental period. Genomic DNA were extracted from the whole blood samples and were used as template for PCR.

All of the PCR products were analyzed by using electrophoresis on 2% agarose gels. Finally, the deletion breakpoints of each α-thalassemia-1 deletion types and the PCR products were the result of the binding of the primers to the intended region were subsequently confirmed by using automated DNA sequencing. For the method of each procedure was given in detail later in this chapter.

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2.2 METHODS

2.2.1 Chemicals and materials

Chemicals and materials used in the study are listed in the Appendix A. Instruments and reagent preparations are also described in Appendix B and C, respectively.

2.2.2 Blood samples

The peripheral venous blood were collected from 114 confirmed cases of patients with Hb H disease who submitted to the Pediatric Thalassemia Clinic, Maharaj Nakorn Chiang Mai hospital, Department of Pediatric, Faculty of Medicine, Chiang Mai University. The 6-ml of whole blood were mixed with EDTA by inverting the tube four or five times then kept in refrigerator until use for DNA extraction.

2.2.3 Genomic DNA preparation

DNA was extracted from whole blood using the Chelex method (9,63). One ml of 0.5% Triton X-100 was added to 50-µl of whole blood in a 1.5-ml microcentrifuge tube, vortexed and centrifuged at 14,000 rpm for 1-min. The supernatant was removed by suction and 1-ml of distilled water was added. After centrifugation as above, the supernatant was removed again. From a Chelex-100 aqueous suspension settled Chelex beads were added so that they cover the nuclei pellet with a 1- to 2-mm thick layer. After adding 120-µl of distilled water, the samples were incubated for at least 3-hr (but usually overnight) at 56°C. after vortexing and centrifugation the samples were boiled for 8-min and again vortexed and centrifuged. The extracts were store at 4°C until they were used as templates in PCR.

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2.2.4 Standard polymerase chain reaction (PCR) systems

2.2.4.1 PCR-bases diagnosis of the ($--^{SEA}$) deletion (64)

Primers SEA-F (5'- GCGATCTGGGCTCTGTGTTCT-3', GenBank Z84721 positions 26119 – 26139) and SEA-R (5'- ACTGCAGCCTTGAACTCCTG-3', GenBank

Z69706 positions 2662 – 2643) were used to amplify a 188-bp fragment than spans the ($--^{SEA}$) deletion junction, while primers SEA-F and α-R (5'-GTTCCCTGAGCCCCGACACG-3', GenBank Z84721 positions 26435 – 26416) amplify a 314-bp fragment that serve as a control for the normal allele. Each 25-μl reaction contained 200-μM of each dNTP; 1.5-mM MgCl2; 1x Q-solution (Qiagen, GmbH, Germany); 1.25 U HotstarTaq DNA Polymerase (Qiagen); 0.4-μM of each primer and 100-200-ng DNA. Reactions were carried out in a Perkin-Elmer 2400 or 9600 thermocycler: initial denaturation at 94°C for 5-min; followed by 40 cycles of 94°C for 30-sec, 58°C for 1-min, and 72°C for 1-min; with a final extension of 72°C for 5-min. The PCR products are analyzed by gel electrophoresis (2% agarose) and visualized by ethidium bromide staining and UV fluorescence.

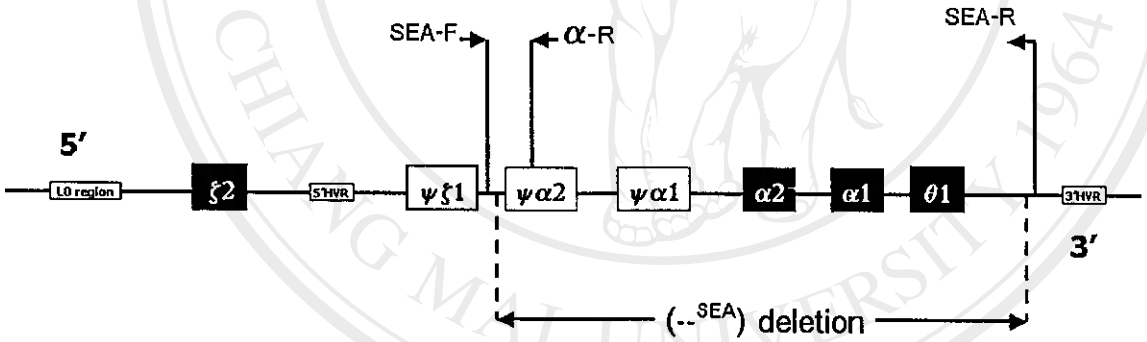

Figure 14. The location of primers for PCR to detect ($--^{SEA}$) deletion

2.2.4.2 PCR-bases diagnosis of the ($--^{THAI}$) deletion (65)

Primers THAI-F (5'-TGACTGCATCATAATTCCAGCAG-3', GenBank Z84721 positions 10504 - 10526) and THAI-R (5'-TGAGGCAGGAGATTCGCTTGA-3', GenBank Z69706 positions 1478 - 1458) were used to amplify a 480-bp fragment than spans the ($--^{THAI}$) deletion junction, while primers THAI-F and THAI-N (5'-GTAGAGATGGTGTTTTGCCATGT-3', GenBank Z84721 positions 11140 - 11118) amplify a 637-bp fragment that serve as a control for the normal allele. Each 25-μl reaction contained 200-μM of each dNTP; 1.5-mM MgCl2; 1x Q-solution (Qiagen,

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GmbH, Germany); 1.25 U HotstarTaq DNA Polymerase (Qiagen); 0.4-μM of each primer and 100-200-ng DNA. Reactions were carried out in a Perkin-Elmer 2400 or 9600 thermocycler: initial denaturation at 95°C for 15-min; followed by 35 cycles of 94°C for 40-sec, 61°C for 20-sec, and 72°C for 2-min; with a final extension of 72°C for 10-min. The PCR products are analyzed by gel electrophoresis (2% agarose) and visualized by ethidium bromide staining and UV fluorescence.

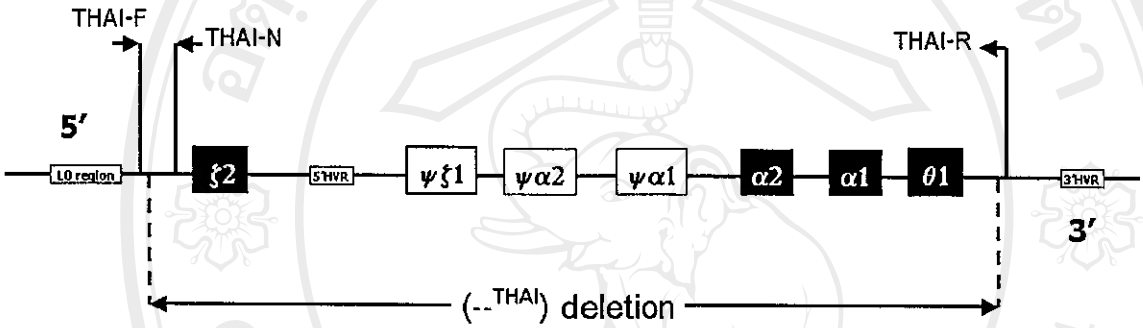

Figure 15. The location of primers for PCR to detect (--^{THAI}) deletion

2.2.4.3 PCR-bases diagnosis of the (--^{FIL}) deletion (65)

Primers FIL-F (5'-CTGCCCTTCACACCTCAGACA-3', GenBank Z84721 positions 11961 - 11981) and FIL-R (5'-GCAATCTTGGCTCACTGCAGG-3', GenBank Z69706 positions 278 - 258) were used to amplify a 597-bp fragment than spans the (--^{FIL}) deletion junction, while primers FIL-F and FIL-N (5'-GAAATGGTATTCTCAAGGTGACAC-3', GenBank Z69706 positions 2 - 25) amplify a 227-bp fragment that serve as a control for the normal allele. Each 25-μl reaction contained 200-μM of each dNTP; 1.5-mM MgCl2; 1x Q-solution (Qiagen, GmbH, Germany); 1.25 U HotstarTaq DNA Polymerase (Qiagen); 0.4-μM of each primer and 100-200-ng DNA. Reactions were carried out in a Perkin-Elmer 2400 or 9600 thermocycler: initial denaturation at 95°C for 15-min; followed by 35 cycles of 94°C for 40-sec, 63°C for 20-sec, and 72°C for 2-min; with a final extension of 72°C for 10-min. The PCR products are analyzed by gel

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electrophoresis (2% agarose) and visualized by ethidium bromide staining and UV fluorescence.

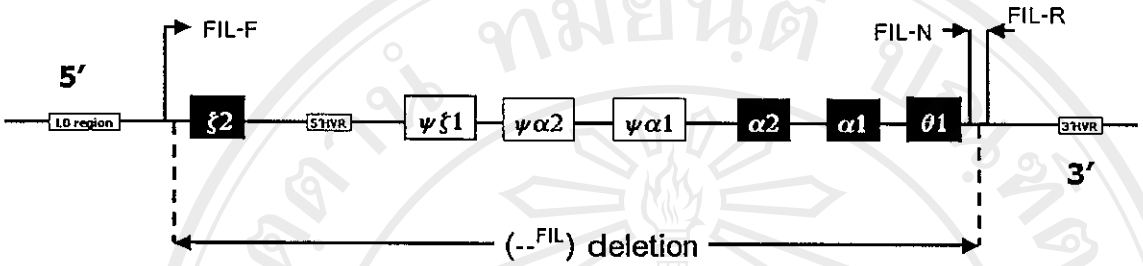

Figure 16. The location of primers for PCR to detect (--^{FIL}) deletion

2.2.5 Multiplex PCR

The concentrations of the primers used in multiplex PCR, their locations and sequences in the α-globin gene cluster are shown in Figure 17 and Table 4. Reaction mixtures of multiplex PCR and cycling programs are detailed in Table 5 and 6.

Table 5. Reaction mixtures of multiplex PCR

Components	Volume/reaction	Final concentrations
PCR buffer*, 10x (supplied with HotstarTaq DNA Polymerase)	2.5-µl	1x
Q-solution, 5x	5-µl	1x
dNTP mix (2-mM of each)	2.5-µl	200-µM
Primer mix (5-µM of each)	2-µl	0.4-µM
HotstarTaq DNA Polymerase (5 U/µl)	0.25-µl	0.05 U/µl
Distilled water	7.75-µl	-
Template DNA	5-µl	100-200-ng
Total volume	**25-µl**	

*contains 15-mM $MgCl_2$

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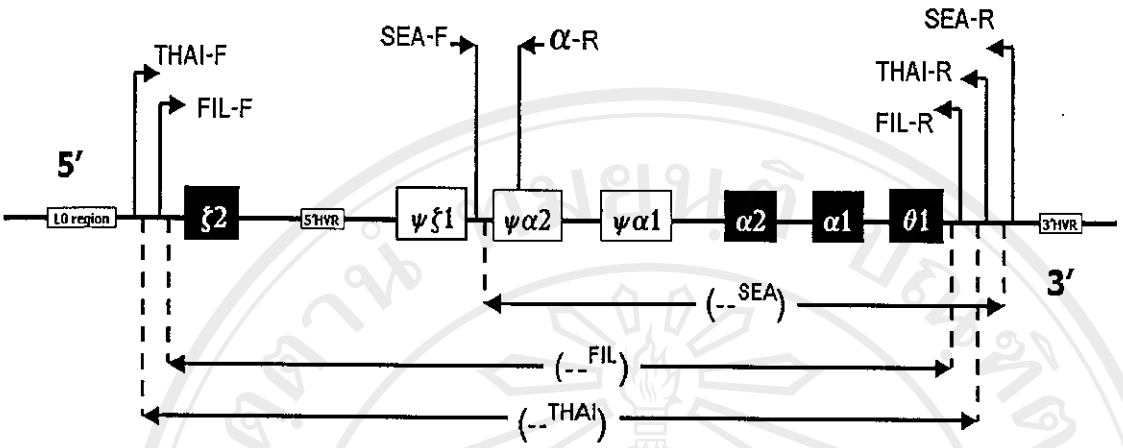

Figure 17 Schematic representation of location of multiplex PCR primers in α-globin cluster. Genes are represented as filled boxes and pseudogenes as open boxes. Hypervariable regions (HVR) are denoted as small boxes. Forward (F) and reverse (R) primers are shown for the $--^{THAI}$, $--^{FIL}$, and $--^{SEA}$ deletions. The SEA-F primer also serves as the forward primer for the normal control α-globin amplification reaction.

Table 4. Primer sequences for α-thalassemia-1 multiplex PCR and expected amplicon sizes

Name	5′→3′ sequence	GenBank ID:Nucleotides	Concentration	Amplicon (size)
FIL-F	CTGCCCTTCACACCTCAGACA	Z84721:11961→11981	0.4-μM	$--^{FIL}$ jxn[a] fragment
FIL-R	GCAATCTTGGCTCACTGCAGG	Z69706:278→258	0.4-μM	(597-bp)
THAI-F	TGACTGCATCATAATTCCAGCAG	Z84721:10504→10523	0.4-μM	$--^{THAI}$ jxn fragment
THAI-R	TGAGGCAGGAGATTCGCTTGA	Z69706:1478→1458	0.4-μM	(480-bp)
SEA-F	GCGATCTGGGCTCTGTGTTCT	Z84721:26119→26139	0.4-μM	$-^{SEA}$ jxn fragment
SEA-R	ACTGCAGCCTTGAACTCCTG	Z69706:2662→2643	0.4-μM	(188-bp)
SEA-F	As above	As above	-	control fragment
α-R	GTTCCCTGAGCCCCGACACG	Z84721:26435→26416	0.4-μM	(314-bp)

jxn, junction

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Table 6. Cycling conditions for multiplex PCR

Initial activation step	15-min	95°C
3-step cycling:		
Denaturation	40-sec	94°C
Annealing	20-sec	60°C
Extension	2-min	72°C
Number of cycles	40	
Final extension	10-min	72°C
End of PCR cycling	Indefinite	4°C

The reaction mixture was soaked at 4°C until it was kept. The PCR products were then analyzed by 2.0% agarose gel electrophoresis stained with ethidium bromide. The 100-bp DNA ladder was used as molecular-size standard.

2.2.6 **Agarose gel electrophoresis** (9,66-67)

The PCR products were analyzed by 2% agarose gel electrophoresis. Agarose was boiled in 0.5x TBE buffer (89-mM Tris-HCl, 89-mM boric acid, and 2-mM EDTA) and the gel was poured when the solution cooled down to 55-60°C. The gel wide 9-cm, thick 1-cm, and long 3-cm. The teeth of the comb were 5-mm wide and 2-mm thick. The gel was soaked in 0.5x TBE contained 0.5-μg/ml ethidium bromide for 20-min. The 0.5x TBE was used as buffer for the horizontal electrophoresis, covering the gel at least 5-mm. Five-μl of PCR products was mixed with 2-μl of a loading buffer (50% glycerol and 0.8% xylene cyanol in water) before loading. Electrophoresis was carried out in at 180-V for 30-min. the DNA bands were detected by UV light and documented using a Biorad gel Doc 1000 system.

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2.2.7 Confirmation of the deletion breakpoints of the ($--^{SEA}$), ($--^{THAI}$), and ($--^{FIL}$) α-thalassemia-1 deletions by DNA sequencing

2.2.7.1 Asymmetric PCR cycle sequencing reaction

The PCR products were purified by using a QIAGEN PCR purification kit (Qiagen, GmbH, Germany) and again electrphoresed on an 2% agarose gel to estimate the quantity before performing cycle sequencing reaction. Five- to 11-μl of the purified PCR product was used as the template for the cycle sequencing reaction depending on the concentration estimates based on agarose gel electrophoresis (10-20-ng of template). Primers FIL-F, THAI-F, and SEA-F were used as the sequencing primer of the 5' region of $--^{FIL}$ junction fragment (597-bp), $--^{THAI}$ junction fragment (480-bp), and $--^{SEA}$ junction fragment (188-bp), respectively. Primers FIL-R, THAI-R, and SEA-R were used as the sequencing primer of the 3' region of $--^{FIL}$ junction fragment (597-bp), $--^{THAI}$ junction fragment (480-bp), and $--^{SEA}$ junction fragment (188-bp), respectively. The reaction mixture contained 8-μl of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Mix, 3.2-μM of sequencing primer. Deionized-distilled water was added up to 20-μl. the reaction mixtures were performed in an automated DNA thermal cycler. The condition for cycle sequencing asymmetric amplification were followed by repeating for 25 cycles of 96°C for 10-sec, 50°C for 5-sec, and 60°C for 4-min. the extension product was stored at 4°C until precipitation.

2.2.7.2 Precipitating the extension product

The extension products were precipitated with ethanol/sodium acetate. First, for each sequencing reaction, prepare a 1.5-ml microcentrifuge tube containing the 2.0-μl of 3-M sodium acetate (NaOAc), pH 4.6 and 50-μl of 95% ethanol (EtOH). Second, the entired contents of each extension product was transferred into a tube of NaOAc/EtOH mixture, then mixed thoroughly and leave at room temperature for 15-min to precipitate the extension products. Third, the tubes were spun in a microcentrifuge for 40-min at maximum speed and the supernatant was discarded. Next, the pellet was rinsed with 250-μl of 70% EtOH and the tubes were spun in a microcentrifuge for 25-min at

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maximum speed and the supernatant was discarded again. After that, the pellet was dried in a heat block at 90°C for 1-min. Finally, the precipitated extension product was stored at -20°C until ready to electrophoresis on the genetic analyzer.

2.2.7.3 Automated DNA sequence analysis

First, the precipitated extension products were re-suspended in 12-25-µl of template suppression reagent (TSR) and the samples were mixed thoroughly and spun down. Next, the samples were heated at 95°C for 2-min following chilled on ice for 10-min. after that, the samples were mixed thoroughly and spun again then transferred to an ABI Prism 310 genetic analyzer. The raw data from capillary electrophoresis on the ABI Prism 310 was collected and analyzed by Macintosh-based software (Sequencing Analysis Software Version 2.1.1). The deletion breakpoints of α-thalassemia-1 mutations were manually compared with the normal α-globin gene cluster sequences (the complete sequences of α-globin gene cluster were derived from GenBank ID: Z84721 and Z69706).

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