CHAPTER III

RESEARCH DESIGNS AND METHODS

Research designs

Whole blood sample from the patient with the unknown hemoglobin was collected and kept at –20 °C during the experimental period. Genomic DNA was extracted by using the Chelex 100 method (Walsh *et al*, 1991;Sanguansermsri *et al*, 1999) and used as template for PCR. The exons of the β–globin gene were amplified by using standard PCR. Each specific PCR product was then purified and used as template in cycle sequencing. The sequencing products were then precipitated and prepared for loading on an ABI PRISM 310 DNA Genetics Analyzer and the point mutation was determined from the sequencing data. Data of sequencing was aliment by computer. In Figure 12 shows a flow chart of the research design.

Chemical and materials

Chemical 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.

Case History

HPLC analysis detected an unknown hemoglobin variant in a child who was a patient of the thalassemia clinic of the Maharaj Nakorn Chiang Mai Hospital.

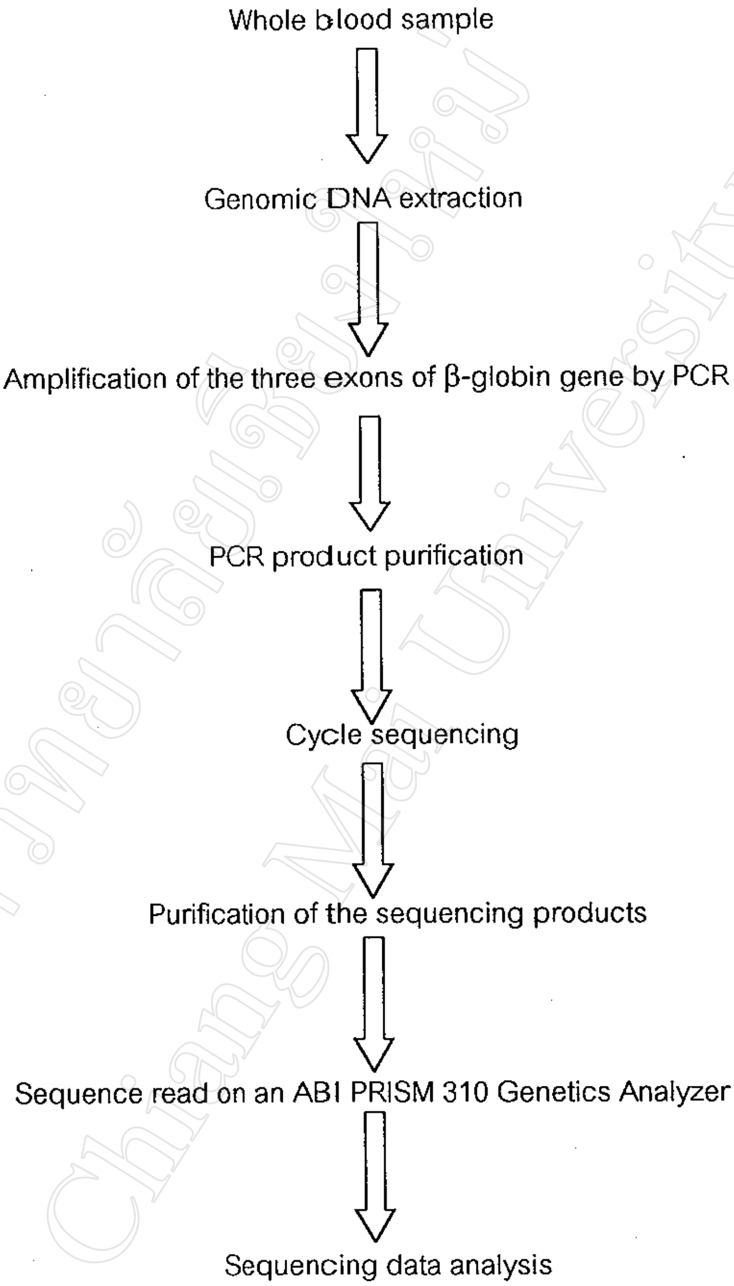

Figure 12. Flow chart of the research design in this study.

1. Laboratory studies

Genomic DNA extraction from whole blood

DNA was extracted from whole blood using the Chelex method (Walsh *et al.*, 1991) with some modifications (Sanguansermsri *et al*, 1999). One ml of aqueous 0.5% Triton X-100 was added to 30 µl of blood in 1.5 ml tubes and vortexed and centrifuged at 14,000 rpm for 1 minute. The supernatant was removed by suction up to about 20 µl, 1 ml of water was added, after centrifugation for 1 minute as above, the supernatant was removed again up to about 20 µl. From a Chelex-100 aqueous suspension settled chelex beads were added so that they bury the nuclei pellet with a 1-2 mm thick layer, also 110 µl of water was added and the samples were incubated for at least one hour or overnight at 56°C. After vortexing and centrifugation were the samples boiled for 5 minutes and then vortexed and centrifuged. The extracts were kept at 4°C until they were used as templates in PCR (Figure 13).

Genomic DNA extraction from blood of patient were suggested use 30-50 µl of the whole blood which equivalent to about 150,000-250,000 nucleated cells (Steger *et al*, 1994).

1.1 Amplification of the β-globin gene exon by PCR

The three exons of the β-globin gene were amplified using the primers shown in Table 3. Primers were synthesized (to see Appendix A). The position of each primer the β-globin gene and the length of the resulting PCR products are shown in figure 14.

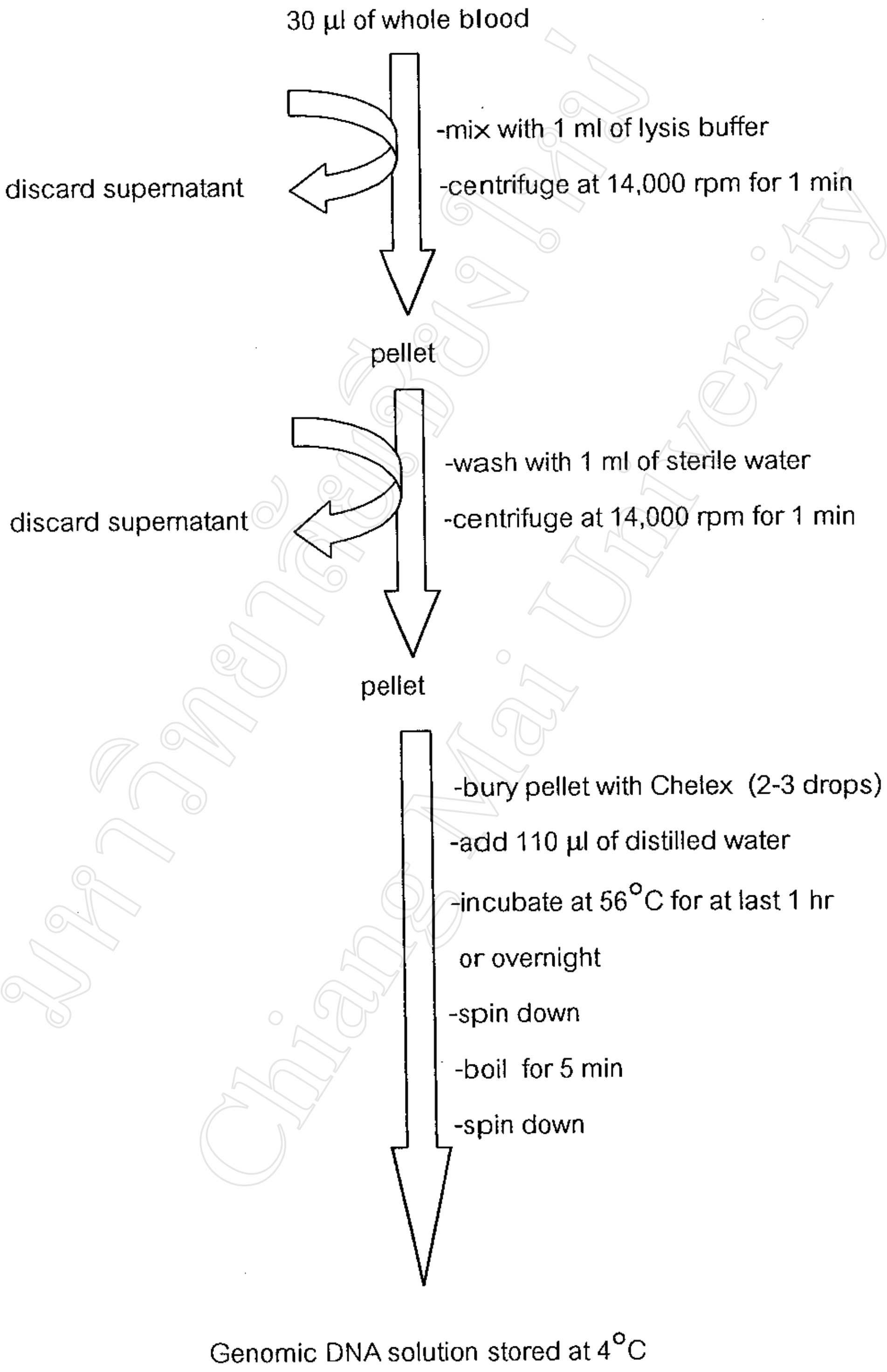

Figure 13. Flow chart of the DNA extraction by the Chelex method.

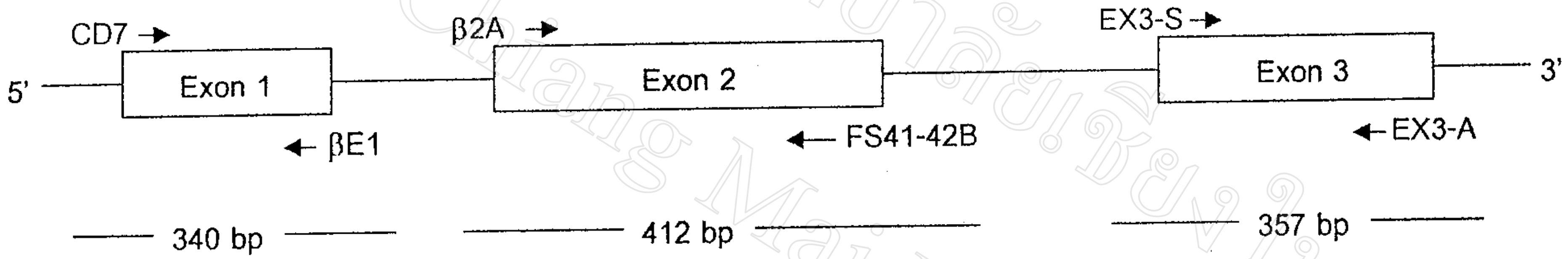

Figure 14. The position of the primers used in the specific amplification of the β-globin gene exons and the size of the corresponding amplicons.

Table 3. Primers used to amplify the β-globin gene exons.

Exon	Amplification Primer	Length
1	**CD7:** TCCTAAGCCAGTGCCAGAAG	20
	βE1: GGTCTCCTTAAACCTGTCTTG	21
2	**β2A:** GACAGAGAAGACTCTTGGGTT	21
	FS41-42B: AATCATTCGTCTGTTTCCC	19
3	**EX3-S:** TAGCAGCTACAATCCAGCTACC	22
	EX3-A: GCAGAATCCAGATGCTCAAGGC	22

For PCR, the following reagents were combined in a 0.2 ml thin wall tube:

Amplification of Exon 1:

H_2O	3.7	µl
100% Glycerol	1.0	µl
10x PCR buffer	1.0	µl
1mM dNTPs	2.0	µl
5µM of primer mix	1.0	µl
0.83 units/µl *Taq* DNA polymerase	0.3	µl
Chelex extract as DNA template	1.0	µl
	Sum=10.0	µl

Amplification of Exon 2:

H_2O	2.9	µl
100% Glycerol	1.5	µl
10x PCR buffer	1.0	µl
1mM dNTPs	2.0	µl
5µM of primer mix	1.0	µl
0.42 units/µl *Taq* DNA polymerase	0.6	µl
Chelex extract as DNA template	1.0	µl
	Sum=10.0	µl

Amplification of Exon 3:

H_2O	3.9	µl
100% Glycerol	0.5	µl
10x PCR buffer	1.0	µl
1mM dNTPs	2.0	µl
5µM of primer mix	1.0	µl
0.42 units/µl *Taq* DNA polymerase	0.6	µl
Chelex extract as DNA template	1.0	µl
	Sum =10.0	µl

PCR conditions for amplification of the exons:

Exon 1:

Step	1 cycle	2-36 cycles	Last cycle
Initial denaturation	94°C :2:30 min	-	-
Denaturation	94°C :0:30 min	94°C :0:30 min	94°C :0:30 min
Annealing	55°C :1.00 min	55°C :1.00 min	55°C :1.00 min
Extension	72°C :1.00 min	72°C :1.00 min	72°C :5.00 min
37 cycles			

Exon 2:

Step	1 cycle	2-36 cycles	Last cycle
Initial denaturatoin	94°C :2:30 min	-	-
Denaturation	94°C :0:30 min	94°C :0:30 min	94°C :0:30 min
Annealing	50°C :1.00 min	50°C :1.00 min	50°C :1.00 min
Extension	72°C :1.00 min	72°C :1.00 min	72°C :5.00 min
35 cycles			

Exon 3:

Step	1 cycle	2-36 cycles	Last cycle
Initial denaturation	94°C :2:30 min	-	-
Denaturation	94°C :0:30 min	94°C :0:30 min	94°C :0:30 min
Annealing	60°C :1.00 min	60°C :1.00 min	60°C :1.00 min
Extension	72°C :1.00 min	72°C :1.00 min	72°C :5.00 min
40 cycles			

PCR was performed in a GeneAmp PCR System 2400 thermal cycle (Perkin Elmer,Cetus, USA). PCR products were analyzed on 3% agarose gels and made visible with ethidium bromide and UV light from a transilluminator. Photographs of the gels were prepared by using a computer-equipped video camera.

1.2 Chain-termination cycle sequencing

Purification of the amplicons

For use as templates in sequencing, the amplicons were purified using the QIA quick PCR purification kit (QIAGEN). The kit was designed to separate single or double-stranded PCR products ranging in size from 100 bp to 10 kb from primers, nucleotides, polymerases and salt, using spin columns. Purified DNA was stored at –20°C.

Step in purification of the amplicons using the purification kit:

A) Add 5 volumes of buffer PB to 1 volume of PCR reaction and mix.

B) Place a QIA quick spin column in a provided 2 ml collection tube.

C) To bind DNA, apply the sample to the QIA quick column and centrifuge at 14,000 rpm for 1 min.

D) Discard the flow-through. Place QIA quick column back into the same tube.

E) Wash with 0.75 ml of buffer PE and centrifuge at 14,000 rpm for 1 min.

F) Discard the flow-through. Place QIA quick column back into the same tube and centrifuge column for an additional 1 min at 14,000 rpm.

G) Place QIA quick column in a 1.5 ml microcentrifuge tube.

H) To elute DNA, add 50 µl Buffer EB to the center of the QIA quick column and centrifuge at 14,000 rpm for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIA quick column, let stand for 1 min and centrifuge at 14,000 rpm for 1 min.

DNA concentration in the purified fractions was determined by measuring the $OD_{260\ nm}$ and assuming that $OD_{260\ nm} = 1$ corresponds to 50 µg/ml of double stranded DNA. A simple equation to estimate the DNA quantity was as follows:

$$\text{DNA (ng/}\mu\text{l)} = OD_{260} \times 50 \times \text{dilution factor}$$

When OD_{260} = the OD measured at 260 nm

50 = DNA co-efficient

The absorbance at 260 and the final concentration of pure PCR product solution are shown in Table 4.

Table 4. DNA concentration of each exons of the β-globin gene.

	Dilution Purified amplicon: H_2O	OD $_{260\ nm}$	DNA Concentration (ng/μl)
Amplicon of exon 1	20 μl : 980 μl	0.006	15.0
Amplicon of exon 2	30 μl : 970 μl	0.013	21.7
Amplicon of exon 3	30 μl : 970 μl	0.038	65.0

Sequencing by chain termination cycle sequencing technique

For sequencing the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer,Cetus,USA) was used.

Cycle sequencing reaction mixture

A) Dye terminator premix

The ready reaction mix was composed of A-Dye,C-Dye,G-Dye and T-Dye terminator, dATP, dCTP, dITP and dTTP, Tris-HCl buffer (pH 9.0) , $MgCl_2$, thermal stable pyrophosphatase and Ampli*Taq*® DNA polymerase, FS. For 20 μl cycle sequencing reaction mixture composed of 8 μl of terminator ready reaction mix. The company did not indicate the final concentration of each component in the stock ready reaction mixture.

B) Sequencing primer

The sequencing primers for the three exons of the β-globin gene as the same as the PCR primers were used to amplify β-globin gene exons. The forward sequencing primer for exon 1 and 3 were CD7and EX3-S, accept exon 2 was change forward sequencing primer from β2A to FSA: CTCTGCCTATTGGTCTATT and the length of sequencing extension product was 353 bp. The reverse sequencing primer for exon 1, 2 and 3 were βE1, FS 41-42B and EX3-A. Source of they was synthesized to see in Appendix A and each stock solution was diluted to 3.2 μl final concentration with water to see in Appendix C.

Chain-termination cycle sequencing technique

The reactions were performed in 0.2 ml PCR tubes (Table 5). The tubes were protected from light to prevent damage to the detection dyes. Cycle sequencing was performed on the GeneAmp PCR System 2400 thermal cycle (Perkin Elmer,Cetus,USA) (Table 6).

Table 5. Cycle sequencing reaction mixture.

Reaction	Final volume or concentration	
Terminator Ready Reaction Mix	8	μl
Amplicon of β-globin gene exon	30-90	ng
Sequencing primer	3.2	pmol
Water	q.s	
Final volume	20	μl

Table 6. Three- step PCR protocol used for cycle sequencing.

Stage	Cycles 1– 25
Denature	96°C: 0.10 min
Annealing	50°C: 0.05 min
Extension	60°C: 4.00 min
25 cycles	

(Note: the reaction tubes were placed in to the PCR machine when the block reached 96°C.)

Ethanol precipitation of the sequencing extension products

The reactions were protected from light exposure throughout the whole process. Step of ethanol purification as follows:

A) For each sequencing reaction, prepare a 1.5 ml microcentrifuge tube containing the follow:

-50 µl of 95% ice-cold EtOH

-20 µl of sequencing extension products.

-2.0 µl of 3M NaAc, pH 4.6

B) Vortex the tube and place on ice for 10 min to precipitate the sequencing products.

C) Centrifuge at maximum speed for 15-30 min.

D) Carefully aspirate the supernatant with a pipette and discard.

E) Rinse the pellet with 250 µl 70% ice-cold ethanol.

F) Centrifuge again at maximum speed for 5 min.

G) Carefully aspirate or decent the supernatant.

H) Dry the pellet at room temperature for least 3 hours.

Preparing and loading the samples on to the sequencer

The pellet from 2.4.5 (H) was resuspended in 25 µl of "Template Suppression Reagent ", TSR (Perkin Elmer, Cetus, USA). The tubes were vortexed and briefly centrifuged. The sample was then transferred to a special tube with a rubber cap and heated at 95 °C for 2 min. After this denaturation was the tube placed on ice until the sample was injected into the ABI PRISM 310 Genetic Analyzer.