CHAPTER 3

METHODS

STEP 1

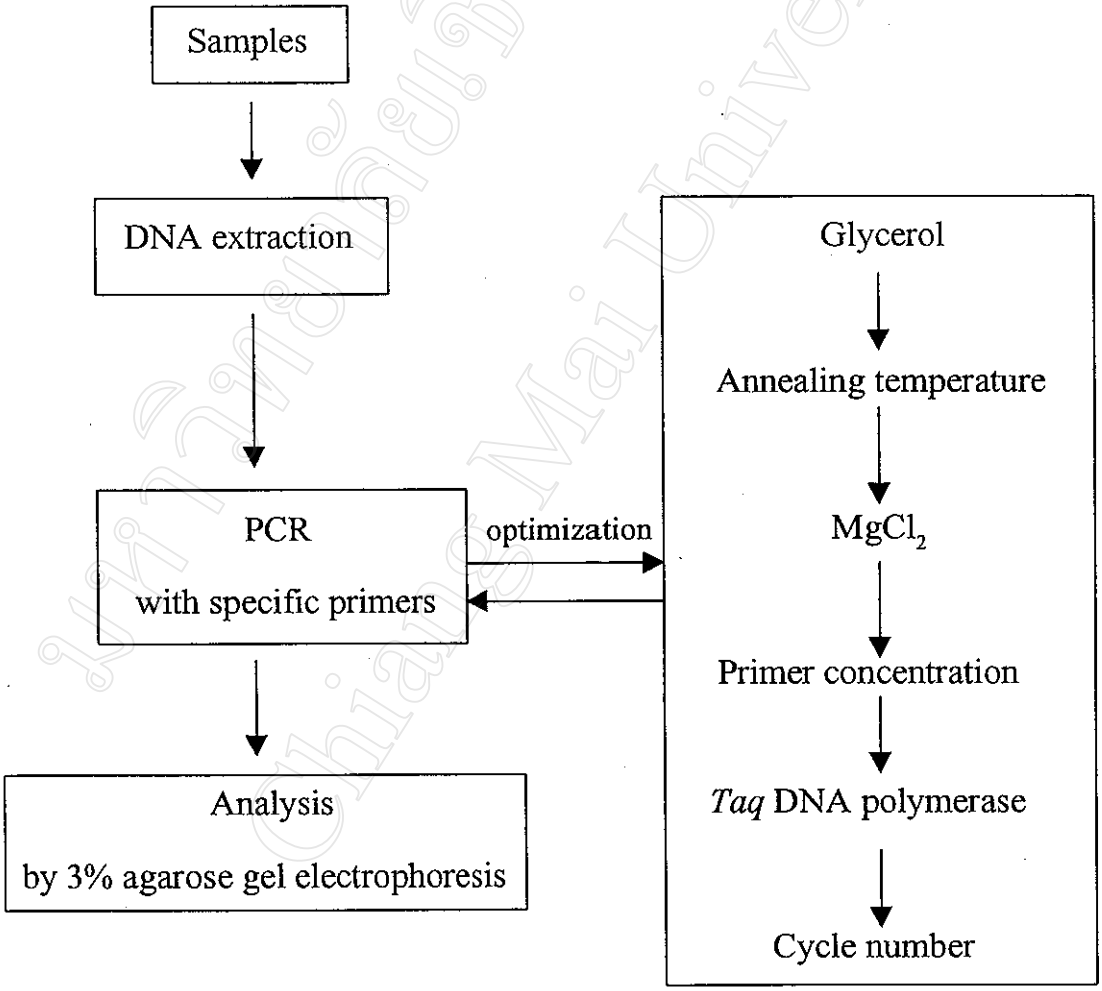

Figure 7 Diagram of step 1 procedure

From Figure 7 the procedures were conducted as following

1. Samples

Blood samples

Blood samples were collected from pregnant women seeking ante-natal care at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand. Ten milliliters of blood were kept in tubes with 0.5 ml of 0.2 M EDTA (ต่อพงศ์, 2539). Aliquots of 30 µl from each sample were transferred into a 1.5 ml eppendorf tube and stored at -20°C for DNA extraction.

Amniotic fluids

Amniotic fluids were collected from women who were at risk of a Hb Bart's hydrops fetalis pregnancy by amniocentesis at week 14-18. Aliquots of 1 ml amniotic fluid were transferred into 1.5 ml eppendorf tubes and stored at –20°C for DNA extraction.

2. DNA extraction

Blood samples

Thirty microliters of whole blood were combined with 1 ml of lysis buffer containing 0.5% Triton X-100 in a 1.5 ml eppendorf tube. The cell membranes were solubilized by Triton X-100. The suspension was vortexed and centrifuged at 14,000 rpm for 1 minute. The supernatant was removed by gentle suction. One milliliters of distilled water was added to wash the pellet containing cell nuclei and centrifuged at 14,000 rpm for 1 minute then the supernatant was removed. The pellet was covered with 2 drops of chelex-100 chelating resin suspension, also 110 µl of distilled water

was added. The tube was incubated at 56°C for 2 hours or more if required until the pellet was clear. The suspension was then mixed and briefly spun down ; the tube was boiled for 5 minutes and vortexed then briefly centrifuged. The sample was stored at 4°C until use.

Amniotic fluids

One milliliters of amniotic fluid was centrifuged in a 1.5 ml eppendorf tube at 14,000 rpm for 2 minutes and the supernatant removed. The pellet was washed with 1 ml of distilled water then centrifuged at 14,000 rpm for 2 minutes. The supernatant was removed by suction. The tube was frozen at –20°C and thawed for break cell membrane. The pellet was resuspended then briefly centrifuged and covered with a two millimeters thick layer of chelex beads ; 110 µl of distilled water was added and the tube was incubated at 56°C for 2 hours, or more if required, until the pellet was clear. After the suspension was briefly centrifuged the tube was boiled for 5 minutes and vortexed then centrifuged. The sample was stored at 4°C until use.

3. Polymerase chain reaction

Primers

Specific primer sequences for detection of the α-thalassemia-1 (SEA) haplotype were obtained from a previous report (primer A and B, Chang *et al.*, 1991) or were newly designed (primer C, Sanguansermsri *et al.*, 1999) using the Primer Detective computer program. Primers were synthesized by the Bio Service Unit, Department of Biochemistry, Mahidol University, Bangkok, Thailand.

Primer sequences :

A : 5′ GCG ATC TGG GCT CTG TGT TCT 3′

B : 5′ GTT CCC TGA GCC CCG ACA CG 3′

C : 5′ GCC TTG AAC TCC TGG ACT TAA 3′

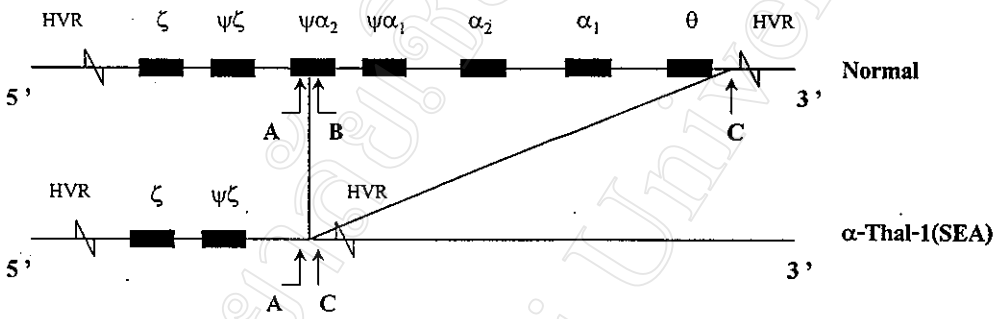

Figure 8 The position of the primers in the α-globin gene cluster for detection of the α-thalassemia-1 (SEA) haplotype by PCR.

Primer A : common upstream primer.

Primer B : downstream primer specific for amplification of normal type DNA.

Primer C : downstream primer specific for amplification of the α-thalassemia-1 (SEA) haplotype.

Primer A and B amplify the normal DNA sequence and give a 314 bp band. Primer A and C amplify the deletion breakpoint and give a 188 bp band.

Optimization

To optimize PCR conditions for the detection of homozygous α-thalassemia-1 (SEA) haplotype. The extracted DNAs were used as template for optimization. The final concentrations of PCR components were varied. The variations were done step by step, while one was varied, the remainders were kept constant. After the optimal concentration was found, the others were varied accordingly. The signal intensity of the expected band was the criterion in optimization experiments. The concentrations of PCR components were varied as in Table 4.

Table 4 Optimization of PCR components

PCR components	Final concentration
Glycerol	5, 10 and 15%
Annealing temperature	58, 59 and 60°C
$MgCl_2$ concentration	1.5, 1.75, 2.0, 2.25, 2.5 and 2.75 mM
Primer concentration	0.125, 0.25, 0.5, 0.75 and 1.0 μM
Taq DNA polymerase	0.06, 0.12 and 0.25 units/10 μl
Cycle number	30, 35 and 40 cycles

Amplification reaction

Two microliters of template was dispensed in a 0.2 ml thin-wall PCR tube containing the following mixture :

H_2O	3.0	µl
10 x PCR buffer	1.0	µl
dNTPs (1 mM each)	2.0	µl
Primer A, B and C mixture (5 µM each)	0.75	µl
Glycerol	0.75	µl
Taq DNA polymerase (0.25 units/µl)	0.5	µl
	Sum = 10.0	µl

The reaction mixture was placed in an automated DNA thermal cycler (Perkin-Elmer system 2400) when the temperature of the block reached 94°C ; 40 cycles of amplification were done.

Step / Cycle	First cycle	2 nd-39 th cycle	Last cycle
Denaturation step	94°C, 3 min	94°C, 30 s	94°C, 30 s
Annealing step	58°C, 1 min	58°C, 1 min	58°C, 1 min
Primer extension step	72°C, 1 min	72°C, 1 min	72°C, 5 min

The PCRs were kept at –20°C for 3% agarose gel electrophoresis.

4. Agarose gel electrophoresis

Agarose gel preparation

A gel tray (9x12x1 cm^3) and three combs with teeth 3 mm wide and 1 mm thick were used. Two grams of agarose was boiled in 66 ml of 0.5 x TBE in an Erlenmeyer flask that was covered. The agarose solution was poured

into the tray with the three combs when the solution cooled down somewhat. After the gel hardened, the combs were pulled out. Three gels about 3 cm long were obtained by cutting out two gel units from the agarose block.

Electrophoresis

Agarose gels were soaked in 0.5 x TBE containing ethidium bromide (0.5 μg/ml) for 5 minutes and horizontal electrophoresis was performed in 0.5 x TBE. In the meantime 8 μl PCR products were mixed with 1 μl of loading buffer. Electrophoresis of the PCR products was carried out at 80 mA for 30 minutes or until the bromophenol blue marker reached the end of the gel. The DNA bands were detected by UV light and documented using a Biorad gel doc 1000 system.

STEP 2

The optimal PCR conditions were applied for the prospective screening for α-thalassemia-1 heterozygotes in expecting couples. During a 12-month period, 3,347 cases were prescreened using the erythrocyte osmotic fragility test (EOF). Of 1,064 cases which had EOF values <60% were analyzed by using PCR. And also 29 cases of amniotic fluid were analyzed.

STEP 3

For testing the sensitivity of the PCR system amplifications were performed with serial dilutions of DNA extracts in PCR buffer. Since whole blood contains 5,000 white blood cells per μl (Kawasaki, 1990), 30 μl of whole blood will contain about 150,000 nucleated cells. The final volume of

DNA extraction by chelex-100 method was approximately 200 µl, thus the 750 nucleated cells contained in 1 µl of template DNA. The template DNA was diluted in PCR buffer until contained approximately 1 nucleated cell in 1 µl. The dilutions of nucleated cells were shown below :

1	750 nucleated cells
1: 2	375 nucleated cells
1: 10	75 nucleated cells
1:100	8 nucleated cells
1:1,000	1 nucleated cell

STEP 4

1. Optimum concentration of proteinase K to digest one cell in PEP step

Assuming that a single cell contains approximately 1 ng of protein. The 10-200 ng/µl of proteinase K was used to digest the excess amount of BSA (10 ng). The complete digestion (in range of 100-200 ng/µl) was shown by 12% sodium dodecyl sulphate polyarylamide gel electrophoresis (SDS-PAGE).

2. Amplification from amniocytes

Since the number of blastomeres was limited, the amniocytes were used in the following procedure. The PEP was performed according to the method of Zhang et al., 1992 and then PCR for detection of the α-

thalassemia-1 haplotype was done by using the optimized conditions from step 1. The DNA contamination was checked by a mock DNA extraction without cells and this resulting extract was used as negative control in PCR. Figure 9 was shown the procedure of step 4 and 5 as a diagram.

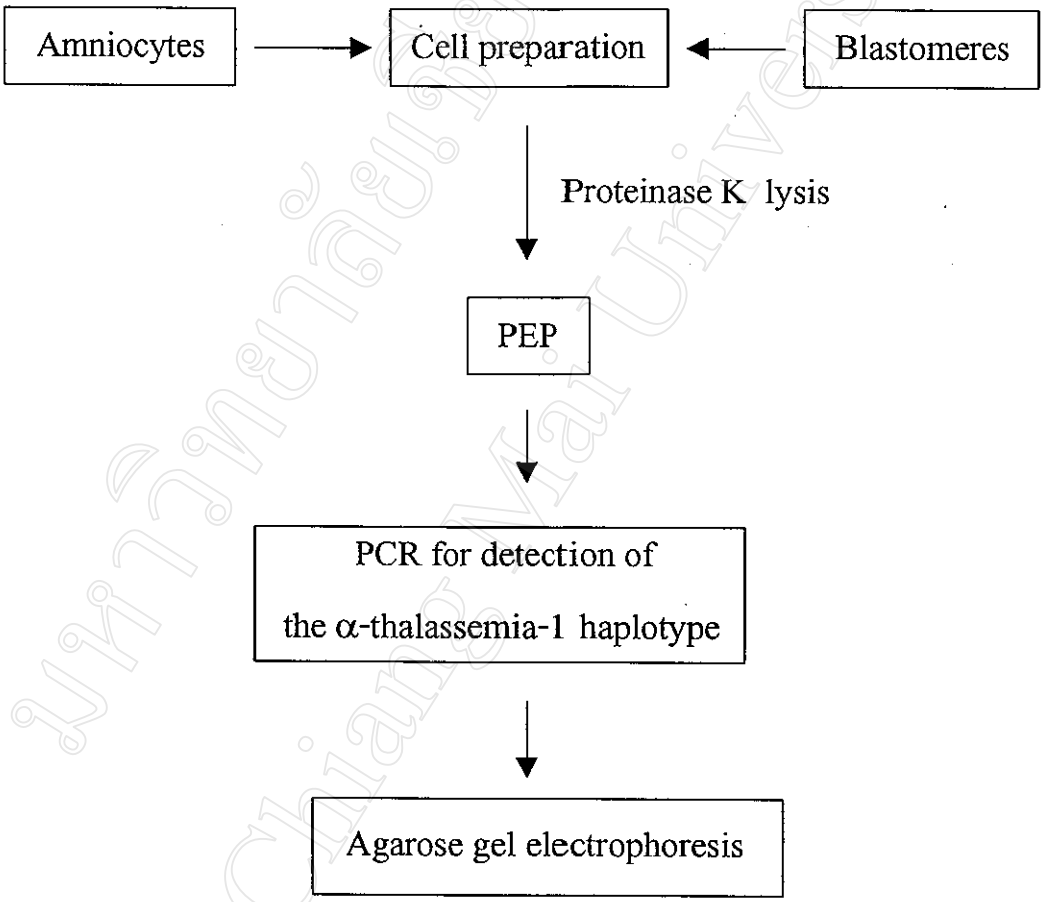

Figure 9 Diagram of step 4 and 5 procedure

Cell preparation

Amniotic fluid was obtained by amniocentesis. One milliliter of amniotic fluid was diluted with distilled water. The cells from that suspension were counted under an inverted microscope and a concentration of 1-2 cells

per μl was adjusted. Five microliters from this suspension (5-10 cells) were transferred into a 0.2 ml thin-wall PCR tube and stored at –20°C until used in PCR experiments.

Primer-extension-preamplification (PEP) and polymerase chain reaction

Random 15 base oligonucleotides were synthesized at Bio Service Unit, Department of Biochemistry, Mahidol University, Bangkok, Thailand and used in PEP. About 5-10 amniocytes in a 0.2 ml thin-wall PCR tube were lysed with 2 μl of 1 μg/ μl proteinase K dissolved in 1% Triton X-100. The volume of the suspension was adjusted to 10 μl with distilled water. The tube was incubated at 37°C for 30 minutes. Proteinase K was inactivated at 95°C for 10 minutes. When the tube cooled down was added :

H_2O	13.4	μl
10 x PCR buffer	6.0	μl
dNTPs (1 mM each)	6.0	μl
Random primers (93.2 μM)	21.2	μl
$MgCl_2$ (25 mM)	2.4	μl
Taq DNA polymerase (5 units/μl)	1.0	μl
	Sum = 60.0	μl

Fifty cycles of PEP were performed.

Step	Temperature/ time
Denaturation step	94 °C, 1 min
Annealing step	37 °C, 2 min
	ramping 37-55 °C, 10 s/degree
Primer extension step	55°C, 4 min

The PEP products were immediately amplified with specific primers for detection of the α-thalassemia-1 (SEA) haplotype or stored at -20°C until use.

One microliters aliquot of PEP product was transferred to fresh tubes containing the same reagent mixture for PCR in step 1. The 40 cycles of amplification were performed. The PCR products were analyzed by 3% agarose gel electrophoresis.

STEP 5

Using the same method as amniocytes step 4 performed detection of the α-thalassemia-1 (SEA) haplotype from blastomeres. Various numbers of 0.2 ml thin wall PCR contained blastomere embryonic cells, obtained by IVF method from the Department of Obstetrics and Gynecology, Faculty of Medicine, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University, Chiang Mai, Thailand. The DNA contamination was checked by a mock DNA extraction without cells and this resulting extract was used as negative control in PCR. The PCR products were analyzed by 3% agarose gel electrophoresis.