CHAPTER 3

RESULTS

3.1 Genomic DNA preparation

The 400 genomic DNA samples were extracted from peripheral venous blood. The yield was approximate 120-180 μg / ml. The OD 260 / OD 280 ratio was between 1.80-1.95.

3.2 Polymerase chain reaction

3.2.1 Optimization of the PCR component

The optimization of PCR reaction such as the concentration of deoxynucleotide 5'- triphosphates, the primer annealing temperature, and the extension times were tritrated to provide the best results. A clear single band at 1.76 kb of amplification products had been observed without any complications neither nonspecific background bands nor the formation of primer - dimer.

In this experiment , the concentrations of deoxynucleotide 5' - triphosphates were varied at 200, 300 , 600 μM (figure. 14) . The best result was found at 200 μM without the formation of primer- dimer, the primer - dimer had been observed at 300 and 600 μM.

The annealing temperatures were varied from 55°C to 70°C (58°C, 62°C and 68°C shown in figures 16.), the best result was found at 68°C.

The extension times were varied for 2.30 , 5 and 7 minutes (figure 15.). The expected bands present in all of these time but the best had been observed in 2.30 minutes without non-specific bands.

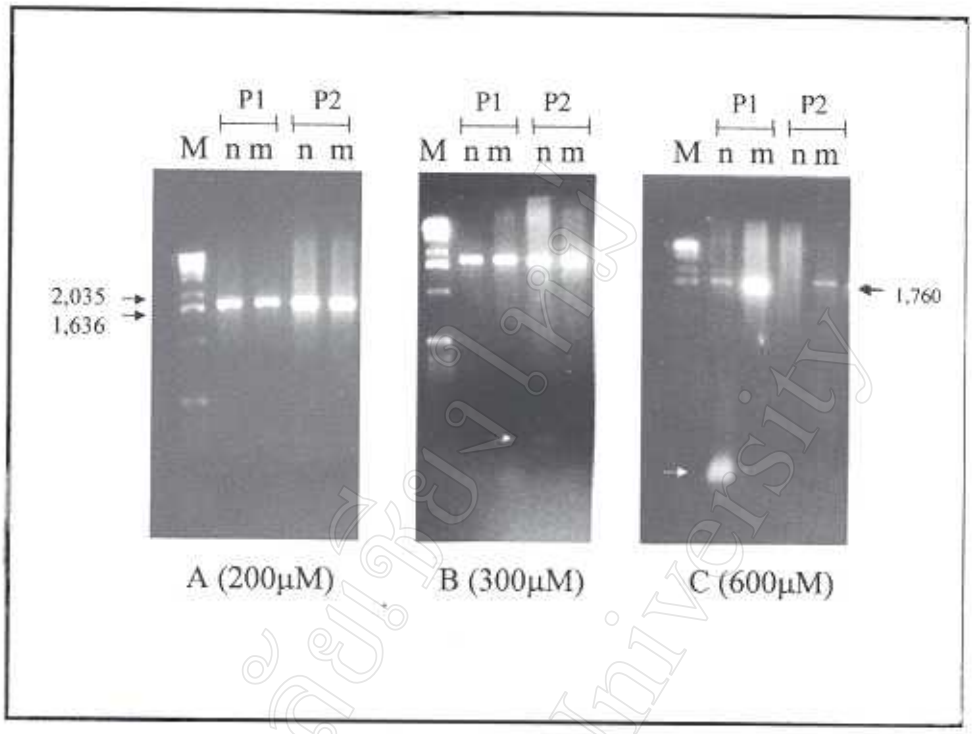

Figure 14. The optimization of the deoxynucleotide 5' -triphosphates concentrations for detection of $-\alpha^{3.7}$ kb deletion by PCR.

The the deoxynucleotide 5' - triphosphates concentrations were adjusted to provide the best result . The concentrations were 200 μM in A , 300 μM in B and 600 μ M in C. The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes n were obtained with AB pair of primers (amplified normal gene) and lanes m were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion). The arrows indicate the bands of primer-dimer

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

P1-P2 = positive control ($-\alpha^{3.7}/\alpha\alpha$)

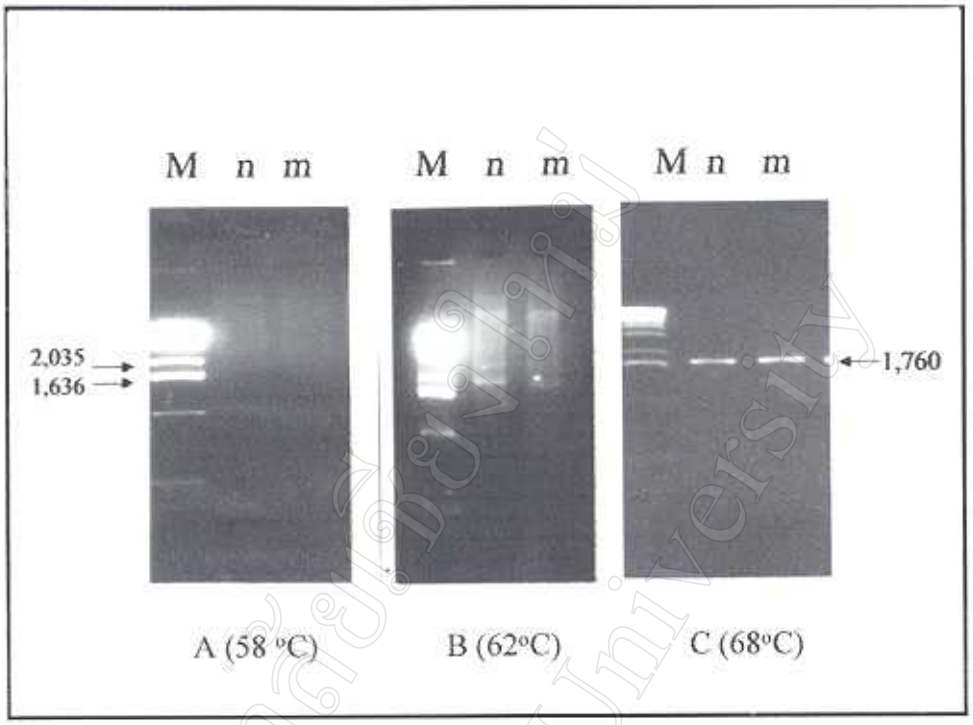

Figure 15. The optimization of the annealing temperature for detection of $-\alpha^{3.7}$ kb deletion by PCR.

The annealing temperatures were adjusted to provide the best result. A was 58° C, 62° C in B , and 68° C in C. The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes n were obtained with AB pair of primers (amplified normal gene) and lanes m were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers
n = normal primers (A+B)
m = mutant primers (A+C)

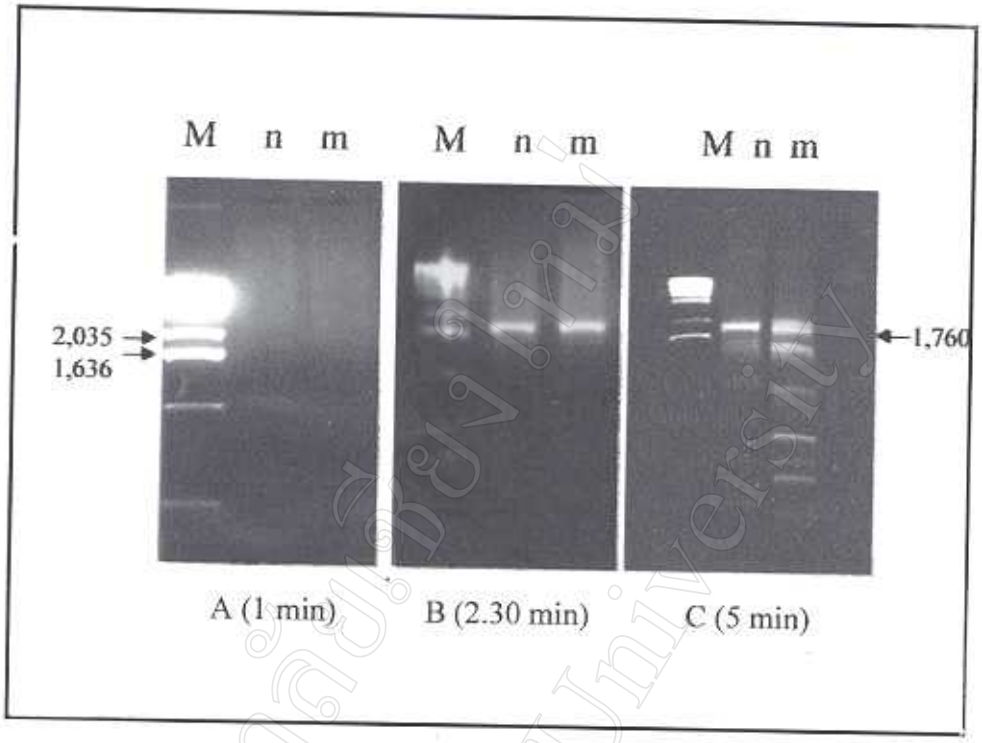

Figure 16. The optimization of extension time for detection of $-\alpha^{3.7}$ kb deletion by PCR technique.

The extension times were adjusted to provide the best result at 1,760 kb. In A the extension time was 5 minutes and 2.30 minutes in B. The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes n were obtained with AB pair of primers (amplified normal gene) and lanes m were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

3.2.2 The identification of $-\alpha^{3.7}$ genotype by PCR technique

This study described a PCR base method for diagnosis of the most common from of α-thalassemia 2, rightward type ($-\alpha^{3.7}$). The samples can be characterized by comparing appearance and disappearance of 1.76 kb bands on gel. The whole results were concluded in figure 17. The PCR products of normal α 2 gene , which were amplified by AB primer pairs are shown in lane "n" and alpha 37 kb deletion which were amplified by AC are shown in lane 'm" . The presence of 1.76 kb bands in both " n" and "m" can be identified the heterozygous of alpha 3.7 kb deletion ($-\alpha^{3.7}$ / αα, P in lane 2, 3). In the homozygous of alpha 3.7 kb deletion ($-\alpha^{3.7}/-\alpha^{3.7}$, Ho in lane 8, 9) and Hb H disease ($--/-\alpha^{3.7}$, H in lane 14,15), the bands of 1.76 kb were observed in " m " only. However, Hb H disease can be simply confirmed by hemoglobin typing (figure 27) and inclusion bodies test (figure 29). In this study, the genotypes in 5 cases of the heterozygous of alpha 3.7 kb deletion and 4 cases of Hb H disease were confirmed by detection of alpha thalassemia 1 by PCR technique in Dr. Luksana Makonkawkeyoon ' s laboratory (results not show).

It was difficult to identify the genotype of the sample which was negative in $-\alpha^{3.7}$. When 1.76 kb band was observed in " n" only, the results may be interpreted to (αα / αα), (—— / αα), (αα / $\alpha^T\alpha^T$) or (αα / $\alpha^T\alpha$) , so that results were reported to (αα /) haplotype.

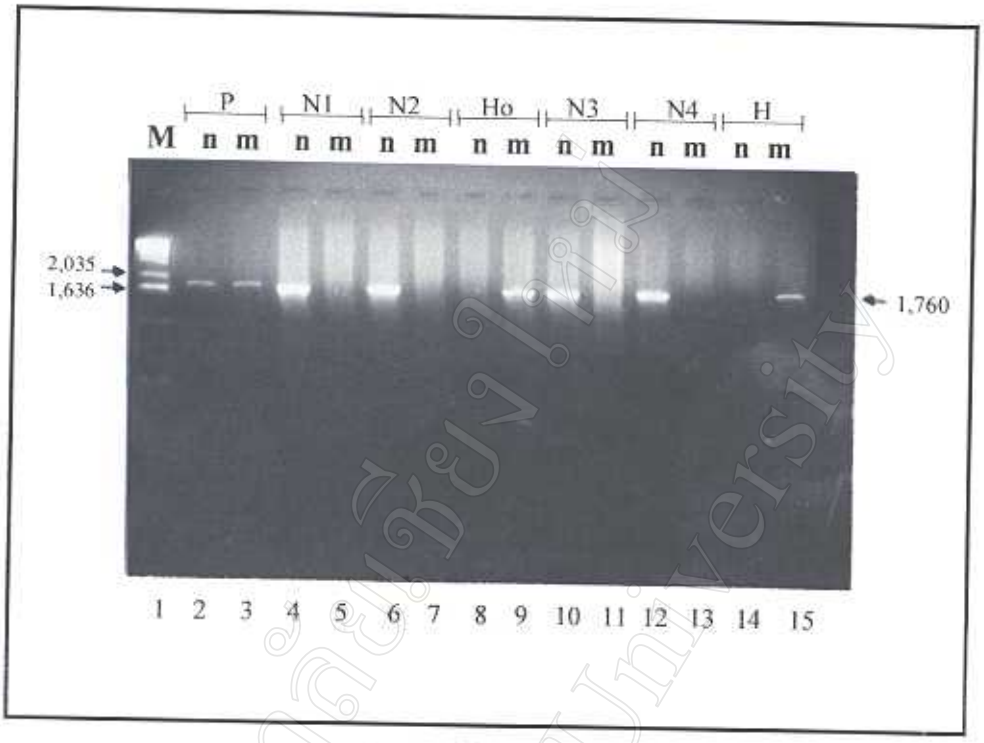

Figure 17. Identification of $-\alpha^{3.7}$ kb deletion in genomic DNA samples by PCR technique.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8, 10 , 12 and 14 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11 , 13 and 15 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

P = ($-\alpha^{3.7}$ / $\alpha\alpha$)

N1-N4 = ($\alpha\alpha$ / $\alpha\alpha$)

Ho = ($-\alpha^{3.7}$ / $-\alpha^{3.7}$)

H = (— / $-\alpha^{3.7}$)

3.2.3 The reliability of the PCR protocol

The reliability of the PCR protocol was checked by identification of the α - globin genotypes in 20 normal genomic DNA samples (samples number 1-7 are shown in figure 18) and 10 heterozygous of the alpha 3.7 kb deletion (samples number 1-7 are shown in figure 19-20) .The genotypes of all samples had been identified by Southern blot hybridization (Makonkawkeyoon et al., 1993a). The results have shown that this method can identify the α-thalassemia 2, rightward type ($-\alpha^{3.7}$) without false positive and false negative.

3.2.4 Detection of $-\alpha^{3.7}$ deletion in two Hb H disease family

The two family of the Hb H children were subjected for identification of the $-\alpha^{3.7}$ phenotype. In the first family, the father possessed of AB primers pair, the mother possessed of both AB and AC while the child possessed in AC (figure 18). Second family , the father possessed of both AB and AC primer pairs, the mother possessed of AB and the child possessed of AC (figure 19). All samples were identified the alpha thalassemia1 by PCR technique (Makonkawkayoon et al., 1993b). The genotypes could be identified as follows. In the first family, the father was heterozygosity of alpha thalssemia 1, the mother was heterozygosity of alpha thalassemia 2 ($-\alpha^{3.7}$ / αα) and the child was Hb H disease (-- / $-\alpha^{3.7}$). In the second family, the father was heterozygosity of alpha 3.7 kb deletion, the mother was heterozygosity of alpha thalssemia 1, and the child was Hb H disease (-- / $-\alpha^{3.7}$).

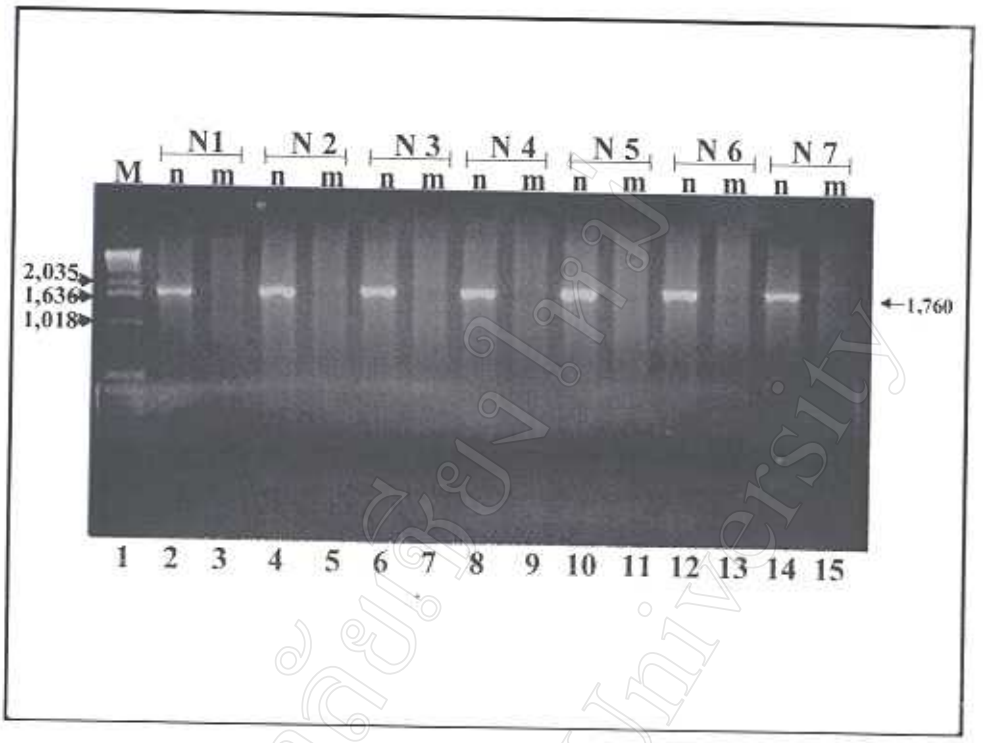

Figure 18. Detection of $-\alpha^{3.7}$ kb deletion in the normal DNA samples by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8,10, 12 and 14 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11, 13, and 15 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

N 1- N 7 = normal DNA sample ($\alpha\alpha$ / $\alpha\alpha$) number 1 to number 7

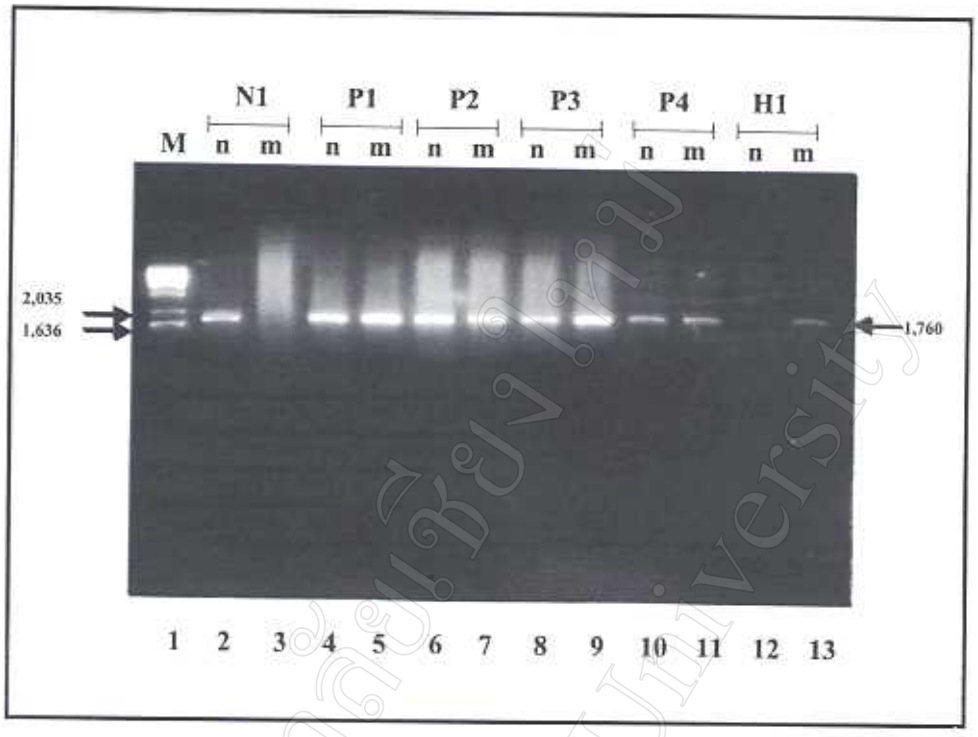

Figure 19. Detection of the $-\alpha^{3.7}$ kb deletion in normal and positive control genomic DNA by PCR technique.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8, 10, and 12 were obtained with AB pair of primers (amplified normal gene) and lanes 3,5, 7, 9,11, and 13 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

N 1 = normal control genomic DNA ($\alpha\alpha$ / $\alpha\alpha$)

P1-P4 = positive control genomic DNA ($-\alpha^{3.7}$ / $\alpha\alpha$)

H 1 = genomic DNA of Hemoglobin H disease (---/ – $\alpha^{3.7}$)

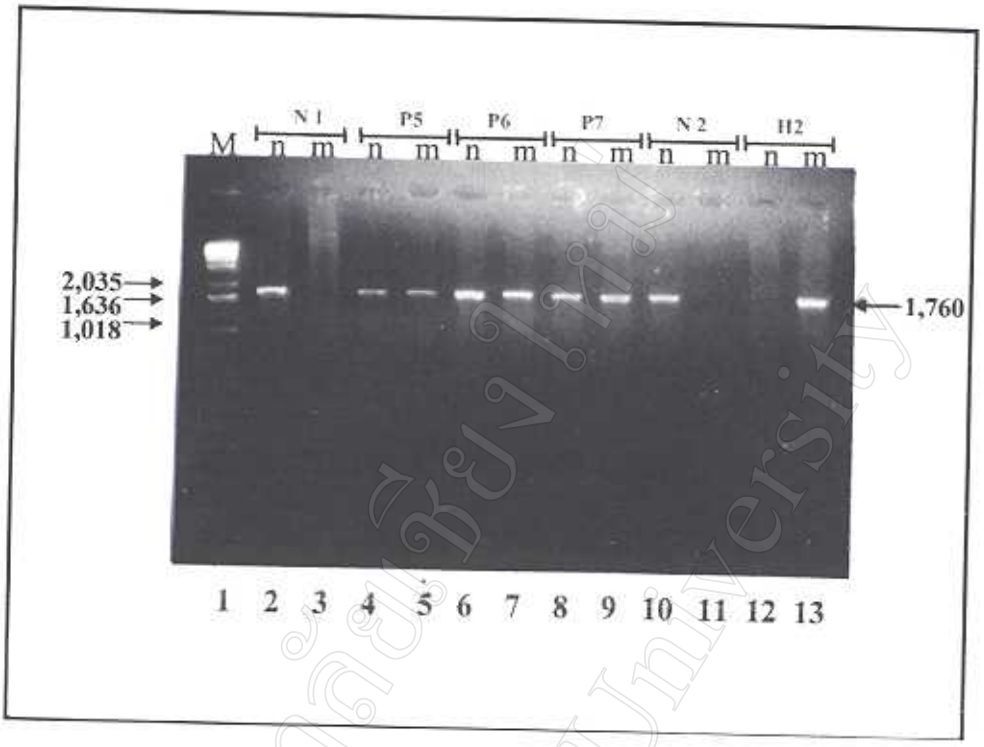

Figure 20. Detection of the $-\alpha^{3.7}$ kb deletion in normal and positive control genomic DNA by PCR technique.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8,10, and 12 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11, and 13 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

N 1 -2 = normal control genomic DNA ($\alpha\alpha$ / $\alpha\alpha$)

P5-P7 = positive control genomic DNA ($-\alpha^{3.7}$ / $\alpha\alpha$)

H 2 = genomic DNA of Hemoglobin H disease (—/ – $\alpha^{3.7}$)

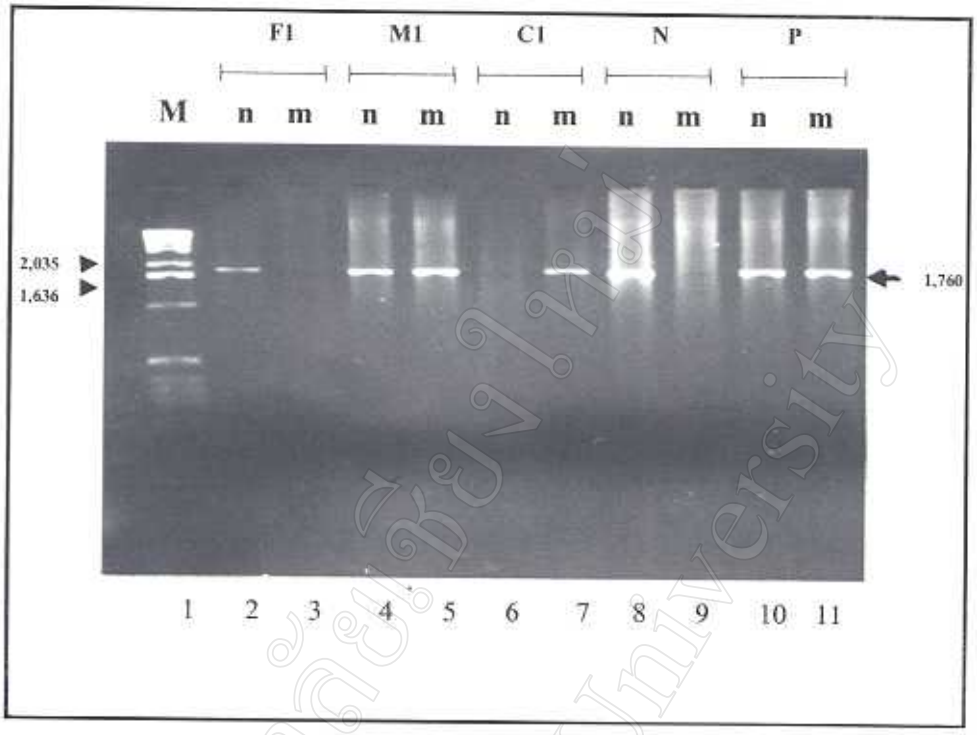

Figure 21. Detection of $-\alpha^{3.7}$ kb deletion in the Hb H family by PCR technique.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, and 8 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9 and 11 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers
n = normal primers (A+B)
m = mutant primers (A+C)
F1 = father of the first family ($--/\alpha\alpha$)
M1 = mother of the first family ($-\alpha^{3.7}/\alpha\alpha$)
C1 = child of the first family ($—/-\alpha^{3.7}$)
N = normal control ($\alpha\alpha/\alpha\alpha$)
P = positive control ($-\alpha^{3.7}/\alpha\alpha$)

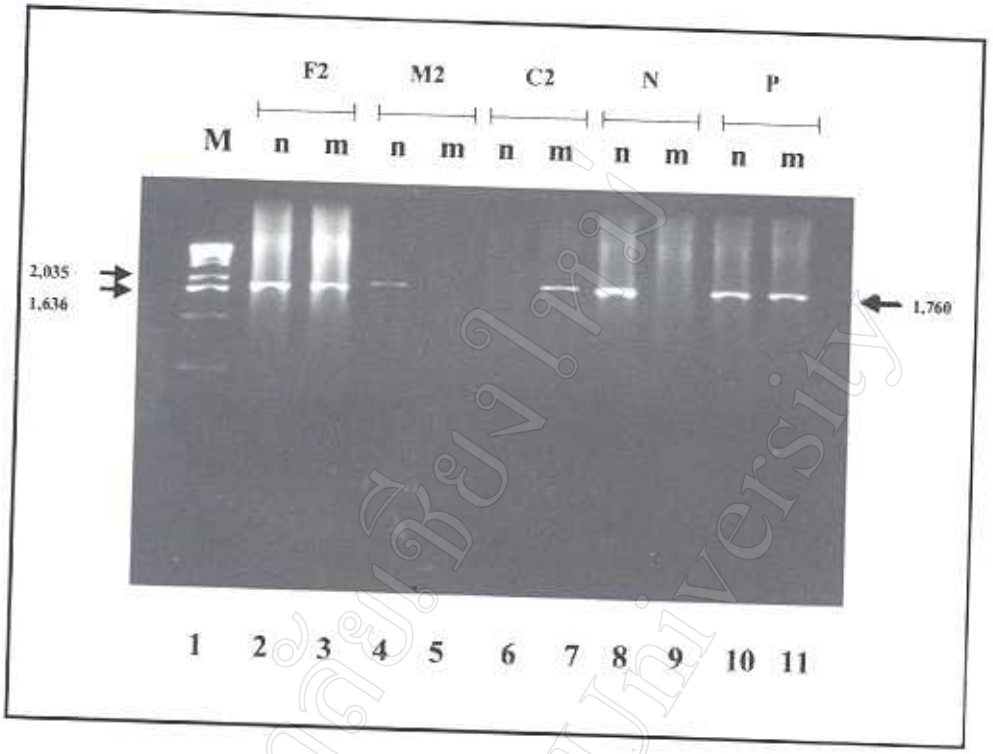

Figure 22. Detection of $-\alpha^{3.7}$ kb deletion in the Hb H family by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, and 8 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9 and 11 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

F2 = father of the second family ($--/\alpha\alpha$)

M2 = mother of the second family ($-\alpha^{3.7}/\alpha\alpha$)

C2 = child of the second family ($—/-\alpha^{3.7}$)

N = normal control ($\alpha\alpha/\alpha\alpha$)

P = positive control ($-\alpha^{3.7}/\alpha\alpha$)

3.2.5 Detection of $-\alpha^{3.7}$ deletion in Northern Thai population by PCR technique

In this study, 400 genomic DNA samples were determined the $-\alpha^{3.7}$ deletion by PCR technique. It has been found that 333 samples possessed of AB primers pair, 58 possessed of both AB and AC, and 9 possessed of AC. In 9 cases which were possessed of AC, the alpha thalassemia 1 were also determined by PCR technique in Dr. Luksana Makonkawkeyoon's laboratory (results not shown). Out of these, 4 cases were possessed in the alpha thalassemia1 , so that the genotype could be interpreted as hemoglobin H disease ($--/-\alpha^{3.7}$). While in 5 cases , which were negative in alpha thalassemia1, the genotype could be interpreted as heterozogosity of alpha 3.7 kb deletion ($-\alpha^{3.7}/-\alpha^{3.7}$). The results were summarized in table 3.

Genotype	subjects	relative frequencies
$-\alpha^{3.7}/\ \alpha\alpha$	58	14.5%
$-\alpha^{3.7}/-\alpha^{3.7}$	5	1.25 %
$--/-\alpha^{3.7}$	4	1 %
negative $-\alpha^{3.7}$	333	83.25 %

Table 3. The results from the detection of alpha 3.7 kb deletion in 400 genomic DNA samples from Northern Thai population by PCR technique.

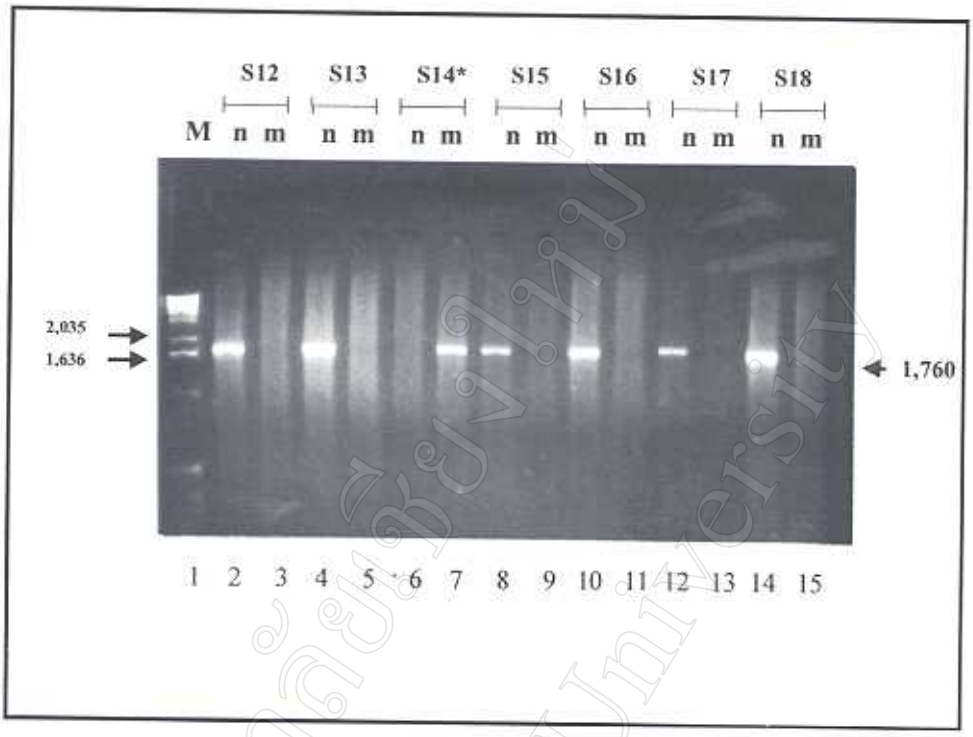

Figure 23. Detection of $-\alpha^{3.7}$ kb deletion in genomic DNA samples by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8, 10 , 12 and 14 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11 , 13 and 15 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

S12, S13, S15, S16, S17 and S18 = genomic DNA samples ($\alpha\alpha$ /)

S14* = genomic DNA samples (—— /– $\alpha^{3.7}$)

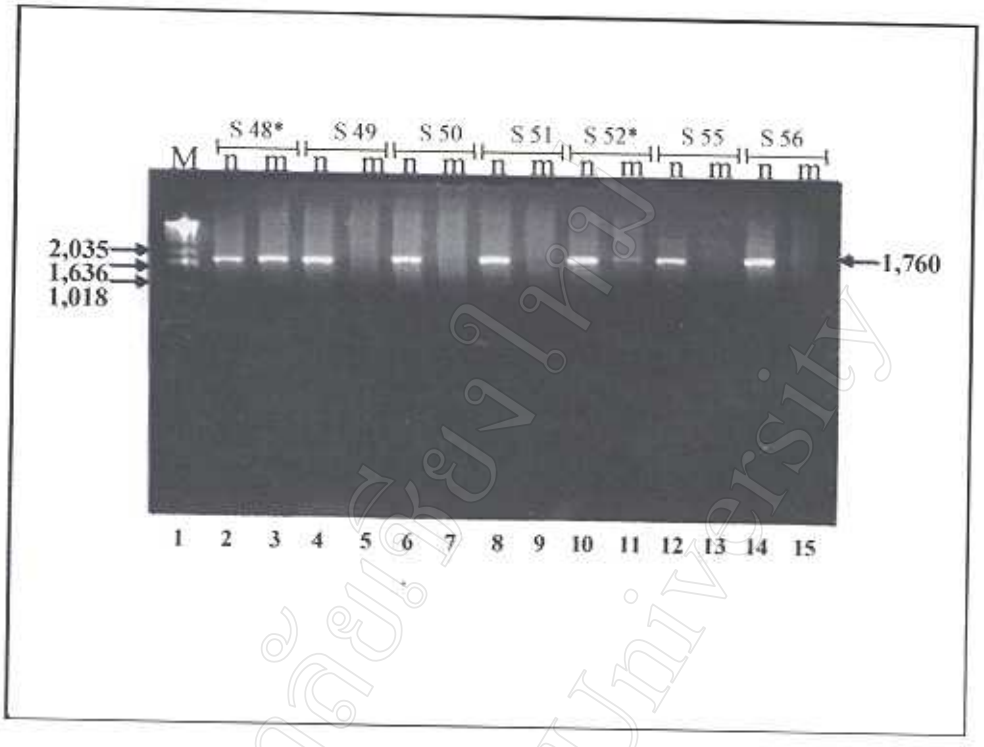

Figure 24. Detection of $-\alpha^{3.7}$ kb deletion in genomic DNA samples by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8, 10 , 12 and 14 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11 , 13 and 15 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

S49, 50 , 51, 55 and 56 = genomic DNA samples ($\alpha\alpha$ /)

S48*, S52* = genomic DNA samples ($-\alpha^{3.7}$ / $\alpha\alpha$)

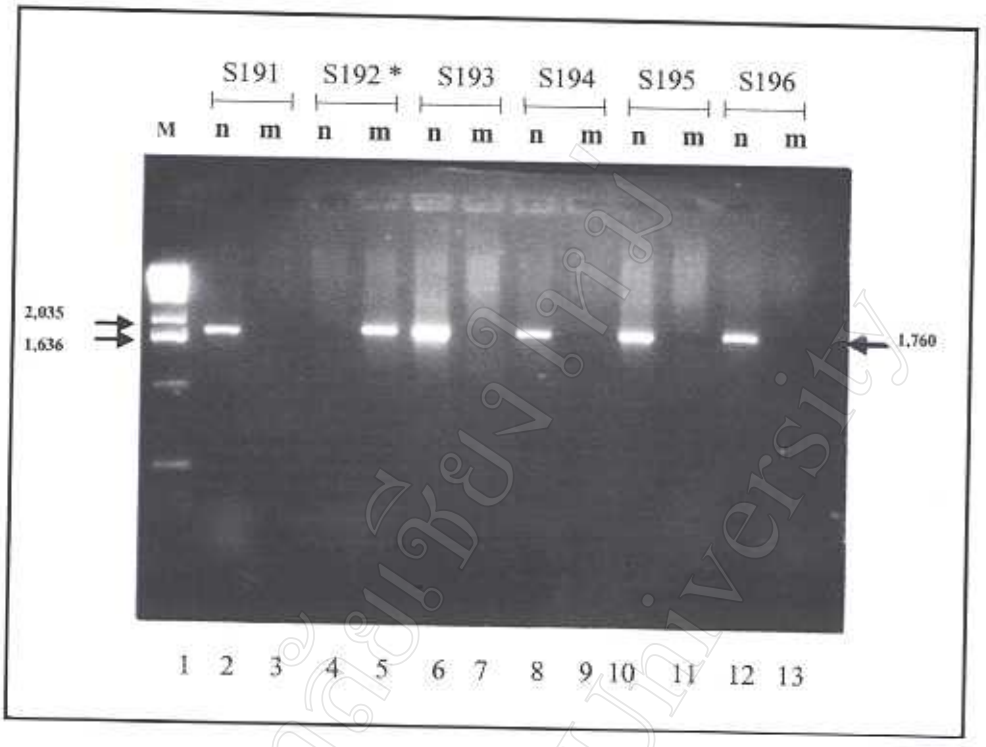

Figure 25. Detection of $-\alpha^{3.7}$ kb deletion in genomic DNA samples by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8 ,10 , and 12 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9,11, and 13 were obtained with AC pair of primers(amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

S191, S193 ,S194,S195 and S196 = genomic DNA samples ($\alpha\alpha$ /)

S192* = genomic DNA samples ($-\alpha^{3.7}$ / $-\alpha^{3.7}$)

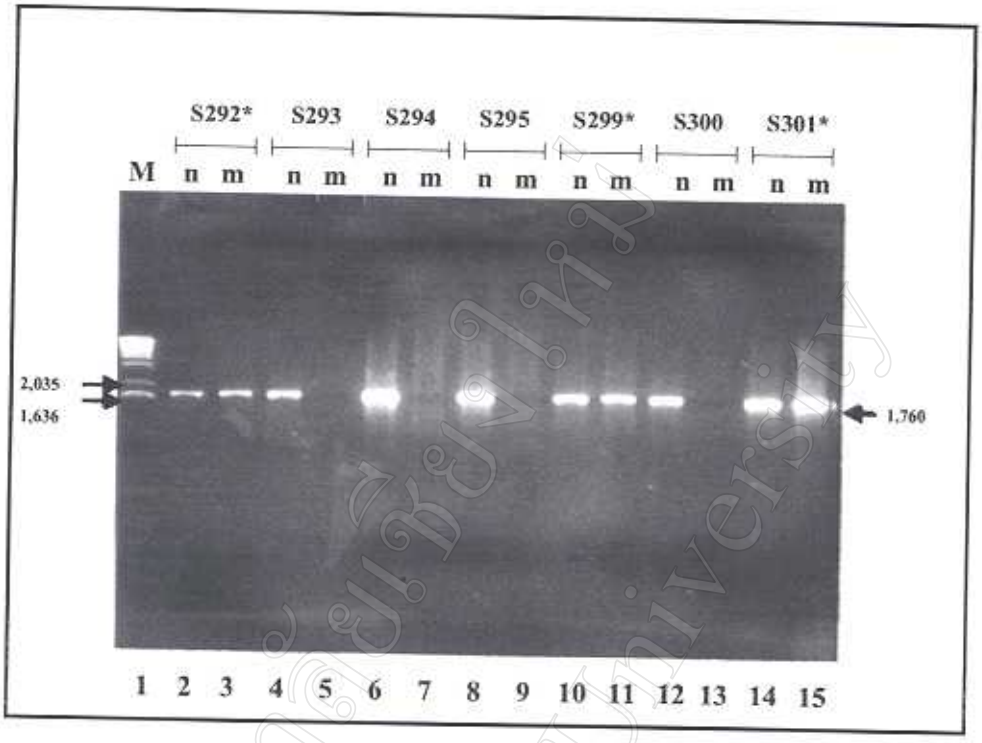

Figure 26. Detection of $-\alpha^{3.7}$ kb deletion in genomic DNA samples by PCR.

The PCR products were subjected to electrophoresis on 1.5 % agarose gel in 0.5 X TBE buffer. The first lane contains DNA size markers. The PCR products in lanes 2, 4, 6, 8, 10 , 12 and 14 were obtained with AB pair of primers (amplified normal gene) and lanes 3, 5, 7, 9, 11 , 13 and 15 were obtained with AC pair of primers (amplified $-\alpha^{3.7}$ kb deletion).

M = DNA size markers

n = normal primers (A+B)

m = mutant primers (A+C)

S293, S294 ,S295,S300 and S301 = genomic DNA samples ($\alpha\alpha$ /)

S292*, S299*, S301* = genomic DNA samples ($-\alpha^{3.7}$ / $\alpha\alpha$)

3.3 Results from hematological screening

3.3.1 The hematological characteristic of α halassemia 2 ($-\alpha^{3.7}$) carriers.

The hematological data : hemoglobin (Hb)concentration, red blood cell count (RBC), percent of hematocrit (Hct), mean corpuscular volume (MCV) and osmotic fragility value (OF) from normals and - $\alpha^{3.7}$ kb deletion carriers are shown in table 4. Comparision of data according to sex did not show any statistically significant differences for MCV and OF (P= 0.05) and these data were pooled. In - $\alpha^{3.7}$ kb deletion, compared with the standard cut off of normal values, no statistical abnormalities can be detected in all parameters (P = 0.05).

sex	Hb (g/dl)	Hct (%)	RBC(x 10^9 /l)	MCV(fl)	OF
Female = 12	12.23 ± 1.12	42.21 ± 6.03	5.07 ± 0.90	86.61 ± 16.95	60.3 ± 13.2
Male =22	13.72 ± 1.36	44.23 ± 3.52	5.21 ± 0.84		

Table 4. The hematological data of the 34 blood samples of α- thalassemia 2 ($-\alpha^{3.7}$) carriers

Normal values

Hb concentration in female = 12 - 16 g/dl , in male = 14 - 18 g/dl
Hct (%) in female = 37 - 47 % , in male = 41 - 51 %
RBC = 4.5-6.0 x 10^9/l
MCV = 80-97 fl
OF = 60>100 %

3.3.2 Results from hemoglobin electrophoresis (Hemoglobin typing).

Hemoglobin types of 400 blood samples were identified by hemoglobin electrophoresis . The normal pattern of hemoglobin A and A2 were found in 370 blood samples (92.5 %). Hemoglobin A , A2 and H were presence in 4 cases of Hb H disease samples (1 %), A and A2 / E were found in heterozygous HbE of 24 blood samples (6%). The A2/E were observed in one case of homozygous Hb E (0.25 %) .One cases of β thalassemia / HbE hemoglobin pattern were A2/E and F. The results are shown in table 5 and figure 27.

	Hb type	subjects	relative frequencies
normal	A + A2	370	92.5 %
Hb H disease	A + A2 + H	4	1 %
heterozygous Hb E	A + A2/E	24	6 %
homozygous Hb E	A2/E	1	0.25 %
β thalassemia / HbE	A2+E + F	1	0.25 %

Table 5. Relative frequencies of hemoglobin types in 400 blood samples.

Figure 27. The results from hemoglobin electrophoresis on cellulose acetate. The hemolysate from 5 subjects were run with knuwns hemoglobin controls in 10 minutes at 160 volts. The controls hemoglobin, Hb A2, Hb F, Hb Bart's and Hb H were run in lane 1 and Hb A were run in lane 7.

Hb H = hemolysate from Hb H sample
N1, N2 = hemolysate from normal sample
β^o = hemolysate from β^o thalassemia sample
Hb E = hemolysate from heterozygous hemoglobin E sample
C = control hemoglobin A = hemoglobin A
A2 = hemoglobin A2 F = hemoglobin F
B = hemoglobin Bart's H = hemoglobin H

3.3.3 Result from inclusion bodies test for hemoglogin H

Hemoglobin H (Hb H) were detected and graded into 0 , trace, 1+, 2+, 3+ and 4+ . In four cases of Hb H disease subjects (--/ $-\alpha^{3.7}$), the red cell inclusion bodies were 2+ (figure 24). Hemoglobin H cloud not detectable in $-\alpha^{3.7}$ carriers and in the remaining subjects.

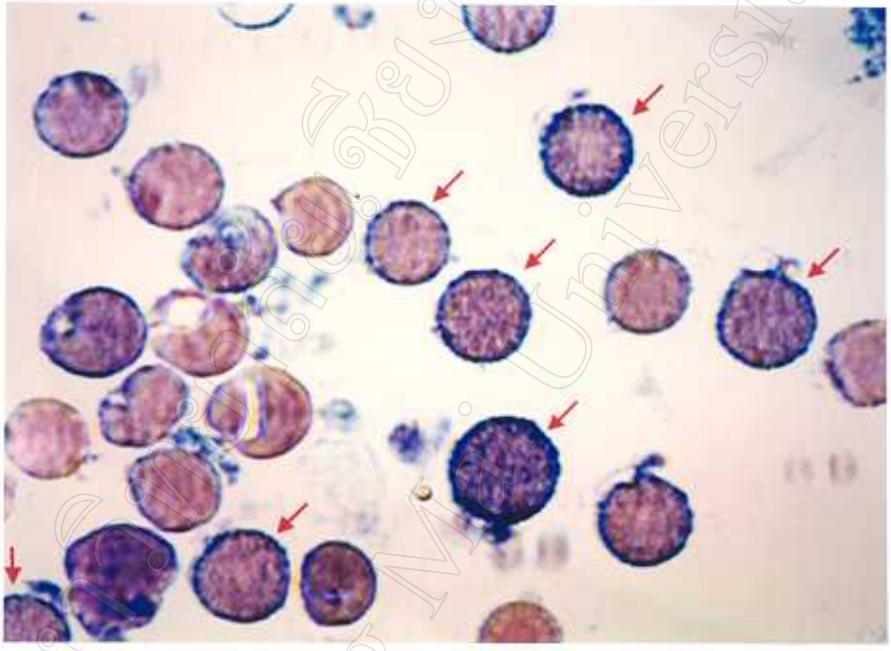

Figure 28. The red cell inclusion bodies (2+) in pheripheral blood of hemoglobin H disease subject (-- /$-\alpha^{3.7}$).

3.3.4 Result from acid elution test for hemoglobin F

Hemoglobin F (Hb F) were detected and graded into normal, trace, 1+, 2+, 3+ and 4+ (data not show). In cord blood sample, Hb F were 4+ (figure 29).

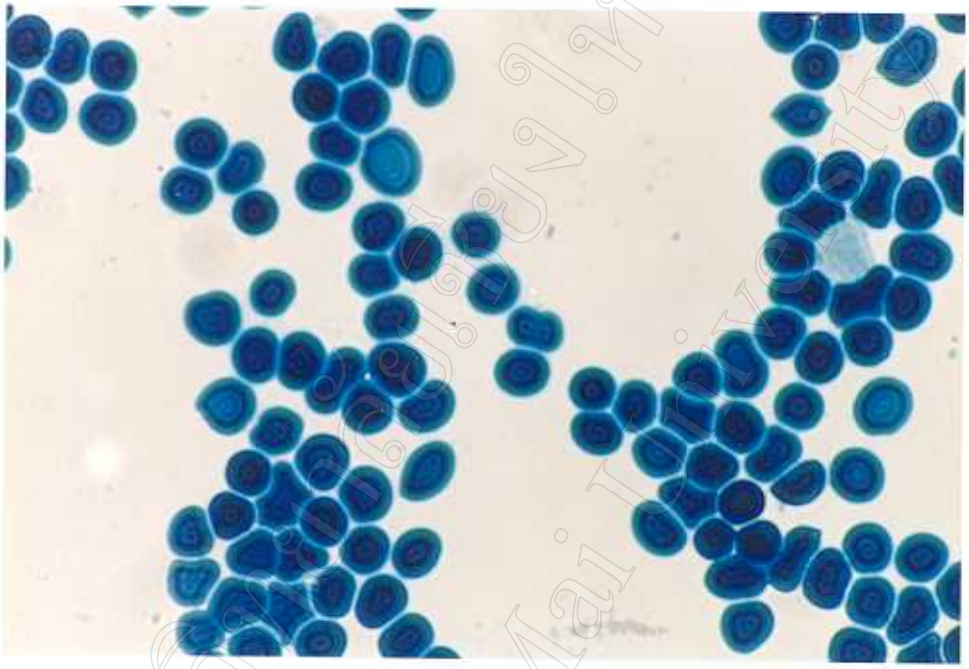

Figure 29. Acid elution preparation of blood film shows hemoglobin F (4+) from cord blood sample.