CHAPTER 2

LITERATURE REVIEWS

2.1 Structure and function of human hemoglobins

Hemoglobin is a red blood pigment. The major function of hemoglobin is the transport of oxygen from the lungs to the body tissues ; an associated function is binding of carbon dioxide and protons by deoxyhemoglobin, thereby serving to buffer the blood on a venous side of the circulation. The hemoglobin is a tetramer with a molecular weight of 64,458 (PhillipsIII and Kazazian, 1984). It is composed of four subunits, each containing a protein globin chain and iron-porphyrin heme moiety. The normal hemoglobin molecule contains two difference types of globin chains, α-like globin chain and β-like globin chain. The α-like globin chains contain 141 amino acids, while the β-like globin chains contain 146 amino acids, which are synthesized from α-globin gene cluster on chromosome 16 and β-globin gene cluster on chromosome 11 (Deisseroth *et al.*, 1978), respectively (Figure 1). Normal hemoglobins and their globin subunits are listed in Table 1.

Table 1 Normal human hemoglobins and their globin subunits (Honig and Adams III, 1986).

Hemoglobin	α-like subunit	β-like subunit	Tetramer composition
Hb A	α	β	$\alpha_2\beta_2$
Hb A_2	α	δ	$\alpha_2\delta_2$
Hb F	α	$^{G}\gamma, ^{A}\gamma$	$\alpha_2\gamma_2$
Hb Gower-2	α	ε	$\alpha_2\varepsilon_2$
Hb Gower-1	ζ	ε	$\zeta_2\varepsilon_2$
Hb Portland	ζ	γ	$\zeta_2\gamma_2$

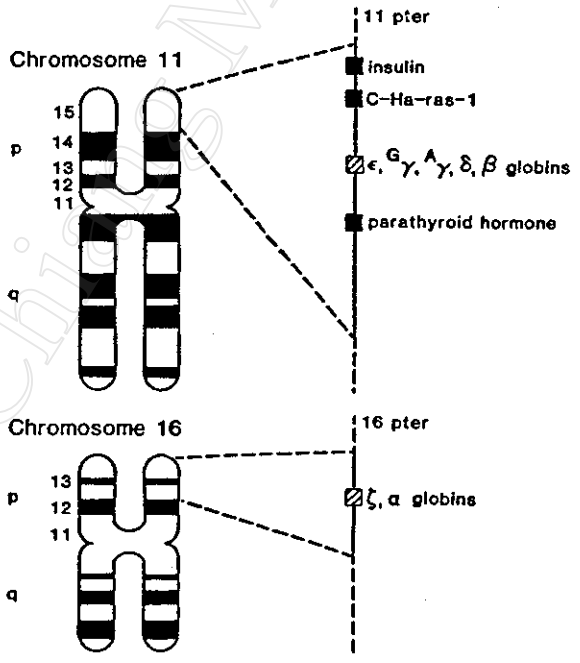

Figure 1 The locations of the β-globin gene group on chromosome 11 and the α-globin genes on chromosome 16 (Honig and AdamsIII, 1986).

The earliest recognizable hemoglobin-containing cells in the human embryo appear during the fourth week of gestation, arising from mesenchyme of yolk sac origin. In embryos of less than 6 weeks gestation Hb Gower-1 ($\zeta_2\varepsilon_2$) and Hb Gower-2 ($\alpha_2\varepsilon_2$) predominate and may account for as much as 66% of the total hemoglobin with Hb Portland ($\zeta_2\gamma_2$) representing as much as 20%. By 8 weeks of gestation, the synthesis of Hb F ($\alpha_2\gamma_2$) increases rapidly, and accounts for at least 90% of hemoglobin in the erythrocytes and remains the predominant hemoglobin form through fetal life and in the neonatal period. Hemoglobin F in normal individuals is a mixture of two molecular species in which the γ-chains have either glycine ($^{G}\gamma$) or alanine ($^{A}\gamma$) at position 136 (Weatherall and Clegg, 1982). The usual ratio of $^{G}\gamma$:$^{A}\gamma$ at birth is about 7:3, and this ratio characteristically reverses to about 2:3 by 6 months of age, where it remains throughout adult life. Hemoglobin A ($\alpha_2\beta_2$) is undetectable before 8-10 weeks of gestation and thereafter accounts for 4-13% of total hemoglobin synthesis. The major switch from Hb F to Hb A takes place in the first few weeks after birth, with Hb F falling to less than 3% of the total by 6 months of age and reaching levels of less than 2% by one year. Hemoglobin A_2 ($\alpha_2\delta_2$) is present in the blood only in trace amounts at birth and increases to 2-3% of the total hemoglobin by one year of age (Figure 2). In normal adult, Hb A makes up about 95% of the total hemoglobin (Rapaport, 1987).

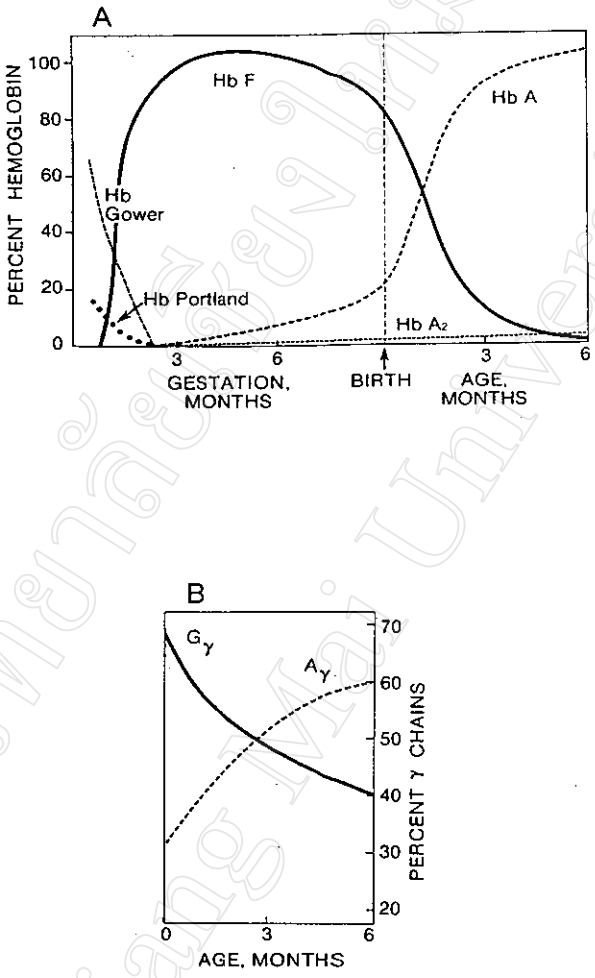

Figure 2 The changes in hemoglobin composition during gestation and development. (A) The hemoglobins of the human embryo, fetus and infant. (B) The changes in the ratio of the $^{G}\gamma$ and $^{A}\gamma$ subunits of Hb F in the early months of life (Honig and Adams III, 1986).

2.2 Molecular genetics and expression of the human α-globin gene cluster

The human α-globin gene cluster, about 40 kb, is localized to the short arm of chromosome 16 (Figure 1). This gene complex includes the duplicated α-genes (α_2 and α_1) (Orkin, 1978), an embryonic α-like gene (ζ_2), several pseudogenes ($\psi\alpha_1$, $\psi\alpha_2$, $\psi\zeta_1$) and a gene of undetermined function (θ_1) arranged in the order 5′-ζ_2-$\psi\zeta_1$-$\psi\alpha_2$-$\psi\alpha_1$-α_2-α_1-θ_1-3′. Serveral regions of the cluster contain tandemly repeated segments of DNA (minisatellites), GC-rich sequences. Organized in hypervariable regions (HVRs) located at 3′-end of complex (α-globin 3′-HVR), between the ζ_2- and $\psi\zeta_1$-genes (interzeta-HVR), within the introns (IVS1 and IVS2) of the ζ-like genes (ζ-intron HVRs) and 5′-end of complex (α-globin 5′-HVR) (Hsu *et al.*, 1988 ; Higgs *et al.*, 1989). The organization of the α-globin complex is shown in Figure 3.

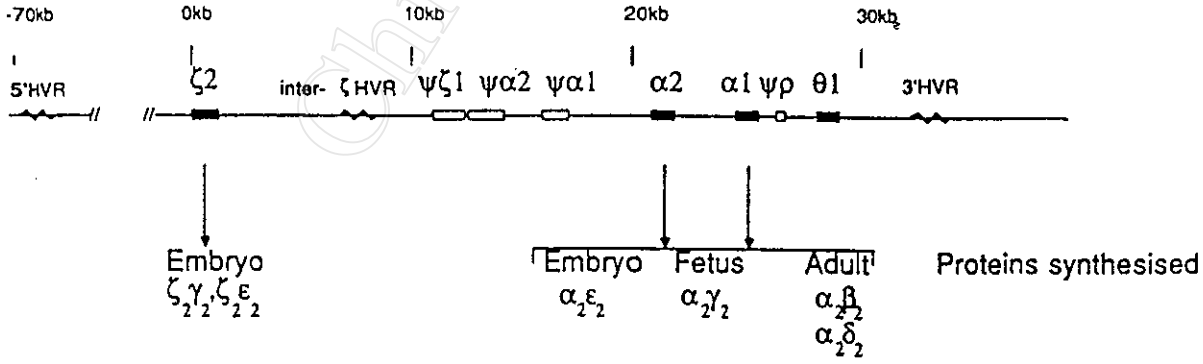

Figure 3 The organization of the α-globin gene complex (Higgs *et al.* ,1989).

The expression of globin genes is regulated during development and differentiation. An embryonic ζ-globin gene, ζ_2, and a pair of almost identical α-globin genes (α_2 and α_1) are expressed in the fetal liver and the adult bone marrow. These active genes are separated by pseudogenes in the gene cluster ; $\psi\zeta_1$ is very similar to ζ_2 but has a premature termination codon, $\psi\alpha_2$ lacks a promotor for transcription by RNA polymeraseII, and $\psi\alpha_1$ is defective in several aspects, including splice junction mutations and premature termination codons. Thus these pseudogenes can not encode a globin polypeptide (Hardison *et al.*, 1986).

Each globin gene includes three exons (coding sequences) separate by two introns (intervening sequences). Comparison of the α-globin gene sequence at the α_1- and α_2-loci reveals no differences in the 5′-flanking region, the 5′-nontranslated segment, the first exon, or the IVS1. Exons 2 and 3 each contain one silent third-base substitution. Codon 54 (Gln) is encoded by CAG in α_1-globin gene and by CAA in α_2-globin gene, and codon 123 (Ala) by GCC in α_1-globin gene and GCT in α_2-globin gene. The IVS2 of the α_1-globin gene is nine bases longer and differs by three bases from the same region of the α_2-globin gene. Position 55 of IVS2 is T in α_2-globin and G in α_1-globin. Comparison of the 3′-noncoding region of the α_1- and α_2-genes defines 18 base differences plus a single-base insertion or deletion, making this region only 84% homologous. The high degree of homology between the α_1- and α_2-globin loci indicate that the protein products of α_2- and α_1-genes are identical (Liebhaber *et al.*, 1981).

2.3 Alpha-thalassemia

The thalassemias are diverse genetic disorders of hemoglobin synthesis, which are due to a reduced rate of synthesis of either the α- or the β-chains of hemoglobin, giving rise to α- or β-thalassemia, respectively (Todd *et al.*, 1970). The α-thalassemias are common genetic disorders in Thailand that result from reduced synthesis of the α-globin chains of fetal and adult hemoglobin. The α-thalassemia most frequently results from deletion of α-genes, and less frequently from mutation involving one or a few nucleotides within the structural gene so-called non-deletional α-thalassemia.

2.3.1 Non-deletional α-thalassemia

Most of non-deletion thalassemia mutations consist of single-nucleotide substitutions (point mutations) involve the canonical sequences that control gene expression. A smaller group of these mutations involve deletions or insertions of one to five nucleotide bases resulting in a shift of the mRNA reading frame. The globin mRNA's in these various mutations may be non-functional, structurally abnormal, unstable, or decreased in a mount. Judging from the symmetric distribution of nondetrimental α-globin missense mutations, this unequal distribution does not appear to reflect a difference in the mutational rates at two loci. The α-globin genes from several different nondeletion α-thalassemia determinants have been characterized in detail (Table 2). The most common nondeletion α-thalassemia mutation is $\alpha_2^{\text{Constant Spring}}$ (α^{CS}). It is particularly prevalent in Southeast Asia with frequencies as high as 8% in Bangkok (Tanphaichitr *et al.*, 1988).

Table 2 Nondeletional α-thalassemia mutations (Liebhaber, 1989).

Mutation		Locus	
Defect in RNA processing			
Intron 1 splice donor 5 bp deletion			α_2
Poly A addition signal		AAUAAA → AAUAAG	α_2
Defect in translation			
Dinucleotide deletion at bases			
-2-3 to the translation initiation codon			$-\alpha^{3.7}$
Initation codon		AUG → ACG	α_2
"		AUG → GUG	α_1
"		"	$-\alpha^{3.7}$
Frameshift	α_{31}	AGG → --G	α_2
" (Wayne)	α_{138}	UCC → UC-	α_2
Nonsense	α_{116}	GAG → UAG	α_2
Antitermination			
Constant Spring	α_{141}	UAA → CAA	α_2
Koya Dora	α_{142}	UAA → UCA	α_2
Seal Rock	α_{142}	UAA → UCA	α_2
Icaria	α_{141}	UAA → AAA	α_2
Unstable protein			
Evanston	α_{14}	Trp → Arg	$-\alpha^{3.7}$
Qong Sze	α_{125}	Leu → Pro	α_2

2.3.2 Gene deletions

Normal individuals have two α-globin genes on each chromosome 16 (αα/αα). Results from a loss of one or more of the four α-globin genes involving different lengths of the α-globin gene cluster can lead to many α-thalassemia syndromes (Fucharoen *et al.*, 1988).

Deletion of a single α-globin gene

The very common one-gene deletion genotype (-α/αα) produces the phenotypically silent α-thalassemia-2 haplotype with no clinical abnormality, and although the erythrocytes of affected individuals may be slightly microcytic, their red cell morphology is usually normal.

The α-globin genes are embedded in a large region of homology which is devided by short divergent regions into three homology subsegments ; x, y, and z. Two common deletion forms of α-thalassemia-2 haplotype that are designated by the size of the deletion as $-\alpha^{3.7}$and $-\alpha^{4.2}$ (Baysal and Huisman, 1994). Both of these deletions result from unequal homologous recombination within the α-globin cluster which produces chromosomes with only one α-gene (α) and triplicated α-gene (ααα). Cross-over in the x box produce a 4.2 kb deletion, while those in the z box give rise to the 3.7 kb deletion (Figure 4). The deletion forms of α-thalassemia-2 haplotype have a worldwide distribution and have reached frequencies ranging from 20 to 90% in different African, Southeast Asian, and Melanesian populations. The incidence of heterozygosity for α-thalassemia-2 haplotype is 12% in Southern Thailand (Srisoongrueng *et al.*, 1997). The $-\alpha^{3.7}$ form seems to be by far the most widely distributed, whereas

the $-\alpha^{4.2}$ variety while it occurs sporadically in many racial groups, has only been found at high frequencies in Papua New Guinea and Vanuatu (Weatherall *et al.*,1988).

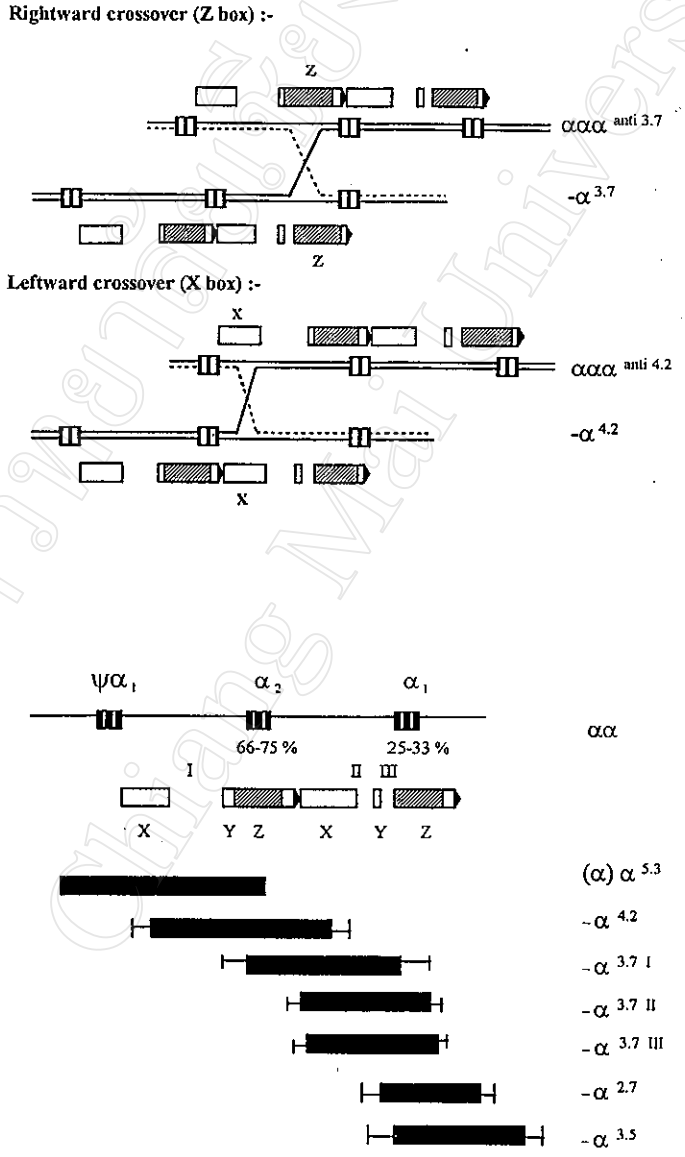

Figure 4 The duplicated xyz box arrangement containing the α-globin genes (Higgs *et al.*, 1989).

Deletion of both α-globin genes

The deletion of both α-globin genes (--/αα) produces the α-thalassemia-1 haplotype. Affected individuals usually are clinically normal, but they typically exhibit microcytosis with abnormal erythrocyte morphology, often accompanied by mild anemia.

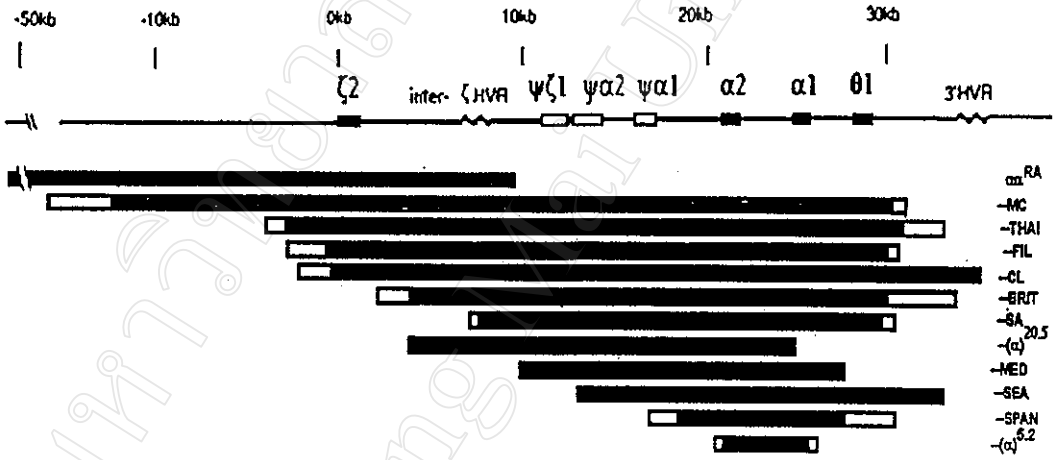

Figure 5 The major deletions in α-thalassemia-1 (Liebhaber, 1989).

These deletions (Figure 5) range in size from rather small ($-(\alpha)^{5.2}$) to those which remove the entire cluster ($--^{FIL}$ and $--^{THAI}$) (Fischel-Ghodsian *et al.*, 1988). The $--^{FIL}$ deletion extends for ~30-34 kb and the $--^{THAI}$ deletion is 34-38 kb long. The $--^{SEA}$ is the common α-thalassemia-1 haplotype observed in Southeast Asia, while the $--^{MED}$ is observed in Mediteranean populations (Kanavakis *et al.*, 1988 ; Bowden *et al.*, 1992).

The $--^{SEA}$ is a deletion of about 20 kb that removes the $\psi\alpha_2$-, $\psi\alpha_1$-, α_2-, α_1-, and θ_1-globin genes. The 5′-breakpoint is in the 5′-end of the $\psi\alpha_2$-globin gene and the 3′-breakpoint is upstream to the 3′-HVR region (Hardison *et al.*, 1986). In Southeast Asia, the frequency of heterozygosity for α-thalassemia-1 haplotype is quite high, ranging from 4% to 14% depending on the population (Eng *et al.*, 2000).

2.3.3 Hemoglobin H disease

Hemoglobin H disease is resulting usually from the combination of the α-thalassemia-1 haplotype and α-thalassemia-2 haplotype (--/-α) which is particularly frequent in Southeast Asia and the Mediterranean (Kan *et al.*, 1975). Other less frequent genotypes can also result in Hb H disease including coinheritance of deletional and nondeletional defects such as α-thalassemia-1 with Hb Constant Spring ($--/\alpha\alpha^{CS}$) (Table 3). This syndrome is expressed as a moderately severe form of thalassemia, characterized by anemia, enlargement of the liver and spleen, and in some cases bone deformities similar to those that are associated with homozygous β-thalassemia. Since these are low levels of the α-globin chains, the excess amount of γ-globin chains form tetramers (γ_4) that are called Hb Bart's. After birth the γ-chain synthesis is switched off and the β-chain synthesis raises. Similar to the γ-chains, the excess β-globin chains form a tetramer (β_4) known as Hb H. Hemoblobin Bart's makes up to 25% of total hemoglobin in affected newborn infants (Thompson and Procter, 1984), and after the first year of life variable levels of Hb H can be detected, accompanied by traces of

hemoglobins which contain ζ-chains. Both Hb Bart's and Hb H are not physiologically useful as oxygen carriers. The β-chain tetramer is unstable and precipitates within the erythrocytes as they age with the formation of inclusion bodies. Hemoglobin H disease is usually not severe enough to interfere with the activities of daily living, longevity, or reproductive function. However, severe exacerbations of the anemia may occur coincident with pregnancy, infections, and exposure to oxidant drugs.

2.3.4 Hemoglobin Bart's hydrops fetalis syndrome

This syndrome results from the inheritance of two α-thalassemia-1 haplotypes (--/--). Usually 80% or more of the hemoglobin is Hb Bart's. Affected infants exhibit severe anemia accompanied by massive enlargement of the liver and spleen. The anemia and resulting heart failure produce generalized edema, and the placenta in this condition is also typically swollen and edematous. Most of these infants are delivered dead or die within a few hours after birth (Lie-Injo *et al.*, 1962). The most common cause of Hb Bart's hydrops fetalis in Southeast Asia is homozygosity for the $--^{SEA}$ deletion which is prevalent in Northern Thailand of approximately 8%. The expected frequency of homozygous α-thalassemia-1 (SEA) is 0.00194 that means there are about 2 cases of hydrops fetalis per 1,000 births (Kitsirisakul, 1996). For the $--^{FIL}$ and $--^{THAI}$ which also involve ζ-globin gene deletions, the embryonic hemoglobins are not present. Embryos homozygous for both deletions therefore can not survive through gestation. This probably is the reason why Hb Bart's hydrops fetalis is not found associated with these types of deletion.

Table 3 Mutations of the α-globin genes found in Thailand (ปราณี และ สุทัศน์, 2541)

Phenotype	Genotype	Remark
Normal	αα/αα	Four α-globin genes on chromosome 16
α-Thalassemia-2 Heterozygote	-α/αα	One gene deletion on single chromosome 16
α-Thalassemia-1 Heterozygote	--/αα	Two genes deletion on single chromosome 16
Homozygous α-Thalassemia-2	-α/-α	One gene deletion on both chromosome 16
Hb Constant Spring (CS) Heterozygote	$\alpha^{CS}\alpha/\alpha\alpha$	Mutation at terminator codon of α_2-gene on single chromosome 16
Homozygous Hb Constant Spring	$\alpha^{CS}\alpha/\alpha^{CS}\alpha$	Mutation at terminator codon of α_2-gene on both chromosome 16
α-Thalassemia-1/ α-Thalassemia-2	--/-α	Compound heterozygote between α-thal 1 and α-thal 2, produce Hb H disease
α-Thalassemia-1/ Hb Constant Spring	$--/\alpha^{CS}\alpha$	Compound heterozygote between α-thal 1 and Hb CS, produce Hb H-CS disease
α-Thalassemia-1/ Hb Suan Dok	$--/\alpha^{SD}\alpha$	Compound heterozygote between α-thal 1 and Hb Suan Dok, produce Hb H disease
α-Thalassemia-1/Hb Q	$--/-\alpha^{Q}$	Compound heterozygote between α-thal 1 and Hb Mahidol (Q), produce Hb H disease
Homozygous α-Thalassemia-1	--/--	No α-globin genes on chromosome 16

2.4 Polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequence. The PCR amplification is achieved by using oligonucleotide primers, also known as amplimers. These are short, single-stranded DNA molecules between 20 and 30 nucleotides in length, which are complementary to the ends of a defined sequence of DNA template. The bound primers are extended on single-stranded denatured DNA (template) by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions (Newton and Graham, 1994). This technique is capable of synthesizing over a millon copies of a specific target DNA sequence in a few hours, significantly facilitating all subsequent analytic procedures (Erlich *et al.*, 1988).

The basic PCR cycle consists of three simple reactions. The conditions of which vary only in the temperature of incubation. All three reactions occur in the same tube in the presence of temperature-stable reagents (Figure 6).

The first step is the heat denaturation of native double-stranded DNA. The target DNA melts at temperatures high enough to break the hydrogen bonds holding the strands together, thus liberating single strands of DNA.

In the second step of a cycle, performed at reduced temperatures, two DNA primers are annealed to their complementary sequences on opposite strands of the target DNA.

The cycle's third step is the actual synthesis of new DNA, which occurs through the extension of each annealed primer by a heat-stable DNA polymerase (e.g. *Taq* DNA polymerase) in the presence of excess dNTPs. A new single strand of DNA is synthesized for each annealed primer. Each new strand consists of the primer at its 5'end trailed by a string of linked nucleotides that are complementary to those of the corresponding template (Eisenstein, 1990).

The cycle of denaturation, annealing and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product.

The products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNAs of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Although longer molecules continue to be produced from the original template DNAs in every round, they accumulate only at a linear rate and therefore do not contribute significantly to the final mass of target sequences (Sambrok *et al.*, 1989). This results in the exponential accumulation of the specific target fragment at approximately 2^n, where n is the number of cycles.

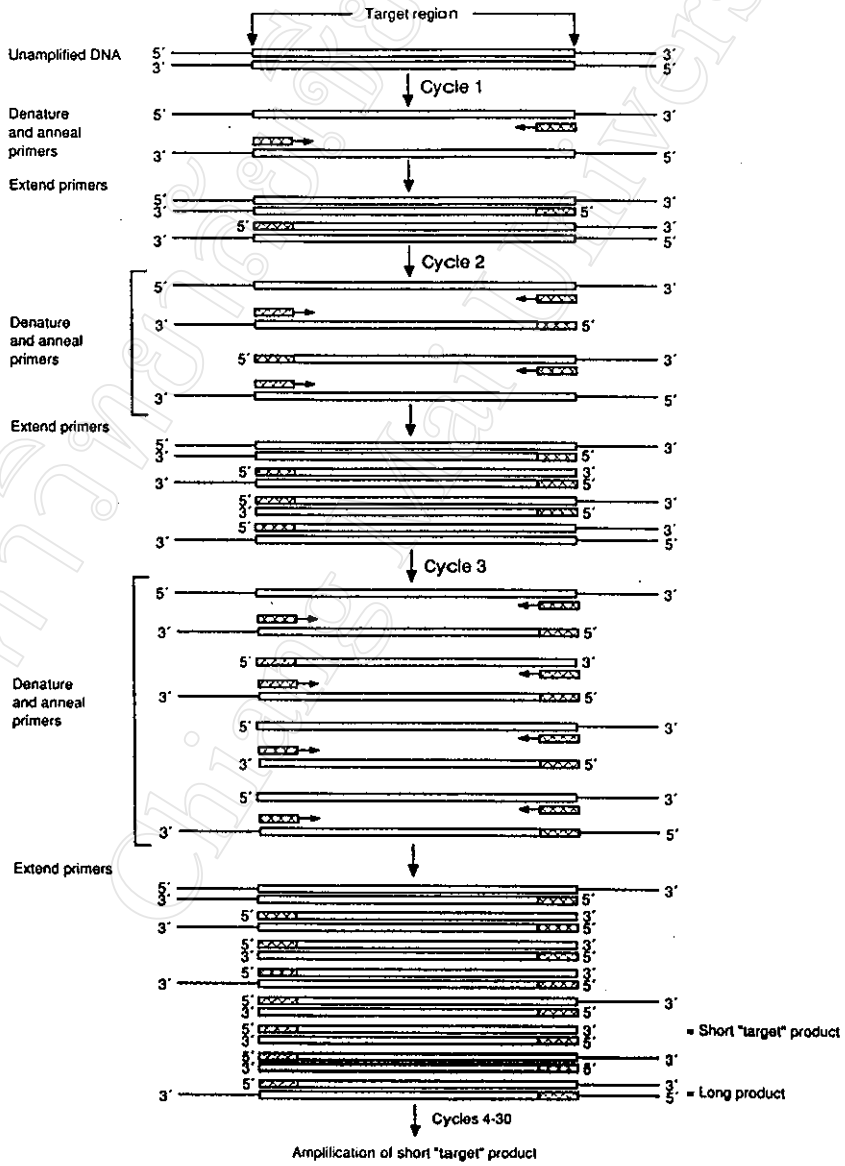

Figure 6 The polymerase chain reaction (Newton and Graham, 1994).

2.5 Standard PCR amplification protocol

Basic components of a PCR amplification is shown below:

	template DNA (10^5-10^6 target molecules)
0.1-0.5 μM	each primer
20 mM	Tris-HCl (pH 8.3 at 20°C)
1.5 mM	$MgCl_2$
50 mM	KCl
0.05 %	Tween 20
100 μg/ml	of gelatin
	or nuclease-free bovine serum albumin (BSA)
200 μM	each dNTP
1 units	of *Taq* DNA polymerase per 50 μl reaction

The reaction components are mixed in the same tube and placed in a thermal cycler when the block reaches denaturation temperature. The 25 to 35 cycles of PCR are performed using the following temperature profile:

Denaturation 90-95°C, 30-60 seconds (an initial time is usually 3 minutes)

Primer Annealing 50-65°C, 30-60 seconds (the exact temperature depends on the composition of the primers)

Primer Extension 72°C, 1.5 minutes (cycling should conclude with a final extension at 72°C for 7 minutes).

2.6 Optimization of PCR

While the standard conditions will amplify most target sequences, it can be highly advantageous to optimize the PCR for a given application, especially routine diagnostic or analytical procedures in which optimal performance is necessary. Many substances will enhance a PCR at a specific concentration but the transfer of these conditions to a different PCR may or may not result in an effect.

Template DNA

Template DNAs can be added to the PCR mixture as a single- or double-stranded DNA or RNA (e.g., genomic DNA, plasmid, amplified DNA, cDNA and mRNA). Template DNAs and RNAs which have been used successfully for PCR should be used in optimized quantity and purification. Usually, the amount of template required is dependent upon the complexity of DNA sample.

Buffer and $MgCl_2$

Standard buffer for PCR contains 50 mM KCl, 10 mM Tris-HCl (pH 8.3, adjusted at room temperature) and 1.5 mM $MgCl_2$. The KCl above 50 mM inhibits *Taq* DNA polymerase activity (Innis *et al.*, 1988). The *Taq* DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and dNTPs. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total dNTP concentration. Generally, insufficient Mg^{2+} leads to low yields and excess Mg^{2+} will result in the accumulation of nonspecific products. Gelatin or bovine serum albumin (100 μg/ml) and

nonionic detergents such as Tween 20 (Molchanova *et al.*, 1994) or Laureth 12 are included to help stabilize the enzyme.

dNTPs

The PCR is normally performed with dNTP concentrations around 200 μM, although at lower dNTP concentrations mispriming at nontarget sites is minimized and the likelihood of extending misincorporated nucleotides is reduced (Innis *et al.*, 1988). However, the optimal concentration of dNTPs depends on: the $MgCl_2$ concentration, the reaction stringency, the primer concentration, the length of the amplified product and the number of cycles of PCR. For optimization of a particular PCR it may be necessary to empirically determine the best dNTP concentration.

Enzyme concentration

The thermostable DNA polymerase from *Thermus aquaticus* (*Taq*) is useful in PCR of amplifying DNA. The high temperature optimum activity of *Taq* DNA polymerase is at 75°C (Lawyer *et al.*, 1989). Although the *Taq* DNA polymerase has very limited activity above 90°C, the enzyme is relatively resistant to denaturation during exposure to high temperature. A recommended concentration range for *Taq* DNA polymerase (Perkin-Elmer Cetus) is between 1 and 2.5 units (specific activity, SA=20 units/pmol) per 100 μl reaction when other parameters are optimal. However, enzyme requirements may vary with respect to individual target templates or primers. If the enzyme concentration is too high, nonspecific background products may accumulate, and if too low, an insufficient amount of desired product is made.

Primer

Some simple rules aid in the design of efficient primers. Typical primers are 18 to 28 molecules in length having 50% to 60% G+C compositions. One should avoid complementarily at the 3′ends of primer pairs as this promotes the formation of primer-dimers artifacts and reduces the yield of the desired product. Primer concentrations between 0.1 and 0.5 μM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent primer-dimer artifacts (Innis and Gelfand, 1990).

Denaturation time and temperature

Typical denaturation conditions are 95°C for 30 seconds, or 97°C for 15 seconds; however, higher temperatures may be appropriate, especially for G+C rich targets. Incomplete denaturation allows the DNA strands to "snap back" and thus reduces product yield.

Primer annealing

The temperature and length of time required for primer annealing depend upon the base composition, length, and concentration of the amplification primers. Annealing temperatures in the range of 55 to 72°C generally yield the best results.

Primer extension

Extension time depends on the length and concentration of the target sequence and on temperature. Primer extensions are usually performed at 72°C, which is the optimum temperature for *Taq* DNA polymerase. However,

longer extension times may be helpful in early cycles if the substrate concentration is very low, and at late cycles when product concentration exceeds enzyme concentration.

Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized. The number of cycles is usually between 25 and 35. With increasing cycles numbers it is common to observe and increase in the amount of unwanted artifactual products and no increase in the desired product. Therefore it is unusual to find reactions that use more than 40 cycles.

2.7 Principle of preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) is the detection of genetic defects in human embryos following *in vitro* fertilization (IVF). After PGD, the unaffected embryos are transferred to the uterus for pregnancy.

Biopsy of a preimplantation embryo can be accomplished at two general stages : the four- to eight-cell embryo, from which one to three blastomeres may be removed, and the blastocyst, from which multiple trophectoderm cells may be removed. Blastomeres are obtained by micromanipulation under a binocular inverted microscope (Buster *et al.*, 1996). After cleavage, these biopsied embryos are returned to the embryo culture medium. Thus the biopsied embryos can be developed to the blastocyst stage by day six in culture (Handyside *et al.*, 1989). However, PGD of IVF-embryos is probably

best performed at the four- to eight-cell stage because development in vitro to blastocyst is compromised by high rates of loss (Handyside *et al.*, 1990).

2.8 Principle of primer-extension-preamplification

The limitation of detection from blastomeres is the limited amount of DNA. In 1992, Zhang and colleagues have developed a PCR method for analysis of single cells that uses random 15 base oligonucleotides as primers and multiple rounds of extension with *Taq* DNA polymerase to amplify the whole genome, the method is called primer-extension preamplification (PEP). Small aliquots from PEP reactions can then be reamplified with specific primers. They studied 12 loci (PTH, LDLR, HBG2, D3S2, D3S11, D3S12, D3S3, D9S52, APOC2, D19S49, X-linked STS locus, and Y-linked STS pseudogene locus) and estimate that at least 78% of the genomic sequences in a single cell can be copied no less than 30 times. This method not only extends the possible applications of single cell studies but also has implications for the analysis of any small amount of DNA sample.