CHAPTER I

INTRODUCTION

1.1 Statement of problems

Thalassemia syndrome is a group of hereditary disorders with defects in the synthesis of globin chains of hemoglobin. These defects lead to the absent or decreased synthesis of the affected globin chains. Erythrocytes of thalassemia, therefore, have a reduction in the hemoglobin content and the size. Thalassemia syndrome has high incidence and affect people living in several countries in Mediterranean, Africa, through the Middle East and South East Asia. (Chong *et al.*, 2000; Krishnsmurti *et al.*, 1998; Weatherall and Clegg, 2001). This disease is now a serious-public health problem of these regions.

Thalassemia syndrome can be divided into two major types, β- and α-thalassemia. β-thalassemia is predominantly caused by point mutations in β-globin genes leading to the decrease of β-globin chain synthesis. The α-thalassemia is usually caused by either the deletion of one or more α-globin genes on chromosome 16 (Old, 2003). The clinical severity of α-thalassemia depending on the number of α-globin structural genes deleted. In normal genotype, there are four α-globin genes with two in tandem on each chromosome (αα/αα). When a single α-globin gene deletion (-α/αα), patient has normal hematological parameters or slightly lower of MCV and MCH and blood smear are unremarkable or describable as silent heterozygote. Deletion of two α-globin genes in *cis* (--/αα) or one from each

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chromosome (in *trans*) (-α/-α) results in microcytosis and hypochromia, while lacking of three α-globin genes (--/-α) causes Hb H disease. The patients with Hb H disease have mild to moderate anemia and precipitation of Hb H is occurred. Complete deletion of all four α-globin genes is known as Hb Bart's hydrops fetalis syndrome. This genotype results in death of fetus and pregnant mother was also suffered with lethal complications.

The α-thalassemia is a common disease throughout Southeast Asia (Higgs *et al.*, 1989). It was widely found in Thailand, Laos, China, Taiwan, Malaysia and Philippines. In Thailand, around 3-4% of population have α-thalassemia 1 gene and 20-30% of them have α-thalassemia 2 gene (Boonsa *et al.*, 2004; Sanchaisuriya *et al.*, 2003). The α-thalassemia 1 with the Southeast Asian deletion (SEA), approximately a 20 kb loss of the α-globin gene cluster, is the most severe form of α-thalassemia 1. The homozygous for α-thalassemia 1 with SEA type ($--^{SEA}/--^{SEA}$) fatally affects the infants causing in-utero death just before birth or die shortly after birth. This condition is conventionally known as Hb Bart's hydrops fetalis. In addition, the combination of α-thalassemia 1 (SEA type) with α-thalassemia 2 gene causes Hb H disease ($--^{SEA}/-\alpha$).

For screening of α-thalassemia 1 heterozygote, in principle, the initial diagnosis was done by Complete Blood Cell Count (CBC) to determine red blood cell indices, especially MCV, MCH, and RDW to further with the combination of red blood cell morphology. One-tube osmotic fragility test (OF test) is also one of the conventional screening method. Microcytosis is a common finding in α-thalassemia heterozygote and iron deficiency persons. In addition, Hb H inclusion bodies are also

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detected in α-thalassemia heterozygote, especially α-thalassemia 1 heterozygote. However, the mentioned screening methods including CBC, OF test and Hb H inclusion body test can not identify all α-thalassemia heterozygote due to their poor sensitivities. Other techniques have been applied and used to screen and diagnose α-thalassemia heterozygote, however, its mostly have to be done in combinations. The most effective method for α-thalassemia heterozygote detection is Polymerase Chain Reaction (PCR) which is used to identify of gene mutations or deletions (Chang *et al.*, 1991). PCR analysis can give more precise information about types of thalassemia, but it normally involves higher technologies and instrumentation. PCR analysis is also expensive and is not suitable to be used as screening method in large population.

Recently, the immunological assays such as Enzyme-Linked Immunosorbent Assay (ELISA) is widely used for screening or diagnosis of various diseases (El-Masry *et al.*, 2006; Nagler *et al.*, 2006; Nuntaprasert *et al.*, 2005; Venturi *et al.*, 2006). The high sensitivity and specificity made this assay reliable. For thalassemia, immunological assays were developed to detect Hbs and globin chains in blood samples. By this technique, trace amount of embryonic ζ-globin chains (Tang *et al.*, 1992) can be detected in blood samples. Detection of α-thalassemia 1 heterozygote with SEA type ($--^{SEA}$) was reported (Chui *et al.*, 1986). Minute amounts of ζ-globin chains in adult hemolysates could be used as a marker and detected by slot blot immunobinding assay. By this assay, the murine anti-ζ globin chain monoclonal antibody was used to detect ζ-globin chains in adult hemolysate (Luo *et al.*, 1988). In addition, the immunocytological test showed its high sensitivity and specificity for identifying adult thalassemia 1 heterozygote with ($--^{SEA}$) deletion using anti-human

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ζ-globin chain antibody to detect ζ-globin chains in adult peripheral blood smear (Lau *et al.*, 1997; Tang *et al.*, 1993). The major equipment required for this technique is a fluorescence microscope which requires sophisticated maintenance and synthesis specialized dark room. To date, it is known that trace amount of Hb Bart's is present in α-thalassemia 1 heterozygote and can be measured by high sensitivity immunological techniques. Two-site immunoradiometric assay was developed for determination of Hb Bart's in blood samples (Makonkawkeyoon *et al.*, 1992a). This immunoradiometric assay using rabbit polyclonal antibody to capture Hb Bart's and demonstrated to be able to detect minute amounts of the substance (Makonkawkeyoon *et al.*, 1992b). However, the major disadvantage of the RIA is the use of short-lived isotope such as ^{125}I and gamma counter.

Therefore, to overcome the disadvantages of RIA, ELISA techniques have been developed to detect Hb Bart's (Garver *et al.*, 1984). For instance, indirect ELISA using anti-Hb Bart's derived from rabbit serum could detect as little as 250 ng of Hb Bart's under the condition specified. Further studies demonstrated that ELISA using the horseradish peroxidase conjugated anti-ζ antibody could detect ζ-globin chains in newborn cord blood (Ausavarungnirum *et al.*, 1998) and could detect heterozygote of the (--^{SEA}) deletion (Ma *et al.*, 2002). The ζ-globin ELISA kit has a high sensitivity and specificity for identifying adult heterozygote of (--^{SEA}) deletion. However the commercial ζ-globin ELISA kit is still too expensive for resource-limited countries.

In this study, a sandwich ELISA was developed to detect Hb Bart's in adult α-thalassemia heterozygote. By using our generated specific monoclonal antibody, an

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inexpensive ELISA with high sensitivity and specificity was made available for screening Hb Bart's in adult α-thalassemia heterozygote.

1.2 Literature review

1.2.1 Hemoglobins

Hemoglobin is the molecule that carries and transports oxygen all through the body. It is composed of tetra globin chains, two α and two non-α chains (Figure 1.1). Each globin chain contains a heme group at its core. The α chains are encoded by the two closely related genes, $\alpha1$ and $\alpha2$, on chromosome 16. The non-α chains, β, γ, and δ are encoded by cluster of genes on chromosome 11. In a fetus, Hb F ($\alpha_2\gamma_2$) level is higher than other type of Hbs. A newborn, Hb F is about 80% of all hemoglobin, then it reaches a normal level in a few weeks after birth. The production of Hb A ($\alpha_2\beta_2$) becomes dominants, then Hb A_2 ($\alpha_2\delta_2$) level is elevated. In normal adult, Hb A is the main type of hemoglobin (96-98%) while Hb A_2 is presented only 2-3% and Hb F is less than 1%, respectively.

The failure in hemoglobin synthesis is the main cause of microcytosis and anemia in many people around the world. Hemoglobin variants (Hb variants) are characterized by the gene mutation. Thalassemia, which is slightly different from Hb variants, that involve the gene mutations, is caused from insufficient production of an amount of normal structure globin chains. All type of thalassemia are considered quantitative hemoglobin disease (Hartwell *et al.*, 2005).

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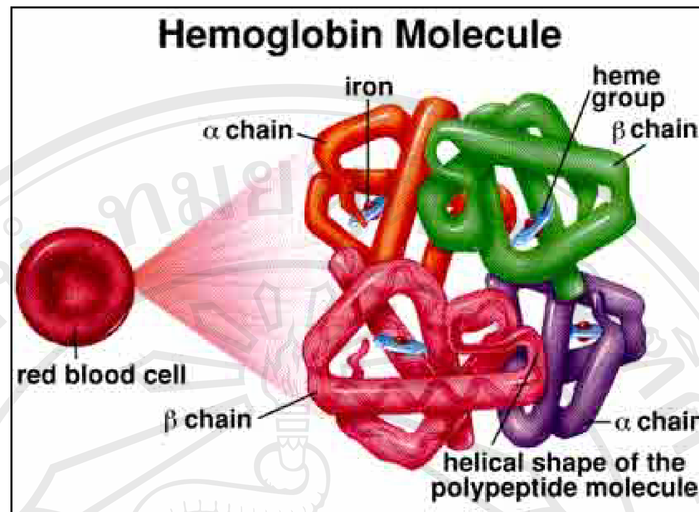

Figure 1.1 Hemoglobin molecule consists of a porphyrin ring with a central iron atom (heme) and two pairs of unlike globin chains, which form a shell around a central cavity containing four-oxygen-binding heme groups each covalently linked to α-globin chain.

1.2.2 The organization of the human α-globin gene cluster

The α chains are encoded by the two closely related genes, α1 and α2 on chromosome 16. As shown in Figure 1.2, α-globin gene cluster includes the duplicated α genes (α2 and α1), an embryonic α like gene (ζ2), three psudogenes (ψζ 1, ψα2, ψα1), and a gene of undetermined function (θ1) are arranged in the order 5′-ζ2 - ψζ1 - ψα2 - ψα1- α2 - α1- θ1- 3′. Several regions of the cluster contain tenderly repeated segments of DNA. They were first identified as hypervariable regions (HVRs) located at the 3′ end of the complex (α-globin 3′HVR), between the ζ2 and ψζ1 genes and with in the introns (IVS1 and IVS2) of the ζ-like genes (ζ-intron

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HVRs) (Liebhaber, 1989). Recently, further hypervariable regions approximately 70 kb upstream of ζ2 (called the α-globin 5′HVR) were identified.

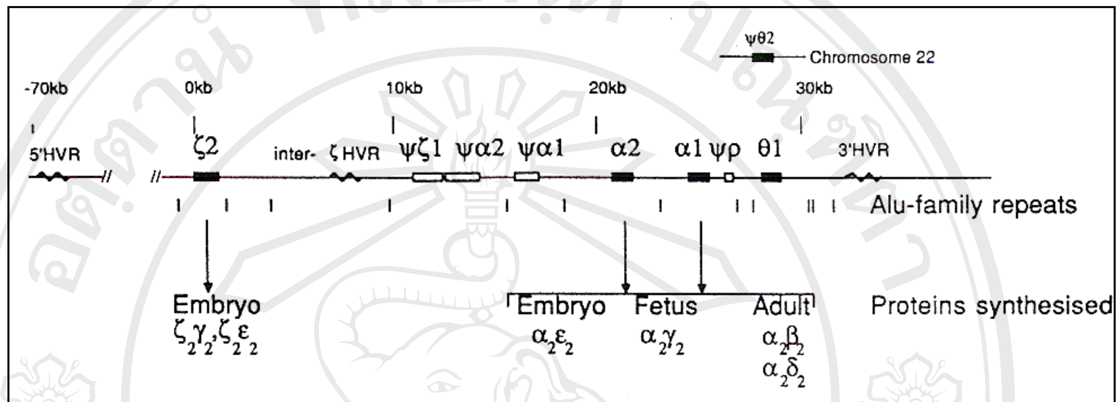

Figure 1.2 Organization of α-globin gene cluster. The filled boxes indicate functional genes and opened boxes are the pseudogenes. Position 0 represents the ζ-globin mRNA CAP site and the hyper variable regions are denoted by zig-zag lines. Each molecule of hemoglobin comprises a tetramer of two α-like (α- or ζ-) and two β-like (β-,γ-, δ- or ε-) globin chains (Higgs *et al.*, 1989).

1.2.3 Hemoglobin production

Human hemoglobin is heterogeneous at all stages of development, beginning with the youngest embryos that have been studied their continuing throughout adult life. In embryos, hemoglobin synthesis is confined in the yolk sac, where Hbs Gower1 ($\zeta_2\varepsilon_2$), Gower2 ($\alpha_2\,\varepsilon_2$), and Portland ($\zeta_2\gamma_2$), are produced. Synthesis of β chain becomes detectable at about 6 weeks, when it comprises ~ 1.5% of the non-α chains, increasing to 5% at 7 weeks. At around 7-8 weeks of gestation, the liver becomes the major site of erythropoiesis, producing large nucleated cells. Throughout most of fetal life, Hb F production is predominates with a small amount

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(< 10%) of Hb A. The different γ chains are produced in a ratio of $^{G}\gamma$ and $^{A}\gamma$ of 3:1, which remains constant until late of gestation. At mid-term, the bone marrow beings to take over as the major site of red cell production, through erythropoiesis are found in the spleen, as well as in other tissue (Figure 1.3). At birth, the cord blood normally contains ~ 70% of Hb F and this will decline to ~ 20% and less than 2% by the age of 1 year. At the same time, there is a differential decline in $^{G}\gamma$ and $^{A}\gamma$ chain production. Both fetal and adult hemoglobin are produced in the same cell until the switching period, a gradual increase in the proportion of cells containing predominantly of Hb A. The proportion of Hb F continues declining throughout childhood and probably adult life. At this time the small amount of Hb F are detectable only 3-5% of red cells, known as F cells (Weatherall and Clegg, 2001).

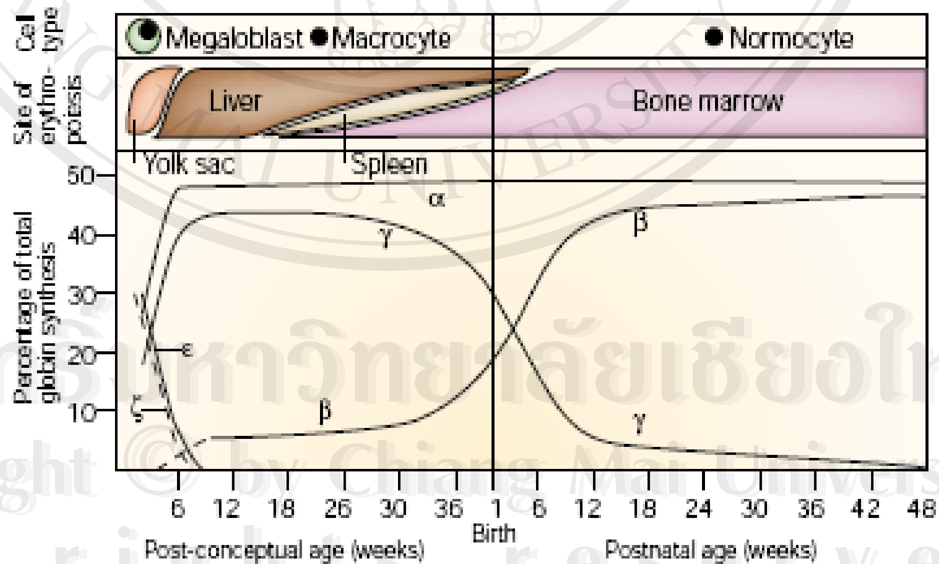

Figure 1.3 Sequence of globin chain synthesis in the embryo and fetus throughout the first year of life (Weatherall, 2001).

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For α chain synthesis, ζ and ε chains are synthesized resulting in the embryonic hemoglobins Gower1, Gower 2, and Portland during the embryonic life. After the first two months of gestation, the production of ζ chains are turn off and start to produce of α chains. Similarly, production of γ chains are synthesized instead of ε chains begins resulting in fetal hemoglobin (Hb F). After birth, the productions of γ chains are gradual decrease and begin synthesis of β and small amount of δ chains from chromosome 11 results in the production of adult hemoglobin ($\alpha 2\beta 2$) (Keren, 2003).

1.2.4 The classification of thalassemia syndromes

Thalassemias are hereditary anemia caused by decreased or absent production of one type of globin chain. They are the most common genetic defect among man. The resulting of hematological disorders ranged from asymptomatic to severe anemia, which can cause the significant morbidity and mortality of humankind.

The thalassemia syndromes are usually classified according to the type of globin chain that is absent or present in decreased amount. The major categories consist of β and α-thalassemia. Although each can be further classified into different subgroups, all these disorders have one thing in common, which is always imbalanced of globin chain synthesis. This is the hallmark of thalassemia, and it is the deleterious consequence of the globin that is produced in excess that are responsible for the ineffective erythropoiesis and shorten red cell survival that characterizes all the severe forms of the disease.

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1.2.4.1 Beta thalassemia (β-thalassemia)

The cause of the β-thalassemia is defective β-globin chain synthesis, which leads to imbalanced globin chain production and excess of α-chains (Nopparatana, 1998). The excess chains aggregate in red cell precursors and cause abnormal cell maturation and their premature destruction in the bone marrow. There is abundant evidence that the severity of β-thalassemia is related to the degree of the globin chain imbalance (Weatherall, 2001). More than 170 different β-thalassemia have now been characterized. The majority of which are point mutations affecting the β-globin gene lead to a reduction (β^+) or absence (β^0) of β-globin gene production resulting in β-thalassemia (Laosombat *et al.*, 2001).

1.2.4.2 Alpha thalassemia (α-thalassemia)

α-Thalassemia is a disease which is a deletion of one or more of the four α-chain genes. Therefore, excess γ and β chains that result from defective α chains production are able to form homotetramers. The γ_4 and β_4 homotetrameric protein are known as Hb Bart's and Hb H, respectively (Kidd *et al.*, 2001). Thus the clinical features of the more severe forms of α-thalassemia are reflection of the properties of Hb Bart's and H and their effects on erythropoiesis, and in particular on red cell survival. As shown in Figure 1.2, there are two α-globin genes per haploid genome, and four in all. For this reason the α-thalassemia is classified according to the total output of each of the α-chain genes that constitutes the haploid pairs. A normal α-globin genotype can be represented as αα/ αα. When both α-globin gene on a chromosome are deleted or otherwise inactivated, the condition is called α^0 thalassemia (α-thalassemia 1); the genotype can be written as --/ αα. This is called

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α^0 thalassemia because there is no output of α-globin from the one affected chromosome. When one of the linked α-genes is inactivated, the conditions is called α^+ thalassemia (α^+ thal trait or α-thalassemia 2), and the genotype is written as - α/α α . In this case, one of α-globin gene is deleted, or if one of them is inactivated by a mutation, genotyping is $\alpha^T\alpha/\alpha\alpha$. Finally, if α^+ thalassemia results from the inactivation of one α-globin gene by a mutation are known, the T can be replaced by the precise molecular abnormality, in the same way as β thalassemia (Weatherall and Clegg, 2001).

The α-thalassemia can also be described at the molecular level. The particular length of the deletion that removes both α globin genes often designs the α^0 thalassemias. The most common α^0 thalassemias deletions are the Southeast Asian ($--^{SEA}$), Mediterranean ($--^{MED}$), 20.5 kilobase (kb) ($--^{20.5}$) and Filipino ($--^{FIL}$). Similarly, there are two common forms of α^+ thalassemias, in which single α-gene deletions are due to loss of 3.7 kb or 4.2 kb of DNA from within the linked pair of α genes and they are written as $-\alpha^{3.7}$ or $-\alpha^{4.2.}$ (Burnini, 2001).

There are different numbers of functional α-globin genes remaining, which is related to the wide range of α-thalassemia clinical spectrum which ranging from a silent trait to a very severe anemia.

A) Heterozygous α-thalassemia 2 or "silent heterozygote" state (-α/αα)

Heterozygous α-thalassemia 2 presents a mild α chain deficiency resulting from the presence of three α chain genes. The hematological parameters are within normal limit.

B) Homozygous α-thalassemia 2 (-α/-α) and Heterozygous α thalassemia 1 (**--**

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/αα)

This thalassemia type presents moderate α chain deficiency resulting from the presence of two α chain genes. There are mild hematological changes and no major clinical abnormality.

C) Hb H disease or heterozygous of α-thalassemia 2 in combination with heterozygous α-thalassemia 1 (--/-α)

Because of their have severe deficiency of α chain which caused by the deletion of three α chain genes, patients with this Hb H disease manifest more severe red cell indices and a 25% to 30% reduction in the hemoglobin and hematocrit values are detected.

D) Hydrops fetalis with Hb Bart's or homozygous α-thalassemia 1 (--/--)

Homozygous α-thalassemia 1 is the most severe form which lacks any functional α genes and is known as hydrops fetalis syndrome. This condition is incompatible with life, and the fetus may stillborn but dies soon after birth (Bunn and Forget, 1986; Tang *et al.*, 2001).

1.2.4.2.1 α-thalassemia heterozygote

1.2.4.2.1.1 heterozygous α-thalassemia 1 (--/αα)

Deletions in α-thalassemia 1 either completely or partially delete both α-globin genes, no α-chain was synthesized in the same chromosome. There are many deletions, which remove two α-globin genes including α-globin gene in *cis* chromosome, i.e., ($--^{THAI}$) deletion in Thailand, the ($--^{FIL}$) deletion in the Philippines and the ($--^{HW}$) deletion found in a Chinese family. Deletion of two α-globin genes but not the ζ- globin gene are ($--^{MED}$), - $(\alpha)^{20.5}$ and ($--^{SEA}$) deletion.

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The Southeast Asian ($--^{SEA}$) α-thalassemia 1 is a common deletion in Thailand and others Southeast Asia countries. Homozygous for this deletion ($--^{SEA}/--^{SEA}$) causes Hb Bart's Hydrops fetalis syndrome. Most of the hemoglobin in hydropic infants is Hb Bart's (γ_4) because of the absence of α-globin genes. The Hb Bart's have very high oxygen affinity; therefore, oxygen is unable to deliver to tissue. The homozygous α-thalassemia 1 fetuses usually die in-uterus or stillborn but die shortly after birth (Chui and Waye, 1998).

1.2.4.2.1.2 heterozygous α-thalassemia 2 (-α /αα)

α-thalassemia 2 or mild α-thalassemia gene consists of a single structure α-globin gene on the chromosome 16. Two different patterns of gene organization responsible for the α-thalassemia 2 genotype were found. The first was the result of a 4.2-kilobase pair deletion involving the normal 5' α-globin gene (leftward deletion α-thalassemia 2 genotype), and the second probably the result of a crossing over deletion of a DNA fragment bridging the two normal α-globin genes (rightward deletion α-thalassemia 2 genotype) (Figure. 1.4) (Embury *et al.*, 1980). As the α-globin genes are embedded within two highly homologous 4 kb duplication units. These regions are divided into homologous sub segments (X, Y, and Z) by nonhomologous elements (I, II and III). Reciprocal recombination between Z segments, which are 3.7 kb apart, produce chromosome with only one α gene ($-\alpha^{3.7}$) which has been called the "rightward deletion" that cause α-thalassemia and others with three a genes ($\alpha\alpha\alpha^{anti3.7}$). It was found that this different type can occur depending on exactly where within the Z box the crossover took place into $-\alpha^{3.7I}$, $-\alpha^{3.7II}$ and $-\alpha^{3.7III}$. These sub regions are defined by sequence differences in the $Z\alpha_2$ and

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$Z\alpha_1$ boxes, which detected by Southern blot hybridization. A deletion approximately 4.2 kb, consistent with a deletion created by recombination between homologous X boxes, which are 4.2 kb apart, also gives rise to an α-thalassemia determinant ($-\alpha^{4.2}$) which has been called the "leftward deletion" and a $\alpha\alpha\alpha^{anti4.2}$ chromosome (Higgs *et al.*, 1989) (Figure 1.5).

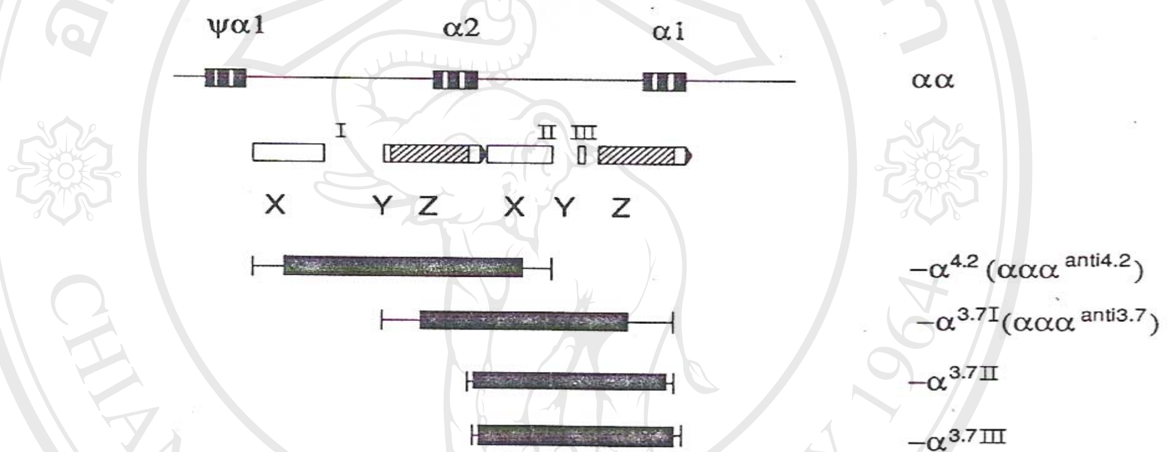

Figure 1.4 Duplicated XYZ box arrangement containing the α genes. Nonhomologous regions (I, II and III) are indicated. The solid blocks indicate the extent of each deletion described in the text and solid lines represent the limits of the breakpoints.

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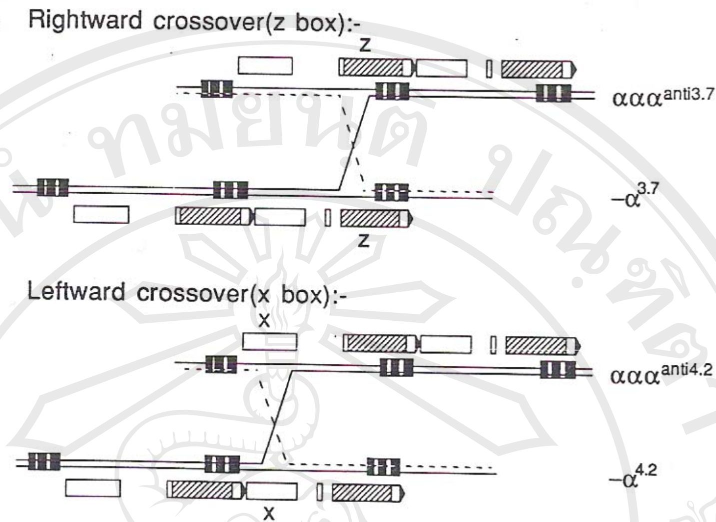

Figure 1.5 Misaligned chromosomes crossing over to produce the $-\alpha^{3.7}$, $\alpha\alpha\alpha^{anti3.7}$ and $-\alpha^{4.2}$, $\alpha\alpha\alpha^{anti4.2}$ haplotypes as examples of how much chromosomes are though to be arise.

1.2.5 Hematological changes in α-thalassemias

In general, those with α gene deletions (-α/αα, - α/- α and --/αα) have lower mean cell hemoglobin concentration (MCHC), mean cell volume (MCV) and mean cell hemoglobin (MCH), but higher red blood cell (RBC) number than normal. These parameters are limited value in distinguishing one genotype from another. However, the - α/αα genotype may not be excluded from normal by these parameters. Most homozygous for deletion types of α-thalassemia 2 or heterozygous for α thalassemia 1 can be distinguished from normal on the basis of their MCH, which is usually below 27 pg and MCV below 80 fl. The peripheral blood film is quite variable between genotype but usually shows hypochromia, with occasional poikilocytes and target cells are presented. The reticulocyte count is usually raised, in

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the range of 2-3% and it is possible to generate Hb H inclusions body in a few red cells in heterozygous α-thalassemia 1 heterozygotes (--/αα), but rarely in heterozygous or homozygous for α-thalassemia 2 (-α/αα) and (-α/-α).

1.2.6 The changing of hemoglobin Bart's levels

During the neonatal development period, Hb Bart's is not detectable in normal infant cord blood while Hb Bart's is presented in cord blood of α-thalassemia heterozygotes (Kyriacou *et al.*, 2000; Rugless *et al.*, 2006). Infants with heterozygous α-thalassemia 2 (-α/αα) have Hb Bart's levels of 1-2% and those with heterozygous α-thalassemia 1 (--/αα) have approximately 5-6% Hb Bart's (Bunn and Forget, 1986; Pootrakul and Dixon, 1970). Therefore, neonatal diagnosis of α-thalassemia 1 heterozygotes may be done by detection of Hb Bart's in cord blood (Ausavarungnirum *et al.*, 1998). By the age of approximately 6 months, the level of Hb Bart's declines and become undetectable by conventional methods.

1.2.7 Hemoglobin analysis

The hemoglobin of α-thalassemia heterozygotes is indistinguishable from normal, although they may have slightly lower levels of Hb A_2 than normal. Hb H inclusion bodies can be detected in up to 65% in heterozygotes for different forms of α-thalassemia (Galanello *et al.*, 1984). In addition, it has been found that by an immunological assay, some α-thalassemia heterozygote adults have trace amounts of Hb Bart's (Tang *et al.*, 1993), and some α-thalassemia 1 heterozygotes have small amounts of embryonic ζ-globin chains (Ma *et al.*, 2002).

The α-thalassemia 1 with the Southeast Asia (SEA) is the commonest deletion of α-thalassemia in Thailand. The homozygous of α-thalassemia 1 ($--^{SEA}$ /--

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^{SEA}), is the cause of hydrops fetalis syndrome. If the parents who are heterozygotes of α-globin genes deletion in *cis* (--/αα), there is a 25% chance to have Hb Bart's hydrops fetalis in each pregnancy. Moreover, mother of infants with this disorder will suffer from severe complications such as pre-eclampsia, oligohydramnios, antepartum hemorrhage and the premature onset of labor. In addition, α-thalassemia 1 in association with α-thalassemia 2 is the cause of Hb H (--^{SEA} /- α) (Smetanina *et al.*, 1996). Therefore, identification of the heterozygotes who are α-thalassemia 1 (--^{SEA} /αα) is important for prevention and control hemoglobin Bart's hydrops fetalis syndrome and severe α-thalassemia. There are many techniques that have been used to screen and diagnose for α-thalassemia, mostly it is done in combinations. The following description is the available methods, ranging from initial screening to extensive analysis for identifying α-thalassemia 1 heterozygotes.

Screening techniques are the group of techniques that can initially indicate a defect in hemoglobin synthesis. Positive results from these tests need confirmation by a more extensive analysis technique. Negative results normally help in cutting down the number of subjects that need to be further diagnosed by more advanced and complicated testing.

Extensive analysis techniques can give more precise information about types of thalassemia. Normally, these involve higher technologies and instrumentation; therefore, they are more expensive than screening techniques. Different laboratories may have different choices of analysis techniques, depending on availability of instrumentation and funding.

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1.2.7.1 Screening techniques

Initial screening techniques are defined as techniques that are simple and relatively low cost, which can indicate the possibility of having thalassemia. These techniques should involve the least sample pretreatment and is rapid sample preparation, and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. They provide a "yes/no" type answer. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing. The red blood cell indicies generated by automated complete blood count (CBC) or the alternative one-tube osmotic fragility test (OF test) can be used to screen for α-thalassemia. The negative result eliminates the possibility of having thalassemia. These screening techniques, however, cannot provide the information on the exact type of thalassemia of the positive persons. (Hartwell *et al.*, 2005).

1.2.7.1.1 Complete blood count (CBC)

Complete blood count, a screening test, involving the measurement of important characteristics of the blood, has an impact to the diagnosis of thalassemia. The main features of the blood normally tested in the CBC are the total white blood cell (WBC) count, red blood cell (RBC) count, hematocrit (Hct), hemoglobin (Hb), red cell distribution width (RDW), peripheral blood smear and other important erythrocyte indices (EI), namely mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Among these parameters, MCV and MCH are the most important. Individual with hypochromic microcytic red blood cell indices that is with an MCH below 27 pg or an MCV below 80 fL should be investigated further (Old, 2003).

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1.2.7.1.2 One-tube osmotic fragility test (OF test)

This simple test utilizes osmosis, the movement of water from lower to higher salt concentration region, to test for the osmotic resistance of the red blood cells. Whole blood is thoroughly mixed with 0.36% buffered saline solution. In a hypotonic condition, the concentration of salt on the outside of a cell is lower than that on the inside, resulting in net water movement into cells. Normal red blood cells are lysed within 1-2 minutes and the mixture then turns clear and reddish. Abnormal red blood cells have deviated osmotic resistances as compared to normal red cells. Red blood cells of thalassemia have higher osmotic resistance and thus have slower rupture rate, therefore the mixture remains turbid even after 1-2 hr. This technique can be carried out in one test tube and it is therefore called one tube method. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution, but all are normally based on the same concept of kinetic osmotic fragility (Hartwell *et al*., 2005). Although the OF test is a quick preliminary and very economic test before performing further studies of the red blood cells and carrying out thalassemia in large populations. However, the specificity of OF test is not so high (74.9%) and its false positive rate is rather high (25%). This means increase the workload of the later conformational PCR analysis (Sirichotiyakul *et al.*, 2004). Recently, the use of a 0.34% modified OF test was suggested for reducing the workload of PCR by about 70% (Panyasai *et al.*, 2002).

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1.2.7.2 Conventional confirmatory test

Hb H inclusion body detection is used as a confirmatory test for thalassemia. This conventional confirmatory test, Hb H inclusion bodies, are relatively low cost

and does not require complicated instrumentation. However, this method may need a high experience technician to translate the results.

1.2.7.2.1 Hb H inclusion bodies

Diagnostic of α-thalassemia carrier relies on the test for Hb H inclusion bodies, tetramers of β-globin chains present in excess in these samples serving as a diagnostic marker for ($--^{SEA}$) deletion. Hb H arises in the setting of α-thalassemia where the decreased production of α-globin chains lead to β-globin excess. Oxidation of these tetramers provokes precipitation, which can be visualized microscopically. Generation of Hb H inclusion is accomplished by staining unfixed cells with an oxidative dye such as New Methylene Blue or Brilliant Cresyl Blue. Blood film examination is undertaken with a search for cells with typical “golf ball” inclusions. Approximately, 30-100% of red cell containing Hb H inclusions was found in Hb H disease (α-/--). However, α-thalassemia minors were found as few as 1 inclusion-containing cell in 1,000-10,000 red blood cells (Clarke and Higgins, 2000). In general, carriers of α-thalassemia are tested by the Hb H inclusion body test. The search for inclusions is laborious, observer–dependent and reported significantly lower sensitivity for the detection of the ($--^{SEA}$) deletion (Lafferty *et al.*, 2000) and all α-thalassemia genotype when compared with the multiplex PCR because Hb H test is seldom positive in α-thalassemia 2 (Bergstrome and Poon, 2002). The absence of Hb H inclusions therefore does not exclude thalassemia trait. In additional, a brisk reticulocytosis can make identification of a rare Hb H inclusion-containing cell difficult (Clarke and Higgins, 2000). The use of a low MCV plus the Hb H test is sufficiently sensitive for the diagnosis of α-thalassemia 1 ($--^{SEA}$) carriers in area with

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a high prevalence of ($--^{SEA}$) deletion and laboratories have no molecular capabilities (Bergstrome and Poon, 2002; Chan *et al.*, 1996). Furthermore, Pan *et al* reported that use of 1% BCB-staining in the detection of α-thalassemia trait can improve sensitivity and specificity (Pan *et al.*, 2005).

1.2.7.3 Instrumental techniques

Several techniques such as electrophoresis or HPLC help in faster and reliable diagnosis of many types of Hb variants. However, these techniques require special instruments. The instrumental techniques have been used mainly for detection of Hb variants rather than for deletion of abnormal of Hbs level in thalassemia.

1.2.7.3.1 Electrophoresis

Electrophoresis is one of the widely used techniques for analyzing hemoglobin variants based on the movement of different hemoglobin, which contain different charges, in the electric field. At an alkaline pH, Hb is negatively charged and move toward the anode (positively charged). Electrophoresis is labor-intensive, and inaccurate in quantification of low concentration Hb variants such as Hb A_2 or in the detection of fast Hb variants such as Hb H, Hb Bart's (Clarke and Higgins, 2000). Detection of Hb Bart's in α-thalassemia carriers using electrophoresis can be carried out only from newborn blood samples (Makonkawkeyoon *et al.*, 1992b)

1.2.7.3.2 High performance liquid chromatography (HPLC)

In high performance liquid chromatography, particles size of the stationary phase packed in the column is quiet small. High pressure is required to force the mobile phase to continuously flow through the column. As the samples solution flows with the liquid mobile phase through the stationary phase, the components of the sample will migrate according to the non-covalent interactions of the compounds

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with the stationary phase. The degree of interactions determines the degree of migration and separation of the components (i.e., the component with a stronger interaction with the mobile phase than with the stationary phase will have a shorter retention time and thus will be eluted from the column first and vice versa) (Hartwell *et al.*, 2005). HPLC has become a very important tool for thalassemia and Hb variants diagnosis because of its ability to accurately and rapidly qualitative and quantitative different types of Hbs. However, in most laboratories, HPLC has been used for diagnosis of Hb variants rather than for quantification of normal Hb or thalassemia, except for the case of prenatal analysis. HPLC is very suitable for prenatal screening diagnosis (Fucharoen *et al.*, 1998). HPLC has been used to detection of α-thalassemia genotypes in cord blood and there were no discrepancies between the result of HPLC testing and the α-globin gene haplotype determined by PCR (Sanguansermsri *et al.*, 2001). In adult blood, HPLC showed the abnormal peaks representing these Hbs, however it cannot measure the concentration of Hb Bart's and Hb H in Hb H disease (Fucharoen *et al.*, 1998).

1.2.7.4 Extensive analysis technique

This advanced technique especially Polymerase chain reaction (PCR) is a standard method that can be used to diagnose heterozygous α-thalassemia. This technique is, however, complicated and expensive. Therefore, it is used to accurately confirm or identify the type of thalassemia.

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1.2.7.4.1 DNA analysis

Currently, with the knowledge of gene deletion breakpoint in α thalassemia, the PCR technique has been increasingly used to identify deletion of α-globin gene in ethnic group. Multiplex PCR has been developed to diagnose the various deletion

forms of α-thalassemia, simultaneously (Fucharoen *et al.*, 2003; Sanchaisuriya *et al.*, 2003; Siriratmanawong *et al.*, 2001). The α-thalassemia alleles consist of either deletion in and around the globin gene cluster or point mutations with in one of the two globins genes. The deletion breakpoints of four of the most common deletions include (--^{FIL}), (--^{MED}), (--^{THAI} and the (--^{SEA}) alleles have now been sequenced and these alleles can now be diagnosed by the PCR known as Gap-PCR (Old, 2003; Weatherall, 2001).

PCR technique allows a small amount of DNA to be amplified *in vitro*. The process is composed of cycles of the three following steps: perform heat denaturing to separate the DNA sequence target in to two strands, anneal each strand to the specific primers and then extend the polymerase chain from the primer termini. Then, agarose gel electrophoresis is commonly done following the PCR to separate different DNA fragments.

The Gap-PCR is also used to diagnose the two common α-thalassemia 2 deletion genes, the - $\alpha^{3.7}$ and - $\alpha^{4.2}$ alleles and five α-thalassemia 1 deletions genes, three Southeast Asia deletions (-^{SEA}, -^{THAI}, -^{FIL}) and two Mediterranean deletions (-^{MED} and - $\alpha^{20.5}$) (Chong *et al.*, 2000; Garshasbi *et al.*, 2003; Old, 2003). This PCR technique, however, is available only in highly specialized laboratories.

1.3 Production of monoclonal antibody

Kohler and Milstein discovered the hybridoma technique in fusible between an antibody-producing spleen cell and an immortal myeloma cell in 1975 (Kohler and Milstein, 1975). To generate a monoclonal antibodies (mAbs) specific for a defined antigen, an immune response of mouse was raised by immunizing the protein of interest as shown in Figure 1.6. The harvested cells from spleen were fused with

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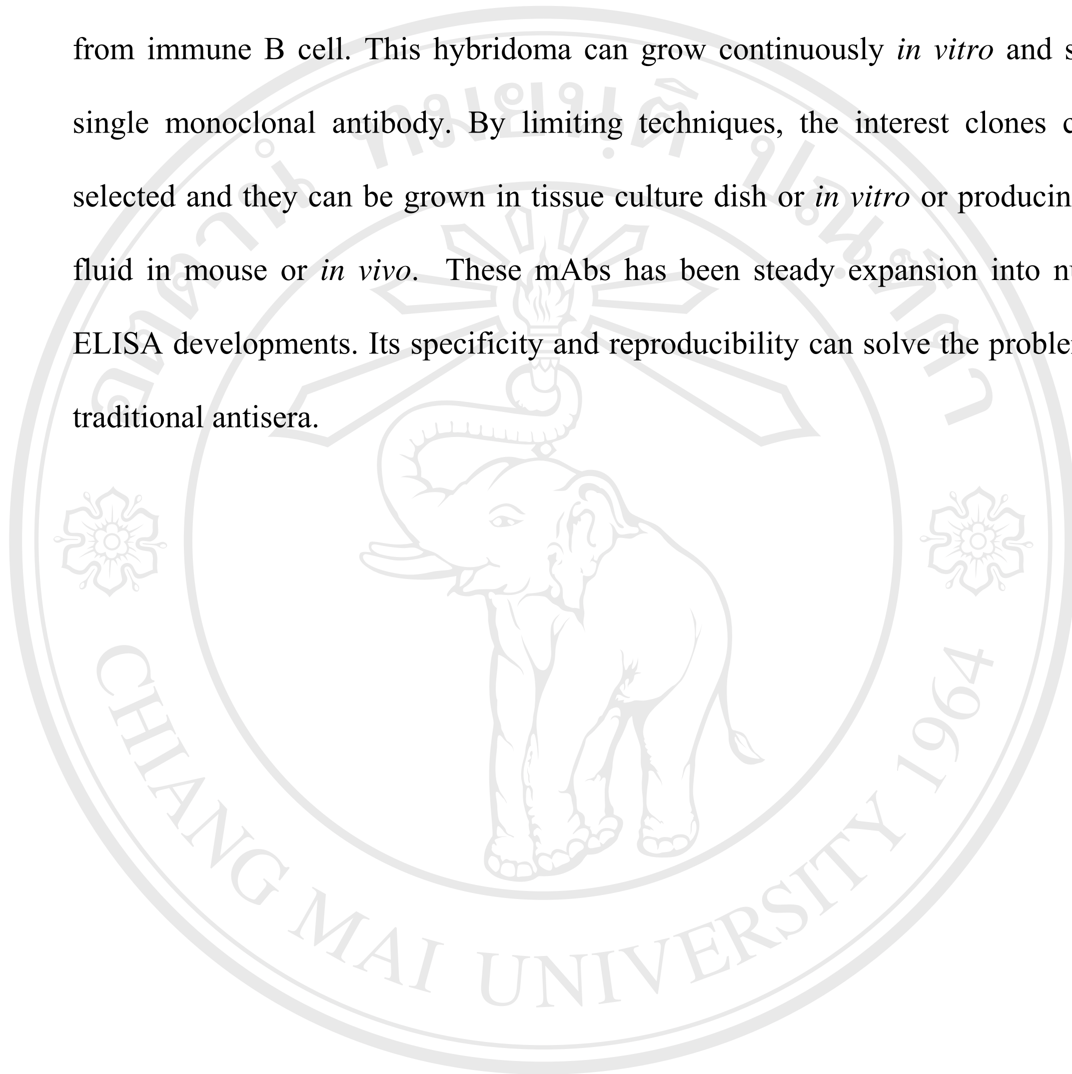
myeloma cells and Hat selection. The survival cells, called hybridoma, are combination advantage nature of myeloma which is immortal cell and HGPRT gene from immune B cell. This hybridoma can grow continuously *in vitro* and secrete a single monoclonal antibody. By limiting techniques, the interest clones could be selected and they can be grown in tissue culture dish or *in vitro* or producing ascitic fluid in mouse or *in vivo*. These mAbs has been steady expansion into numerous ELISA developments. Its specificity and reproducibility can solve the problems from traditional antisera.



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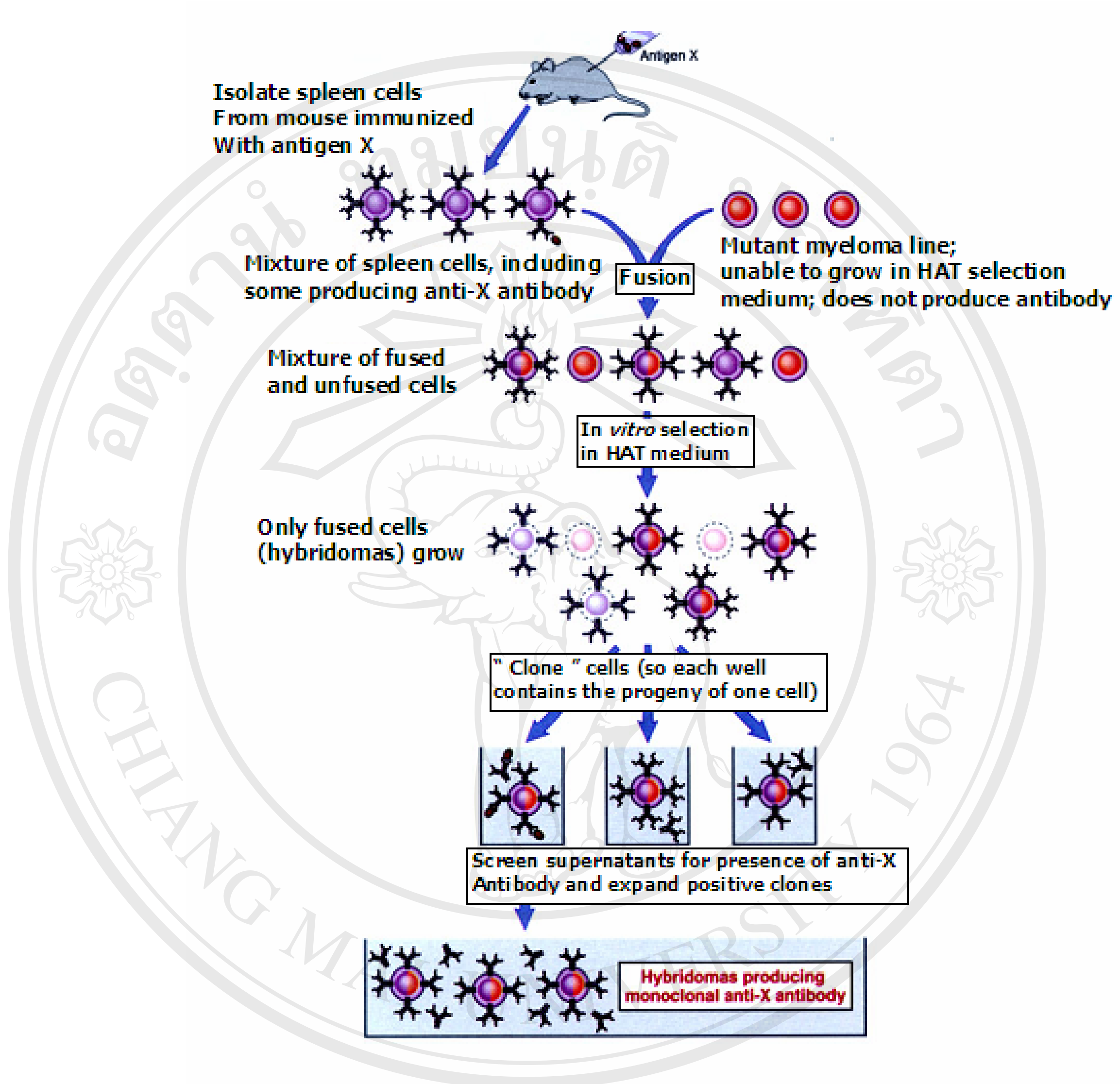

Figure 1.6 Monoclonal antibodies production (adapted from http://www.uccs.edu)

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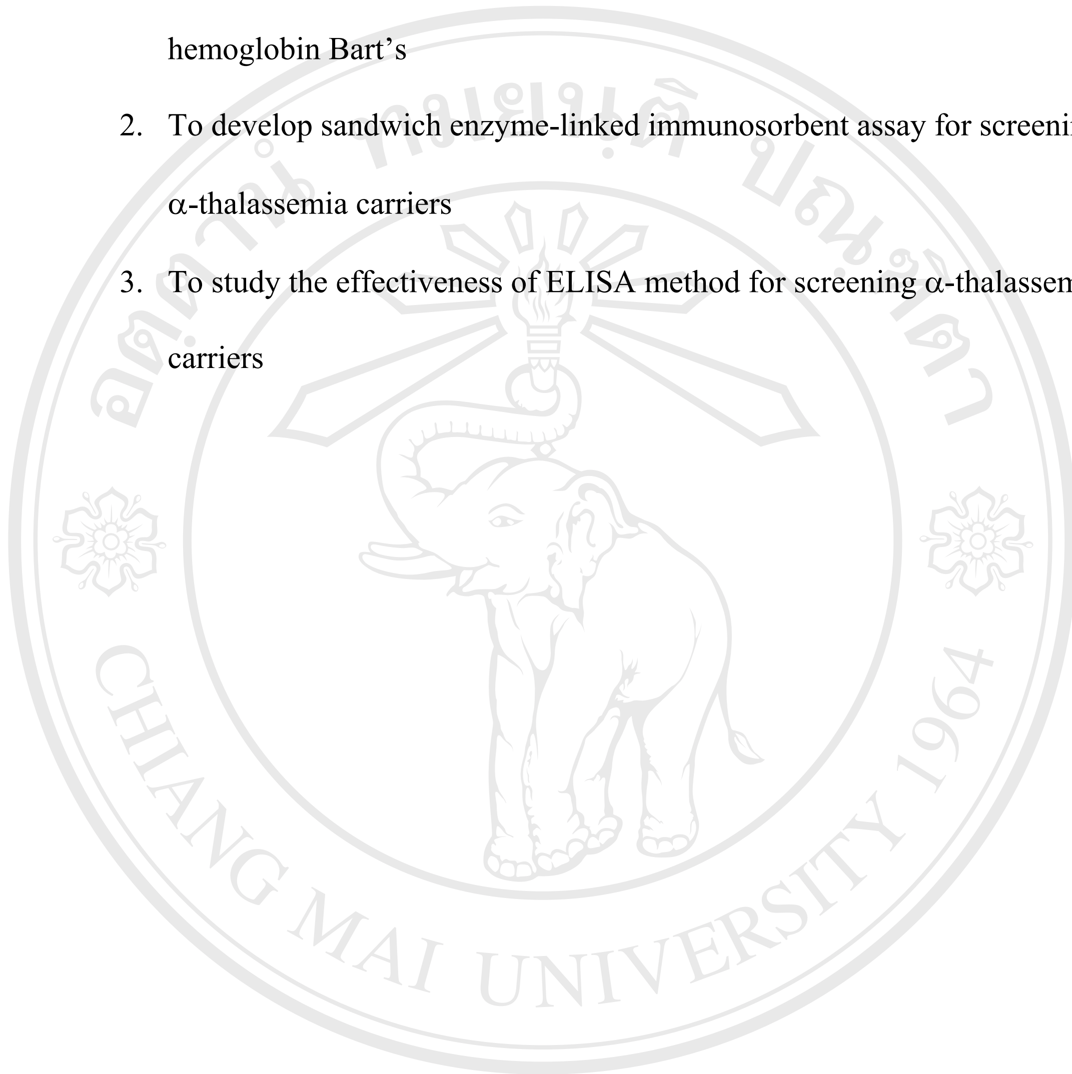
1.4 Objectives

1. To study the specificity of monoclonal antibodies produced against hemoglobin Bart's
2. To develop sandwich enzyme-linked immunosorbent assay for screening α-thalassemia carriers
3. To study the effectiveness of ELISA method for screening α-thalassemia carriers



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