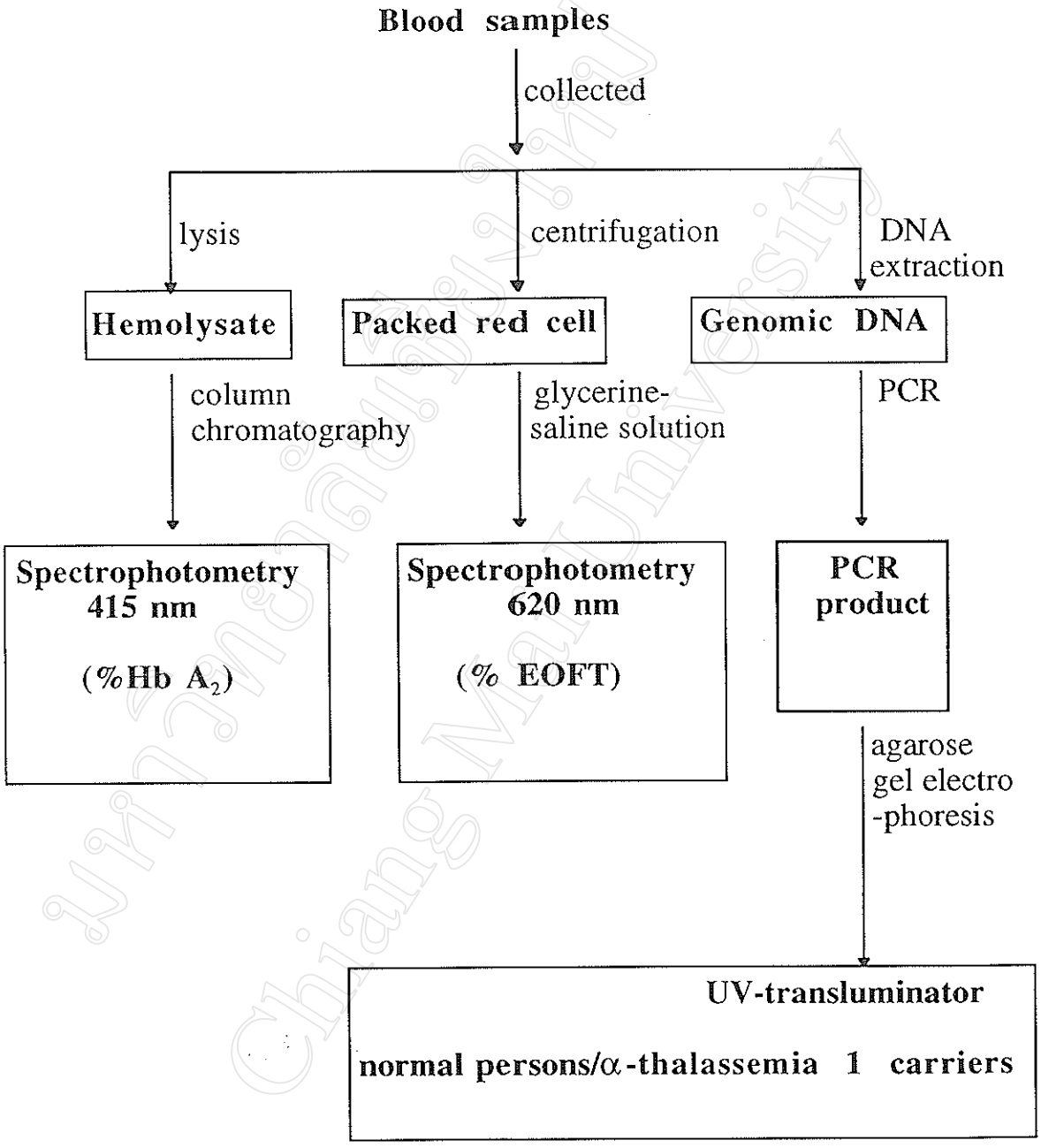
CHAPTER II

RESEARCH DESIGNS AND METHODS

2.1 Research Designs

Blood samples were separated into 3 parts for HbA_2 determination, erythrocyte osmotic fragility test and polymerase chain reaction. The first part was lysed for micro-column chromatography. HbA_2 which eluted from the column was detected by spectrophotometry at 415 nm. The second part of blood sample was centrifuged and packed cells were swelled in glycerin-saline solution. The percentage of erythrocyte osmotic fragility was detected by spectrophotometry at 620 nm. The last part was extracted for genomic DNA and applied for PCR. PCR was used to detect α-thalassemia 1 carriers and normal persons. PCRs were analysed by agarose gel electrophoresis. The percentage of HbA_2 and erythrocyte osmotic fragility were compared between normal persons and α-thalassemia 1 carriers.

The method of each procedure is given in detail later in this chapter. The diagram below shows the whole procedure.


Blood samples
collected
lysis
centrifugation
DNA extraction
Hemolysate
Packed red cell
Genomic DNA
column chromatography
glycerine-saline solution
PCR
Spectrophotometry 415 nm
(%Hb A2)
Spectrophotometry 620 nm
(% EOFT)
PCR product
agarose gel electro -phoresis
UV-transluminator
normal persons/α-thalassemia 1 carriers

2.2 Method

2.2.1 Blood Samples

Blood samples were collected from 500 pregnant women seeking ante-natal care at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, during April to July 1995.

10 ml blood were kept in tubes with 1 ml of 0.5 M EDTA and stored at 4°C for quantitation of Hb A_2 and erythrocyte osmotic fragility test. Aliquots of 65 µl from each sample were transfered into 1.5 ml Eppendorf tubes and stored at -70 °C for genomic DNA extraction.

2.2.2 Hb A_2 Determination

Preparation of hemoglobin solution

2-5 ml of blood sample was put in test tube (diameter 1.5 cm, length 10 cm) then 10 ml of 0.154 M NaCl was added. The solution was mixed and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded. Red cells were washed as described for 3 times. The packed red cells were lysed with an equal volume of distilled water. The lysed cells were mixed and an equal volume of CCl_4 added. Cell ghosts and other debris were removed by centrifugation (3000 rpm, 30min) and the supernatant (hemolysate) was collected. Prior to chromatography, hemolysate was diluted 100 fold with 0.05 M Tris-HCl containing KCN buffer, pH 8.5.

Column preparation

The anion exchanger known as diethyl aminoethyl (DEAE)-Sephadex A50 by Phamacia was used. The dried powder was suspended in a large volume of 0.05 M Tris-HCl buffer, pH 8.5, which contained 100 mg of KCN per 1000 ml. The suspension was stored at room temperature.

The eluting buffers were prepared from 1.0 M stock Tris-HCl buffer, pH 9.0. They were 0.05 M Tris, contain 100 mg of KCN per 1000 ml, and were adjusted with 4 M HCl to the desired pH 8.5 and 8.2, respectively.

Pasteur pipettes (diameter 0.5 cm, length 10 cm) served as chromatographic mini-column. The pipette was loosely packed with a small plug of cotton, and the pipette was placed vertical. The cotton was moistened before the addition of the Sephadex to the column. The column was then attached with rubber tubing to funnels which acted as areservoir for the buffer. The pasteur pipettes were filled with slurry of the above gel up to 8-9 cm. The remaining space of about 1-2 cm was filled with 0.05 m Tris-HCl containing KCN buffer, pH 8.5, and the tube was capped and stored until use. The chromatographic equipment shown as Figure 5.

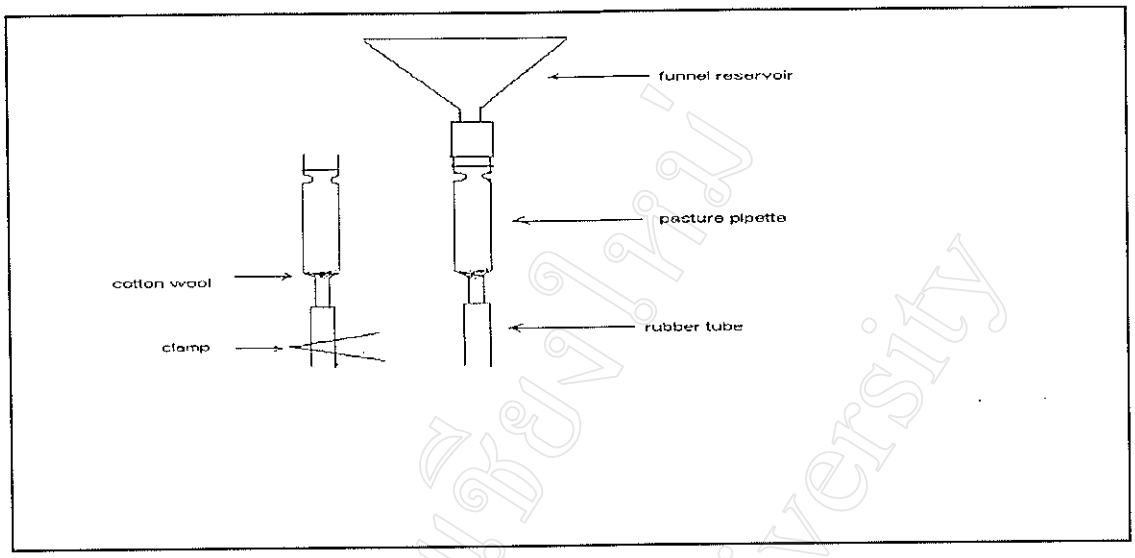


Figure 5 Micro-column chromatography equipment (Sanguansermsri 1994)

Chromatographic procedure

The DEAE-Sephadex A50 packed tube which described above carefully uncapped and all layer of buffer was removed. 10 ml diluted hemolysate was slowly and evenly applied to the column from a pipette. After the hemolysate had entered the gel, 10 ml of 0.05M Tris-HCl buffer, pH 8.5 was added gently on a top, and the tube was then attached to the funnel. The effluent was discarded and 30 ml of 0.05M Tris-HCl, pH 8.2 buffer was added on the top of the gel. The effluent was collected in a beaker. The absorption of the effluent was measured by spectophotometry at 415 nm as OD1. 10 ml diluted hemolysate which

described in preparation of hemoglobin diluted with water to 30 ml final volume. The absorbance of diluted hemolysate at 415 nm as OD2.

Calculations of percentages :

$$\%Hb\ A_2 = \frac{OD1}{OD2} \times 100$$

2.2.3 Erythrocyte Osmotic Fragility Test

Preparation of the glycerine saline solution

The 0.45% glycerine saline solution contained 180 mM glycerine, 10 mM Na_2HPO_4, 2 mM $NaH_2PO_4 2H_2O$, and 48 mM NaCl. The solution was stored at room temperature and prepared freshly everyday.

EOFT procedure

The hemolysis of the red blood cell using glycerine-saline condition was simultaneous monitored the absorption using computer connected to the spectrophotometer. The 0.5-1 ml of blood which contain EDTA was collected and centrifuged at 3,000 rpm for 5 minuses. Plasma was discarded and 20 µl of packed red cells were suspended in 20 ml of 0.45% glycerine saline solution. Cell suspension was transferred to a cuvette and the hemolysis was monitored at 620 nm after 20 seconds in time intervals of 20 seconds for 120 seconds. The percentage of EOF was calculated using the following formula :

$$\%EOFT = \frac{(OD_{20} - OD_{120})}{OD_{20}} \times 100$$

OD_{20} : Absorbance 20 seconds after transfer of the cell suspension to the cuvette.

OD_{120} : Absorbance 120 seconds after transfer of the cell suspension to the cuvette.

2.2.4 Genomic DNA Preparation

50-65 µl of whole blood which is equivalent to about 250,000 nucleated cells was mixed with 500 µl of lysis buffer which consist of 10 mM Tris-HCl buffer (pH 7) containing 1% Triton X-100 in a 1.5 ml Eppendorf tube. The cell membrane was solubilized by Triton X-100. The suspension was mixed and then centrifuged for 10 seconds at 14,000 rpm in an Eppendorf microcentrifuge. The resulting supernatant was discarded by gentle suction. The pellet containing cell nuclei was washed twice in 500 µl of 10 mM Tris-HCl buffer, pH 7.0 until it became nearly colorless. The pellet was then resuspended in 45 µl of 100 mM KOH and incubated for 10 minutes at 70 °C. Finally, the sample was neutralized by the addition of 45 µl of 100 mM HCl and 10 µl of 100 mM Tris-HCl buffer (pH 7). The denatured proteins and cell debris precipitate were spun down by brief centrifugation. The samples

were stored at -20°C until use. The samples were thawed, mixed and centrifuged when needed (Steger *et al.*, 1994). 500 DNA samples extracted from blood of pregnant women had the yield around 50-100 ng/μl. The OD 260/280 ratio were 1.1-1.3.

2.2.5 Polymerase Chain Reaction

2.2.5.1 Components of the PCR

Primers

The sequences of the primers for PCR to detect α-thalassemia 1 of Southeast Asia type were obtained from previous report (Chang *et al.*, 1991). They were synthesized from Bio Service Unit, Department of Biochemistry, Mahidol University, Bankok, Thailand as the following sequences :

A : 5' GCG ATC TGG GCT CTG TGT TCT 3'

MW : 6852.2

B : 5' GTT CCC TGA GCC CCG ACA CG 3'

MW : 6481.0

C : 5' ACT GCA GCC TTG AAC TCC TG 3'

MW : 6510.0

Primer A and C were used for detection of α-thalassemia 1 carrier which showed a specific band at 194 bp. The concentration of these

primer were 0.5 μM in a 15 μl reaction mixture. Whereas, primer A and B were used for detection of the normal DNA sequence which gave a specific band at 314 bp. The concentration of these primer is 0.5 μM in a 10 μl reaction mixture.

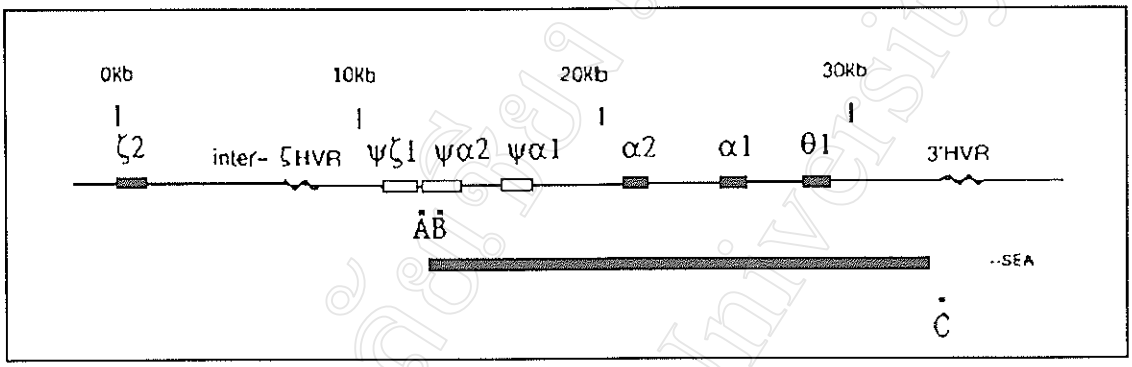


Figure 6 The position of primers for PCR to detect α-thalassemia 1 (Southeast Asia type) (Chang *et al.*, 1991) The solid bar indicates the size of the 20 kb deletion in α-thalassemia 1 SEA type.

Deoxynucleotide Triphosphates (dNTPs)

From dATP, dCTP, dGTP and dTTP powders, a 4 mM solution was made by solving each of the dNTPs in water and neutralizing with NaOH, Tris-HCl (pH 7.2), final concentrations 6 mM and 10 mM respectively. So neutralized solutions of 4 mM dATP, 4 mM dCTP, 4 mM dGTP and 4 mM dTTP were obtained. An equal aliquot of each of these dNTPs is combined to give a ready to use stock solution of 1 mM of dNTPs (Steger, unpublished).

Taq DNA Polymerase

Taq DNA polymerase was obtained from Promega. The concentration of *Taq* DNA polymerase was 5 U/µl. The enzyme was diluted 15 fold in 50% glycerine and 1×PCR buffer. The final concentration was 0.025 U/µl in the PCR.

DNA Template

The 1.5 µl of each sample was dispensed in each reaction for primer A and C. Whereas, the reaction for detection of the normal persons used 1 µl of each sample.

PCR Buffer

The PCR buffer consisted of 50 mM KCl, 20 mM Tris-HCl buffer pH 8.4, 1.5mM $MgCl_2$, 0.1 mg/ml BSA and 0.05% Tween 20. The buffer was prepared as a 10 fold concentrate.

2.2.3.2 PCR Procedure

Detection α-thalassemia 1 carriers with primer A and C

Each amplification reaction was carried out in a 0.5 ml Eppendorf tube containing :

H_2O	5.45 µl
10×PCR buffer	1.05 µl

dNTPs 1 mM each 3.00 µl

Primer A/C 7.5 µM each 1.00 µl

The mixture was irradiated with germcidal UV lamp 15 minutes for destroy the amplicon from previous PCR in the mixture that caused contamination (Sarkar *et al.*, 1991). The genomic DNA template was added to the tube and two beads of paraffin wax (Sparkman *et al.*, 1992) approximate bead volume 15 µl, to perform hot start PCR (Kitsirisakul *et* al.,1996). Hot start PCR had to be used to prevent amplification failure due to a selfhomology of primer C. The reaction mixture was heated for three minutes at 94 °C. Then the reaction were allowed to cool down to room temperature and *Taq* DNA polymerase was added on the top of the hardened wax layer. The sample were then kept in a thermocycler at 85 °C for 2 minutes followed by 40 cycles of amplification in Hybaid Omnigene thermal cycler. Denaturation, annealing and extension were as follows.

Cycle	1st-39th Cycle	Last Cycle
Denaturation	94 °C, 1 min	94 °C, 1 min
Annealing	57 °C, 1 min	57 °C, 1 min
Extension	72 °C, 1 min	72 °C, 5 min

Detection of normal type with primer A and B

Each amplification reaction was carried out in 0.5 ml Eppendrof tube containing :

H_2O	3.3 µl
10×PCR buffer	0.9 µl
dNTPs 1mM each	2.0 µl
Primer A/B 7.5 µM each	0.7 µl

1.5 µl 100% glycerine was added to the mixture. The genomic template were added to the Eppendrof tube and followed by 2 drops of paraffin oil (approximate 20 µl) to prevent the evaporation of water. *Taq* DNA polymerase was added at last. The reaction mixture was taken to the thermocycler and the 40 amplification cycles started as described below. The amplification mixture was kept at 4 °C before analysis by gel electrophoresis.

Cycle	First cycle	2nd-39th cycle	Last cycle
Denaturation	94 °C, 3 min	94 °C, 1 min	94 °C, 3 min
Annealing	57 °C, 1 min	57 °C, 1 min	57 °C, 1 min
Extention	72 °C, 1 min	72 °C, 1 min	72 °C, 5 min

2.2.5.3 Agarose Gel Electrophoresis

PCR products were analyzed by 3% agarose gel electrophoresis. Agarose was boiled in 0.5×TBE (45 mM Tris, 45 mM boric acid and

1mM EDTA) and the gel was poured when the solution cooled down to 60-70 °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 0.5×TBE containing 0.5 μg/ml ethidium bromide for 10 minutes. 0.5×TBE was used as buffer for the horizontal electrophoresis, covering the gel at least 5 mm. 8 μl PCR product was mixed with 1 μl of gel-loading buffer (0.5% bromophenol blue, 0.5% xylene cyanol FF, 30%w/v sucrose in water) before loading. Electrophoresis was carried out in at 50 mA until the bromophenol blue marker reached the bottom of the gel. The DNA bands were visualised on a transilluminator using UV-light.

2.2.5.4 Photography

The gels were photographed using a UV-transillumimator (SPECTROLINE) and camera (PENTAX). The film was a black and white negative film (KODAK). The exposure time was 30 seconds.