CHAPTER 1

INTRODUCTION

1.1 Overview

Thalassemia is a genetic disorder and frequently results from globin gene mutations or gene deletion that control globin production [1]. The frequency and severity of the several types of thalassemia depend on race of the population. For example, β-thalassemia is commonly found in Africa and the Mediterranean area, whereas α-thalassemia and hemoglobin E disease are common in Southeast Asia [1-3].

Many thalassemia screening techniques have been reported and a few widely used ones will be briefly explained here. For example, Osmotic Fragility Test (OFT) [4], electrophoresis and chromatographic technique [5-7]. The technique that is commonly used for thalassemia screening is the High Performance Liquid Chromatography (HPLC) but it requires an expensive instrumentation [8-9]. However, many countries where thalassemia has been found are developing countries that may have some problem with economic restriction and are not able to use the latest technologies involving expensive instrumentation. Effective screening techniques are needed to help cut down the cost of unnecessary extensive tests. The main objective of this study is to develop the micro-DEAE-Sephadex ion exchange column for thalassemia screening. Advantages of this low cost online system over the conventional batch DEAE-micro column system are very

small amount of sample consumption, short analysis time and low possibility of sample contamination.

1.2 Hemoglobin and Thalassemia

1.2.1 Hemoglobin

The term " hemoglobin" was first introduced by Hoppe-Seyler (1864) to describe the red blood cell [10]. Hemoglobin is made of two similar proteins that stick together which called globin chains. The molecule of hemoglobin is a tetramer of two α globin chains and two non-α globin chains as shown in **Figure 1.1**. The pairing of the globin chain produces three types of hemoglobin: hemoglobin F (Hb F; $\alpha_2\gamma_2$), hemoglobin A (Hb A; $\alpha_2\beta_2$) and hemoglobin A_2 (Hb A_2; $\alpha_2\delta_2$). A failure of α chain production can result in hemoglobin H, which has four β globin chains (β_4) [1, 11-12].

Globin gene expression varies with the age of human development. During the fetal development, red blood cell contains only Hb F [10, 13]. In the first several months of life, γ gene expressing is suppressed and both β and δ globin chain production are activated. In normal adults, red blood cells contain high amount of Hb A (96-97%), small amount of Hb A_2 (2.5%) and very minute amount of Hb F (less than 1 %). The production and development of different of globin chains is shown in **Figure 1.2**.

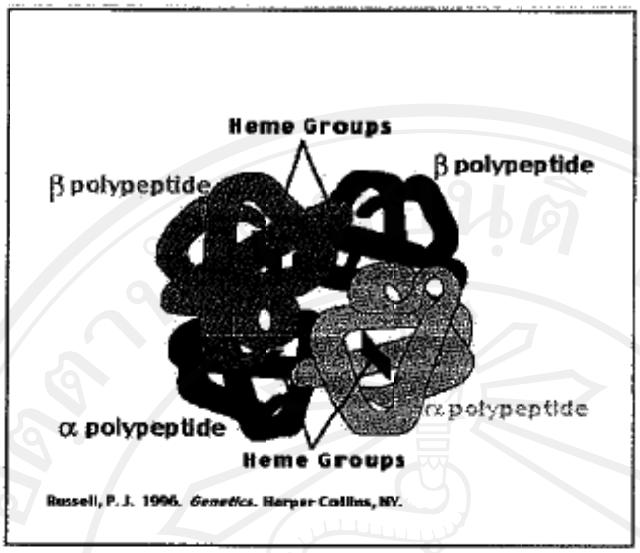

Figure 1.1 Hemoglobin molecule

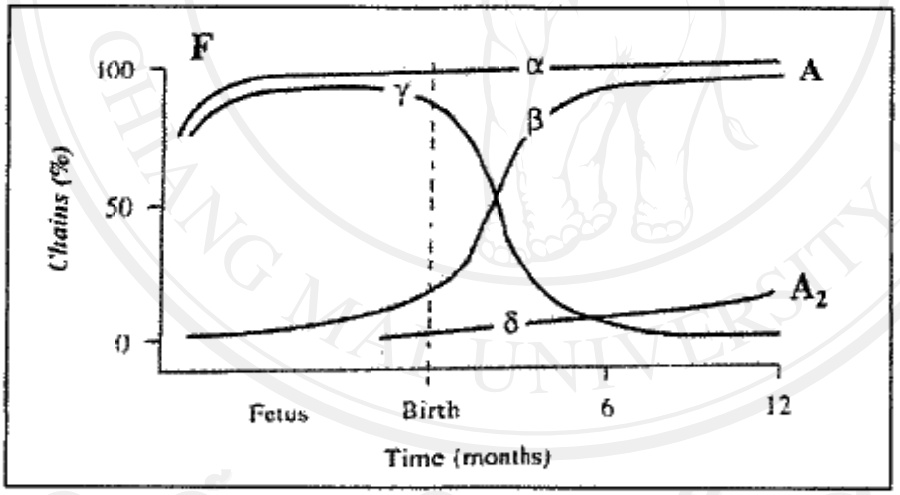

Figure 1.2 Globin chain production and devlopment. Fetal red blood cells contain primarily Hb F ($\alpha_2\gamma_2$). Soon after birth, γ chain synthesis is suppressed and β while δ chain production increase, which results in the production of Hb A ($\alpha_2\beta_2$) and A_2 ($\alpha_2\delta_2$) [1].

Since the gene mutations are associated with a quantitative failure in globin chain production [10], the quantity of each hemoglobin type is important as it can be used in diagnosis of thalassemia and other blood disease as summarized in **Table 1.1**.

Table 1.1 Human hemoglobin [10]

Hemoglobin	Structure	Percentage in normal adult Hemolysate	Increased System	Decreased System
A	$\alpha_2\beta_2$	92	-	-
A_2	$\alpha_2\delta_2$	2.5	β-thalassemia Megaloblastic anemia	Iron deficiency Sideroblastic anemia
A_{1a} A_{1b} A_{1c}	Not Known Not Known $\alpha_2(\beta\text{-N-glucose})_2$	<1 2 5	Diabetes mellitus	Hemolytic anemia
F F_1	$\alpha_2\gamma_2$ $\alpha_2(\gamma\text{-N-acetyl})_2$	<1 <1	Fetal red cells β-thalassemia Sickel cell anemia, Pernicious anemia, etc.	-
Gower 1 Gower 2 Portland	$\zeta_2\varepsilon_2$ $\alpha_2\varepsilon_2$ $\zeta_2\gamma_2$	0 0 0	Early embryo	-
H Bart's	β_4 γ_4	0 0	α-thalassemia	-

1.2.2 Thalassemia

Hemoglobinopathies include the thalassemia syndromes and the inherited structural hemoglobin variants. Hemoglobinopathy is a genetic defect that results in abnormal structure of one of the globin chains of the hemoglobin molecule. Although the suffix "-pathy" would conjure an image of "disease," most of the hemoglobinopathies are not clinically apparent. The abnormal hemoglobins can have a variety of physiologically significant effects, discussed below in greater depth, but the most severe hemoglobinopathies (Hb S and Hb C diseases) are characterized by hemolysis. Thalassemia is genetic defect that results in production of an abnormally low quantity of a given hemoglobin chain or chains [11-12]. This defect causes absent or decreased synthesis of the affected chain. In alpha-thalassemia (α-thalassemia), the production of α globin chain of normal adult hemoglobin (Hb A) is damaged, whereas in beta-thalassemia (β-thalassemia), the systhesis of beta globin chain is reduced or absent [11, 14]. Note that these two definitions of hemoglobinopathy and thalassemia are not mutually exclusive. Some hemoglobinopathies may also be thalassemias, in that structurally abnormal hemoglobin (hemoglobinopathy) may also be under produced (thalassemia). Some, but not all, hemoglobinopathies and thalassemias are hemolytic anemias. Thalassemia is most common in the Mediterranean, the Arabian Peninsula, Turkey, Iran, India, Southeast Asia and South of China, as shown in **Figure 1.3** [15].

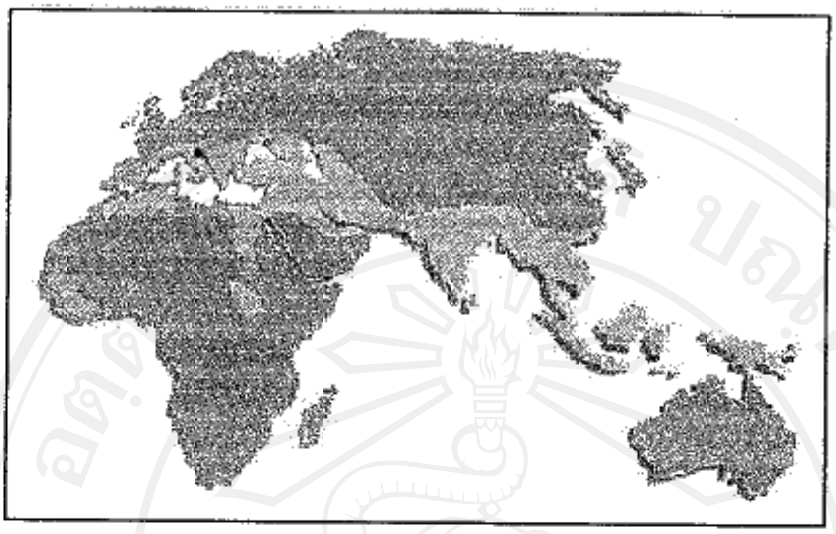

Figure 1.3. The geographic of thalassemia distribution, the yellow area is the area where thalassemia is commonly found [15].

1.2.3 Classification of thalassemia

Thalassemia is normally classified according to the type of the globin chain that is absent or present in decreased amount. The different types of thalassemia syndromes are listed in **Table 1.2**. Each type can occur in both the heterozygous and homozygous forms [11].

Table 1.2 Classification of thalassemia [1]

Clinical	Genotype	Severity
Thalassemia minor		
Alpha-thal-1	—/αα	Microcytosis, hypochromia mild anemia (Hemoglobin 10-14 g/dl)
Hemoglobin Constant Spring	$\alpha\alpha^{CS}/\alpha\alpha^{CS}$	
Alpha-thal-2	−α/−α	
Beta thalassemia trait	$\beta/\beta^{0,+}$	
Lepore trait	β/β^{Lepore}	
Delta-beta thal	β/β^{+}–$Delta^{+}$	
Thalassemia intermedia		
Beta thalassemia	$\beta^{+Africa}/\beta^{+Africa}$	Microcytosis, hypochromia moderate anemia F (Hemoglobin 6-10 g/dl)
Alpha-beta talassemia	β/β^{0}, −α/−α	
Beta thalassemia major with high Hb F production	β^{0}/β^{0} increase	
Thalassemia major		
Beta thalassemia major	β^{0}/β^{0}	Severe microcytosis, hypochromia, and severe anemia (Hemoglobin 3-6 g/dl)
Beta thalassemia-medit	$\beta^{+Medit}/\beta^{+Medit}$	
Beta-thal-Hemoglobin disease	β^{0}/β^{E}	
Hemoglobin H disease		
Alpha-thal-2/alpha-thal-1	—/−α	Microcytosis, hypochromia moderate anemia with reticulocytosis
Alpha-thal-1/Constant Spring	$—/\alpha\alpha^{CS}$	

1.2.3.1 Alpha thalassemia

Alpha thalassemia occurs when one or more of the four α globin chains gene fails to function. The most common patterns for α-thalassemia are summarized in **Table 1.3** [1, 11-12, 16]. α-thalassemia can be associated in four clinical syndromes that will be described as follows [1, 11];

1) The lost of one gene reduces the synthesis of alpha globin chain only slightly, causing α-thalassemia silent or heterozygous α-thal-2. The hemoglobin level in α-thalassemia 1-trait may be slightly depressed or nearly normal. The α-thalassemia 1-trait can be discriminated from β-thalassemia trait by the presence of normal levels of Hb A_2 and Hb F. Diagnosis can be done inferred from family study [11-12, 16].
2) The loss of two genes (two-gene deletion α-thalassemia) produces a condition with small red blood cells, and at most a mild anemia. People with this condition look and feel normal. This type of α-thalassemia involves three cases; homozygous α-thal-2, heterozygous α-thal-1 and hemoglobin constant spring (Hb CS). This condition can be detected by routine blood testing such as the mean corpuscular volume (MCV) [1, 11, 17].
3) The loss of three α genes produces a serious hematological problem (three-gene deletion α-thalassemia). Patients with this condition have a severe anemia, and often require blood transfusions to survive. The severe imbalance between the α chain production (now powered by one gene, instead of four) and β chain production

(which is normal) causes an accumulation of β chains inside the red blood cells. Normally, β chains pair only with α chains. With three-gene deletion α-thalassemia, however, beta chains begin to associate in groups of four, producing an abnormal hemoglobin, called "hemoglobin H". The condition is called "hemoglobin H disease". Hemoglobin H induces two problems. First it does not carry oxygen properly, making it functionally useless to the cell. Second, hemoglobin H protein damages the membrane surrounding the red cell, accelerate cell destruction. The combination of the very low production of α chains and destruction of red cells in hemoglobin H disease produces a severe, life-threatening anemia. Untreated, patients mostly die in childhood or early adolescence [1, 11].

4) The loss of all four α genes produces a condition that is incompatible with life. The gamma chains produced during fetal life associate in groups of four to form an abnormal hemoglobin called "Hydrop fetalis". Most people with four-gene deletion α-thalassemia die in utero or shortly after birth. Four-gene deletion α-thalassemia has been detected in utero. In utero blood transfusions have saved some of these children but they require life-long transfusions and other medical support [11-12].

Table 1.3. Hemoglobin patterns in α-thalassemia [1]

	Hemoglobin (%)			
	A	H(β^4)	Bart's (γ^4) (at birth only)	Constant Spring
Normal	97	0	0	0
α-thalassemia silent				
–α/αα (heterozygous alpha-thal-2)	98-100	0	(0-2)	0
α-thalassemia trait				
–α/–α (homozygous alpha-thal-2) or —/αα (heterozygous alpha-thal-1)	85-95	Red Blood cell inclusions	(5-15)	0
$\alpha\alpha^{CS}/\alpha\alpha^{CS}$ (Constant Spring)	85-95	0	(5-15)	5
Hemoglobin H disease				
—/–α (alpha-1/alpha-2)	70-95	5-30	trace	trace
—/$\alpha\alpha^{CS}$ (alpha-1/constant Spring)	60-90	5-30	trace	5-10
Hydrops fetalis				
—/— (alpha-1/alpha-1)	0	5-10	90-95	0

1.2.3.2 Beta Thalassemia

The fact that there are only two genes controlling the beta chain of production makes β-thalassemia a bit simpler to understand than α-thalassemia . Unlike α-thalassemia, β-thalassemia rarely arises from the complete loss of a β globin gene [12]. The β globin gene is present, but produces little β globin protein. The degree of suppression varies. Many causes of suppressed β globin gene expression have been found. In some cases, the affected gene makes essentially no β globin protein (β-0-thalassemia). In other cases, the production of β chain protein is lower than normal, but not zero (β-(+)-thalassemia) [12, 18]. The most common patterns for β-thalassemia are shown in **Table 1.4** [17]. The severity of β-thalassemia depends in part on the type of β-thalassemic genes that a person has inherited as discussed here;

1) One-gene β-thalassemia is the type of thalassemia that has one normal β globin gene on affected β globin gene that causes a variably reduced production of β globin. The degree of imbalance with the α globin depends on the residual production capacity of the defective β globin gene. Even when the affected gene produces no β chain, the condition is mild since one β gene functions normally. The red cells are small and a mild anemia may exist. People with this condition generally have no symptoms. The condition can be detected by a routine laboratory blood evaluation. (Note that in many ways,

the one-gene β-thalassemia and the two-gene α-thalassemia are very similar, from a clinical point of view. Each results in small red cells and a mild anemia) [12, 18-19].

2) Two-gene β-thalassemia produces a severe anemia and a potentially life-threatening condition. The severity of the disorder depends in part on the combination of genes that have been inherited; (1) β-0-thal/ β-0-thal, (2) β-0-thal/ β-(+)-thal and (3) β-(+)-thal/ β-(+)-thal which shown severity in the order from low to high. The β-(+)-thalassemia genes vary greatly in their abilities to produce normal hemoglobin. Consequently, the clinical picture is more complex than might otherwise be in the case of another 2 genetic possibilities outlines [16, 18].

Table 1.4 Hemoglobin patterns in β-thalassemia [17]

	Hemoglobin (%)				
	A	F	A_2	Lepore	S
Normal	97	<1	2-3	0	0
β-thalassemia minor					
Trait β/β^0 or $^+$	90-95	1-5	4-7[a]	-	-
Lepore trait β/β^{Lepore}	80	1-5	2-3	15	-
β-thalassemia intermedia					
$\beta^{+Africa}/\beta^{+Africa}$	30-50	50-70	0-5	-	-
β-thalassemia major					
β^0/β^0	0	95-100	0-5	-	-
$\beta^{+Medit}/\beta^{+Medit}$	10-20	70-80	0-5	-	-
$\beta^{Lepore}/\beta^{Lepore}$	0	80	0	20	-

[a] Iron deficiency can lower the Hb A_2 level to normal

1.2.4 Methodology for detection and characterization of thalassemia

1.2.4.1 Screening methods

Determination of the mean corpuscular volume (MCV) as a part of a complete blood count (CBC) has given a primary indicator for the presence of α- or β-thalassemia trait (carrier state) [3, 5]. Carriers of β-thalassemia usually have a high concentration of Hb A_2 (>3.5%), with or without the high concentration of Hb F (>1.5%), as determined by hemoglobin electrophoresis. In contrast, α-thalassemia carriers have normal hemoglobin electrophoresis [3, 5, 14, 20-21]. Cellulose acetate electrophoresis at alkaline

buffer pH 6.0-6.2 [3], or thin layer isoeletric focusing are the preferred screening tests for hemoglobin disorder. Citrate agar electrophoresis is used in many laboratories to confirm the presence of abnormal hemoglobin after it has been detected by another technique. In 1980, another screening technique, called Osmotic Fragility Test (OFT), was developed to be used for preliminary test. It is based on the slower rupture rate of red blood cells of patients with thalassemia (positive) in hypotonic salt solution as compared to normal red blood cells [4].

Chromatographic procedures had never played a significant role for screening until micro procedures were developed. Column chromatographic fractionation of human hemoglobin and also of animal hemoglobins has been performed with the use of weak cation exchangers as for instance, Amberlite IRC-50 and carboxymethylcellulose [20-22]. The cellulose anion exchangers, such as dimethylaminomethyl (DEAE) cellulose, triethylaminoethyl (TEAE) cellulose and epichlorohydrin/triethanolamine (ECTEOLA)-cellulose have proved to be useful chromatographic media for many proteins, particularly serum proteins [20, 23]. In the fractionation of serum proteins on DEAE cellulose, application of buffer gradients of increasing concentration and decreasing pH results in separation of many components. High performance liquid chromatography (HPLC) is a newer chromatography technique that offers higher resolution than electrophoresis [4, 24]. It becomes the most commonly used technique in the present day because it can be used for quantitative and qualitative analysis of hemoglobin. However, it requires an expensive instrumentation. In many cases, using only one technique cannot complete

diagnosis of thalassemia. Several different tests are normally done in combination, depending on suitability and availability of materials and instrumentation. An example of the stepwise approach in thalassemia diagnosis involving different techniques is shown in **Figure 1.4**.

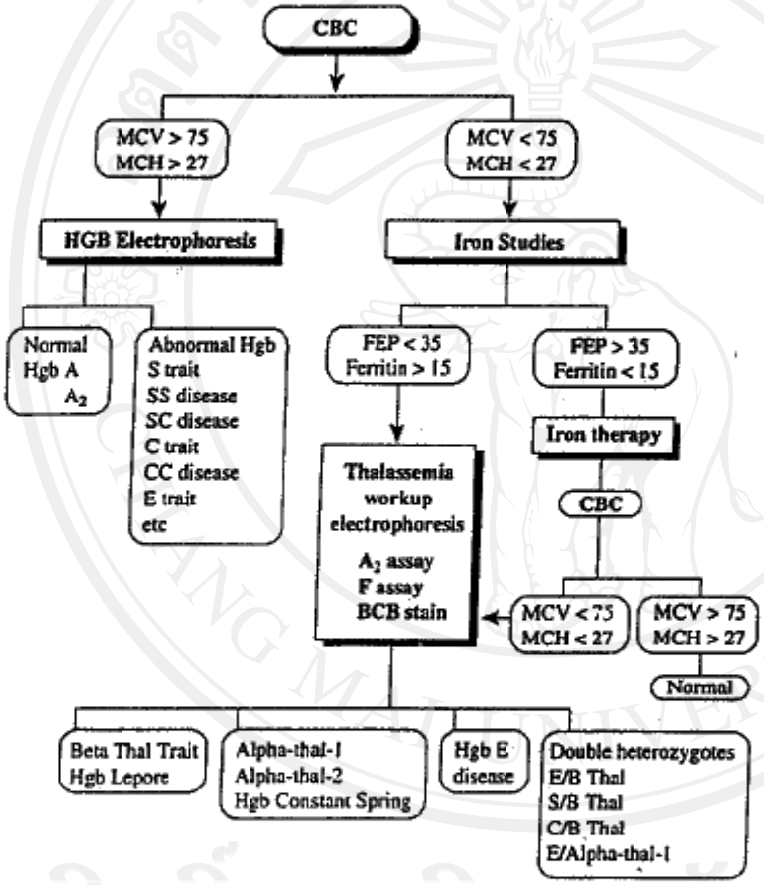

Figure 1.4 Stepwise approach to the diagnosis of thalassemia [1]

1.2.4.1.1 Complete Blood Count (CBC)

Structural hemoglobinopathies may have an impact on the red cell indices, which the key components of the CBC are critical to diagnosis of thalassemia, these indices include Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), red cell distribution width (RDW) [5].

The precise automated cell counters, such as the counter Model S [5]. CBC directly measures red cell volume has become a valuable tool for detecting patients with thalassemia. Many hospitals and clinics use these instruments for performing their routine blood counts, and have detected asymptotic microcytosis of thalassemia minor. The association of microcytosis with thalassemia is so strong that several population-screening programs have used the measurement of mean corpuscular volume (MCV) as the initial screening procedure [3, 5, 24].

To evaluate screening tests used in the laboratory, three important parameters are test sensitivity, test specificity, and the predictive value of a positive result [5]. Sensitivity is the percentage of patients that show a positive result and really have the disease. Specificity is the percentage of patients that show a negative result and are truly without the disease. The predictive value of a positive result is the percentage of patients that show positive results and really have the disease.

The best parameter of the CBC in screening thalassemia is the MCV. This parameter can be directly measured, but the values are dependent on the method of calibration. A decrease in MCV is a sensitive detector for α- and β-thalassemia minors.

When children are screened for thalassemia, different criteria are needed to characterize microcytosis. Normal children have a normal MCV range of 95 to 120 fl. Schmaier and coworkers [5] have recommended performing hemoglobin electrophoresis on all newborns who have MCV values less than 94 fl to screen for α-thassemia. The predictive value of a low MCV for thalassemia screening is dependent on the prevalence of various other diseases that are also associated with microcytosis. Some of these other diseases are iron deficiency, lead poisoning, chronic disease, sideroblastic anemia, and various hemoglobinopathies. In most populations, iron deficiency is the most prevalent. Several mathematics manipulations of the routine blood counts have been proposed to improve specificity and predictive value in screening for thalassemia. These procedures generally attempt to separate thalassemia from iron deficiency, the other common cause of microcytosis [5].

The proposed mathematics functions for separating thalassemia and iron deficiency are based on the different relationships of blood cell parameters in these two disorders. Representative distributions of various cell parameters from two studies of iron deficiency and thalassemia trait are illustrated in **Figure 1.5** and **1.6**. Note that iron-deficient patients are more anemic, have relatively larger cells, and have fewer cells than the thalassemia patient. Also note that the distributions of the mean corpuscular hemoglobin concentration (MCHC) are similar in the two disorders. The proposed mathematics formulas serve to quantitate and accentuate the differences in hemoglobin, MCV, and red cell counts.

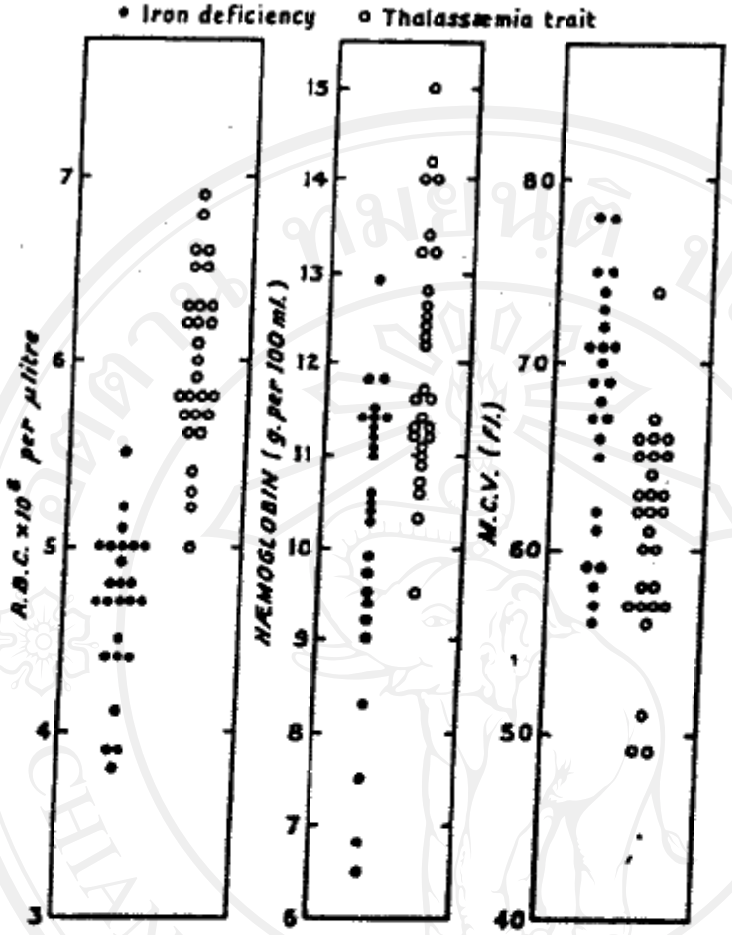

Figure 1.5 Distribution of erythrocyte counts (RBC), hemoglobin concentration and MCV in patients with iron deficiency (solid circles) and thalassemia trait (open circles). This study includes only patients with microcytosis. The thalassemia patients have relatively higher erythrocyte counts and are less anemic than the iron-deficient patients [5].

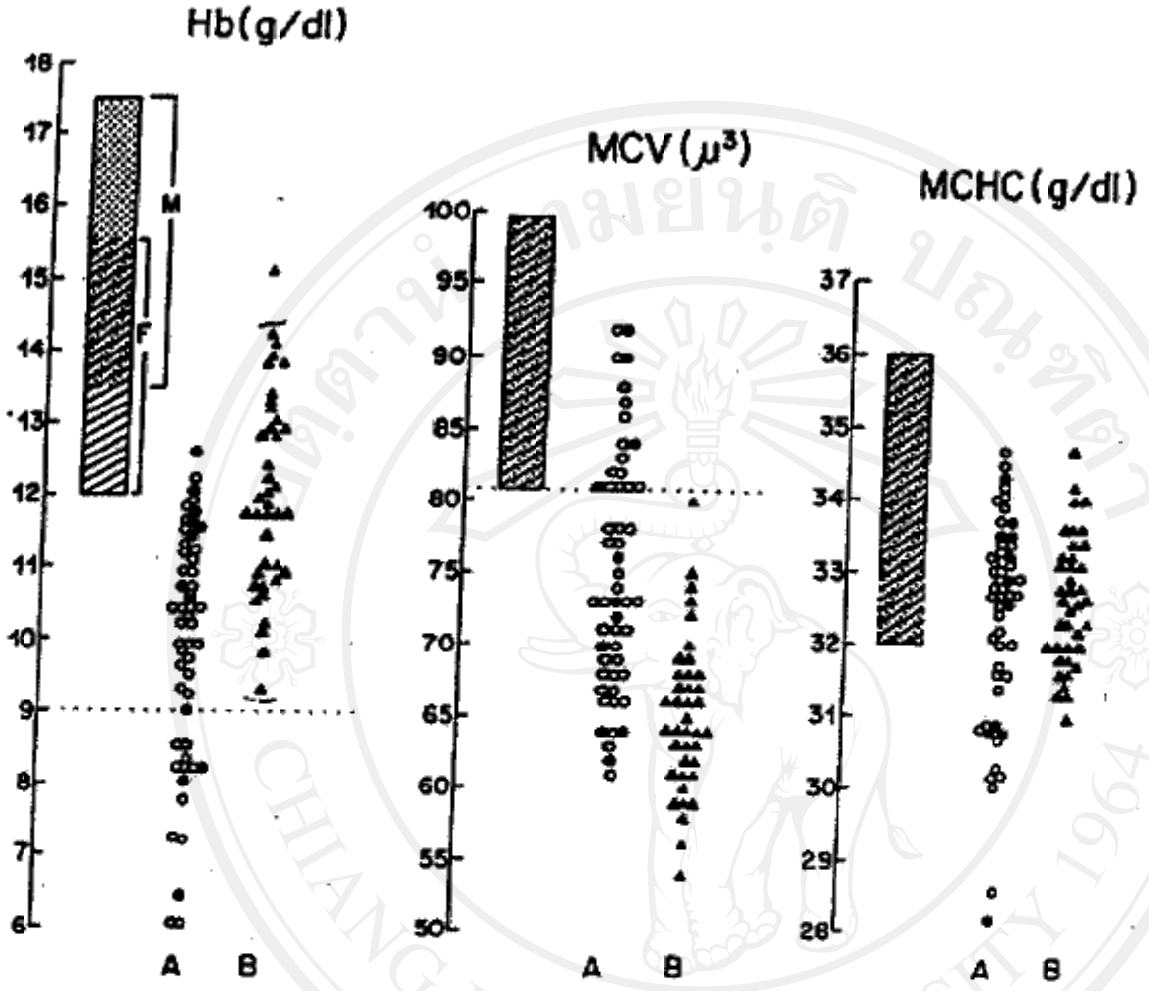

Figure 1.6 Hemoglobin concentrations, MCV and MCHC in uncomplicated iron deficiency (A) and uncomplicated thalassemia minor (B). Solid symbols represent males and open symbols represent females. Dashed lines represent limits used in defining the category of microcytosis without marked anemia. The reference values for healthy adults are indicated by the shaded areas [5].

In conclusion, it can be said that the MCV parameter from electronic blood counters is a sensitive screening test for thalassemias and aids in the detection of other hemoglobinopathies. The application of various mathematics functions helps to improve the specificity of the screening tests, but even with these refinements they are not sufficiently reliable to permit diagnosis with a high level of confidence. The additional information from platelet counts, erythrocyte anisocytosis, ferritin, and FEP helps to further classify these disorders. Direct quantification of hemoglobin components or α/β chain synthesis rates are also needed to unequivocally characterize the thalassemias and hemoglobinopathies [5].

1.2.4.1.2 Osmotic Fragility Test (OFT)

Not all laboratories have the facility of electronic cell counters to measure red cell index [4]. In such cases Osmotic Fragility Test (OFT), a rapid, simple and cost effective screening test could be used for thalassemia screening [25-29]. The principle of OFT is based on the limit of hypotonicity which the red cell can withstand. In this procedure, 2 ml of 0.36% buffered saline is taken in a test tube, 20ml of whole blood is added to it, and is allowed to stand at room temperature. After 20 minutes, reading is taken on a OFT stand on which a thin black line is marked. If the line is visible through the solution, the test is considered as negative and if line is not visible it is considered as positive. Positive test is due to the reduced osmotic fragility of red cells (**Figure 1.7**).

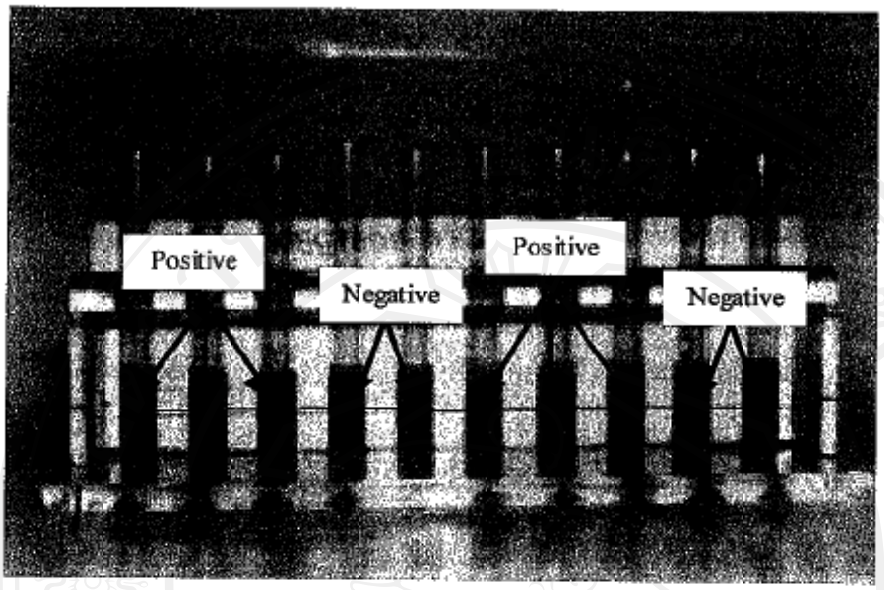

Figure 1.7 OFT stand showing positive and negative samples in different tubes. Tubes from L to R: Tubes 1-3 and 6-8 are positive samples where black line is not visible through the solution. Tubes 4-5 and 9-10 are negative samples where black line is visible through the solution.

OFT has sensitivity ranging from 94 to 99 per cent [26-27, 30]. A lower positive predictivity suggested false positive results probably due to associated iron deficiency which requires confirmation by estimation of HbA_2 levels. Based on high negative predictive values, the use of OFT has been recommended for mass screening due to its low cost and simplicity. Though OFT is a simple and rapid test, combination of OFT and red cell indices increases the sensitivity and negative predictive value to almost 100 per cent [4].

1.2.4.1.3 Microcolumn Chromatography

This method is used for determination of Hb A_2 and the results can be used for diagnosis of β-thalassemia trait [21, 23-24]. The anion or cation exchanger is used for chromatographic resin to separate different Hb types. Relative amount of each Hb type can be estimated from peak areas. For example, diethylaminoethyl (DEAE) cellulose is used for the adsorption of HbA_2. The reactive functional group of anion exchanger is a strongly basic amine [21, 31-33] as shown in **Figure 1.8.** The performances of various chromatographic resins for Hb A_2 quantification are compared in **Table 1.5** [5, 7].

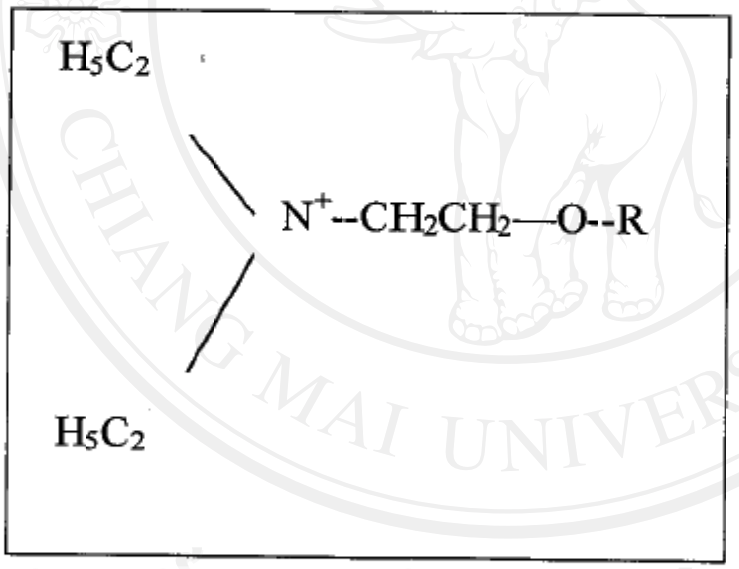

Figure 1.8 The reactive functional group of the DEAE anion exchanger. R is the fourth carbon of glucose in the cellulose chain. The COO^- of hemoglobin binds to the N^+ of the DEAE.

After applying the sample into the column, a buffer solution of higher pH is introduced then Hb A_2 is first eluted. In the second step, when the buffer solution of a lower pH is introduced then Hb A is eluted. The two fractions are collected separately and the absorbances of hemoglobin in the elutents are measured spectrophotometically, usually detected at 415 nm.

Table 1.5 comparison of various chromatographic resins for Hb A_2 quantification [5].

Resin	Advantages	Disadvantages
DEAE Sephadex	Realiable	- Very time consuming and requires expertise - Cannot distinguished C, E or O from A_2
DEAE Cellulose	Realiable	- Very time consuming and requires expertise - Cannot distinguished C, E or O from A_2
CMC (carboxymethyl cellulose)	Realiable	- Very time consuming - Cannot distinguished S from A_2

1.2.4.1.4 High Performance Liquid Chromatography (HPLC)

Recently, HPLC has become the most widely utilized method for hemoglobin analysis [3]. It can be used for the quantification of Hb A_2, Hb F and other Hb variant [3-4, 9, 24]. It provides precise quantification of Hb A_2 and is therefore suitable for the

diagnosis of beta thalassemia trait. However, Hb A_2 may not be accurately quantified in the presence of Hb S [34-36]. In addition, Hb A_2 cannot usually be separated from Hb E. The cation exchange and reverse phase HPLC are mostly employed. The cation exchange HPLC has been developed in recent years for hemoglobin variant studies. It has been shown to be a sensitive, specific and reproducible alternative to electrophoretic and other techniques for screening and quantifying Hbs of clinical significance. The Bio-Rad variant (Bio-rad laboratories) is an automated cation exchange HPLC instrument that has been used to quantify Hb A_2, Hb F, Hb A, Hb S and Hb C [9, 24, 34]. This method has the great advantage that the minor Hb A_{1c} (i.e. glycosylated Hb A) and fetal Hb (Hb F) are completely separated allowing the quantitation of Hb F even in samples from normal persons. **Figure 1.9** shows the separation of Hb types in hemolysate of a normal by using cation exchange column. The reversed phase HPLC has been used for a rapid separation of the polypeptide chains of human Hbs; Hb A, Hb A_2, Hb F and the embryonic Hbs at a low pH. Separation of the β, δ, α, $^{G}\gamma$ and $^{A}\gamma$ polypeptides is possible. Variation in the pH gradient is desirable to improve the separation of the γ- chain from the α-chain. **Figure 1.10** shows the separation of polypeptide chain in hemolysate of a 3 months old normal infant by using C-18 as a reverse phase column [3-4, 34-36].

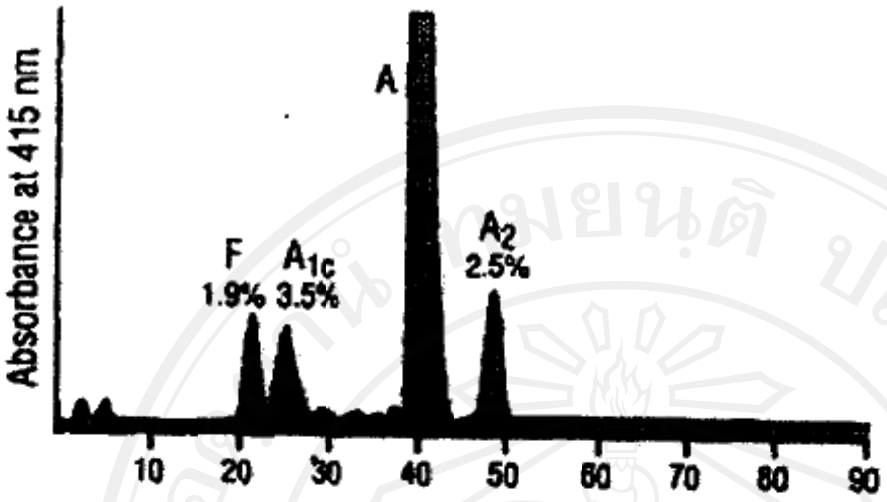

Figure 1.9 Separation of normal Hb types by cation exchange HPLC [36]

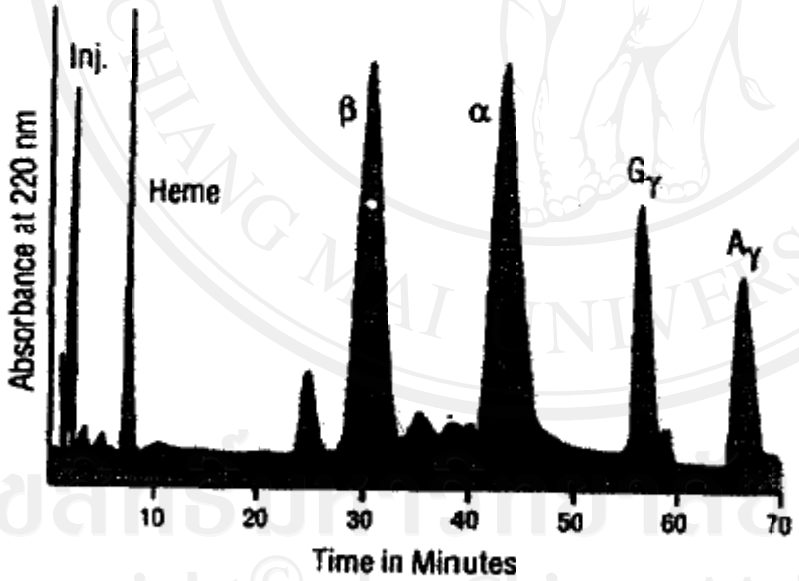

Figure 1.10 Separation of normal polypeptide chain by reversed phase HPLC [36]

Interpretation of HPLC data sometimes can be difficult because Hbs may co-elute or may elute before instrument peak integration. It may need to be used with other methods such as electrophoresis and mass spectroscopy for characterization of other Hb variants that cannot be completely separated by HPLC [34-36].

1.2.3.1.1 Electrophoresis

Electrophoresis, an alternative separation method, is used to separate charged particles in a conductive medium using an externally applied electric field. In this method, an external electric field is applied to a sample causing positively charged cations to migrate toward the negatively charged electrode, or cathode, and negatively charged anions to migrate toward the positively charged anode. In this manner, compounds move toward the anode or cathode under the influence of the electric field. In the absence of any other influences, all compounds will travel through the electrolyte buffer as discrete zones, or bands, based on differences in solute mobility [37].

Electrophoresis can be used to measure the different types of hemoglobin in the blood [3, 5-6]. It is performed when a disorder associated with abnormal hemoglobin is suspected. The test can use for diagnosis of sickle cell, anemia and thalassemia. **Figure 1.11** shows the hemoglobin electrophoresis in the most prevalent sickle hemoglobinopathies [12, 14, 24]. In the experiment, net charge is not only determinant of electrophoretic mobility of globin chains but it also depends on the size of Hb [37]. Each globin is readily distinguished by its mobility which is different in acidic and alkaline

buffers. At alkaline pH, the migration of electrophoretic of Hb C, Hb E, Hb A_2 and Hb O is similar. Hb S, Hb D and Hb G also comigrate. At acid pH, electrophoretic separation of Hb C from Hb E, and Hb O and Hb S from Hb D and Hb E is accomplished [3, 5-6, 14]. It is not possible to differentiate between Hb E and Hb O and Hb D and Hb G using electrophoretic method. The early electrophoresis method was time consuming and laboratory intensive due to the preparation of globin and medium support. It was inaccurate in the quantification of Hb variant's concentration or in the detection of fast Hb variant.

Recently, electrophoresis technique has been developed into a technique called isoelectric focusing (IEF) [24]. IEF is an equilibrium process that the migration of Hb is occurred in pH gradient to a position of zero net charge. The migration of Hbs in IEF is the same as in the alkaline electrophoresis but with much better resolution of Hb C from Hb E; and Hb O and Hb S from Hb D and Hb G; Hb A from Hb F are also clearly resolved. It offers excellent resolution due to narrower bands as compared to the conventional electrophoresis, therefore IEF can be used to effectively quantitate and qualitate Hbs. However, it is time consuming and labor-intensive [12].

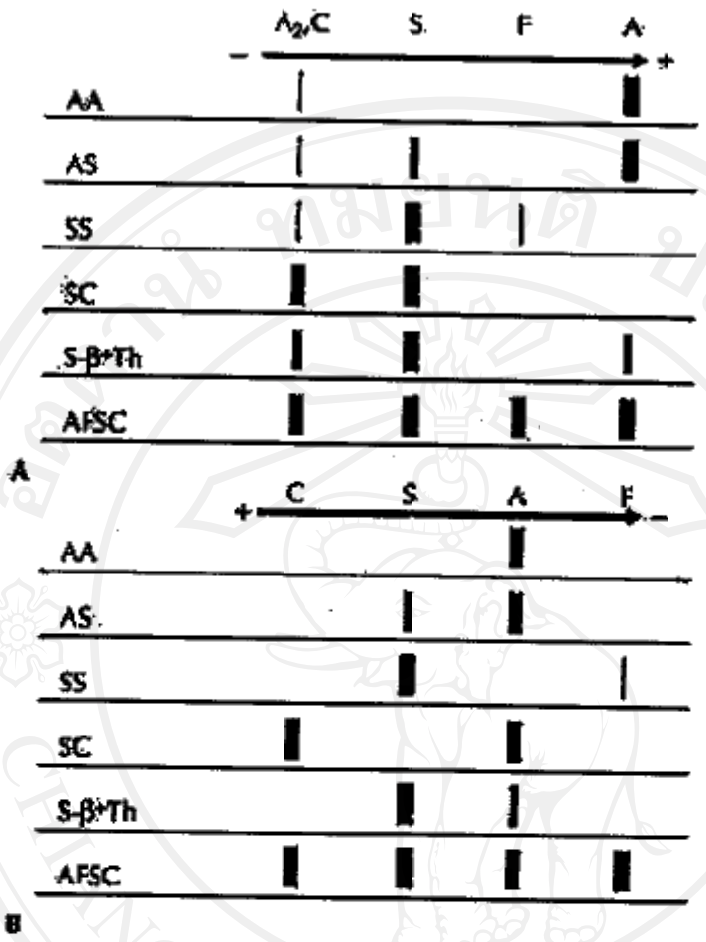

Figure 1.11 Hemoglobin electrophoresis of the most prevalent hemoglobinopathies [6]

(A) Cellulose acetate electrophoresis at alkaline pH causes hemoglobins to move from the cathode toward the anode. AA, normal sample; AS, sickle cell trait; FASC, control containing Hb A, F, S, C; Hb S-$^{+}$ Th, HbS, β^{+}-thalassemia; SC, HbSC disease; SS, sickle cell anemia.

(B) Citrate agar electrophoresis, at acidic pH. Note that the hemoglobins now travel from anode to cathode.

1.2.4.2 Characterization method [14]

Identification of an amino acid replacement in a Hb variant requires the isolation of the abnormal Hb and of the abnormal chain (α or β or γ or δ), digestion with a proteolytic enzyme (usually trypsin), separation of the smaller fragments, determination of the composition of each peptide with an amino acid analyzer, and sequencing of the peptide with the amino acid replacement. Isolation of the Hb is usually done by chromatography [37] such as cation exchange HPLC, preparative starch-block electrophoresis or preparative IEF [3, 14] . The chromatographic procedure on CM-cellulose with phosphate-8 M urea developers is most popular for the separation of the polypeptide chains [37-39], although more recently (semi-) preparative reversed phase HPLC methodology is preferred by some laboratories, particularly when micro methodology is available for the entire structural analysis.

The first method available for the separation of the proteolytic fragments was the fingerprinting technique, i.e. a combination of an electrophoretic and chromatographic separation which are previously described in the screening section. This technique was slowly replaced by macro-chromatographic methods with cation exchangers , and more recently, reversed phase HPLC methodology [40-41]. The latter method is fast, accurate, and reproducible. There are numerous suitable HPLC columns on the market that are excellent for qualitative of Hb type and polypeptide chain. Amino acid analysis can be done using automated amino acid analyzers; some 40 years ago one such analysis took

two weeks and can at present be accomplished in 30-60 minutes. Instrumentation for automatic sequencing of peptides and proteins are also available.

Prenatal diagnosis of sickle cell disease, thalassemia and other hemoglobinopathies in the fetus has been aided by advances in techniques of obtaining and analyzing specimens. Early test involved the analysis of fetal blood obtained by fetoscopy or placental aspiration. Recent genetic advances have provided a safer and more practical method to identify the gene mutation in globin chain directly through recombinant DNA technology; polymerase chain reaction (PCR) methodology and sequencing technique. These techniques are highly accurate in detecting sickle cell disease and certain forms of thalassemia. However, the disadvantage of these techniques is that DNA cannot be performed safely until about 16 weeks' gestation, thus delaying diagnosis and potentially intervene until late in the secondmester [3, 39-41].

1.2.4.2.1 Polymerase Chain Reaction (PCR) [3]

The application of molecular biology techniques to the study of genetic disease has provided a wealth of information about their molecular basis. The haemoglobinopathies were the first genetic disorders to be studied extensively at the molecular level, and probably all the common mutations. In this study, presents the techniques currently in general use for the screening of the globin gene mutations. The defects are regionally specific with each local population having its own combination of structural haemoglobin variants and thalassemia mutations [42]. Therefore, knowledge of the ethnic origin of a patient is often need to enable the quick identification of the globin gene defect by molecular biology techniques based on amplification of DNA by the polymerase chain reaction (PCR). Mutation identification is achieved by screening first for the expected known mutations, using one or more PCR-based techniques such as gel electrophoresis, restriction endonuclease analysis, allele-specific probe hybridization and allele-specific primer amplification. In the few cases where these techniques fail to reveal the genetic defect, characterization may be achieved by the application of non-specific detection methods such as denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, single-stranded conformational polymorphism analysis (SSCP) and direct sequencing of amplified DNA. Some laboratories are using DGGE as their first approach to mutation detection because of their need to identify a large number of different mutations arising from a variety of ethnic groups in their at-risk population.

The strategy for characterizing β-thalassemia mutations in most laboratories depends upon knowing the most prevalent mutations likely to be encountered in the ethnic group of the individual being screened. These are then tested by using one of the many methods now available such as Allele-specific oligonucleotides that is based on the use of two oligonucleotide probes for each mutation, one complementary to the mutant DNA sequence and the other complementary to the normal β-gene sequence at that position [43]. The amplification refractory mutation system (ARMS) [44], based on the principle of primer-specific amplification, which is that a perfectly matched PCR primer is much more efficient in annealing and directing primer extension than one containing one or two mismatched bases. Gap PCR. Deletion mutations in the β-globin gene sequence may be detected by PCR using two primers complementary to the sense and antisense strand in the DNA regions which flank the deletion. deletion β-thalassaemia, Hb Lepore and a number of δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) deletion mutations can be diagnosed by this method [45]. The alpha-thalassemia can also be diagnosed by this method.[46-47] These techniques simply pinpoint the presence of a mutation or DNA polymorphism in the amplified target sequence. Sequencing of the amplified product is then required to identify the localized mutation. This can now be done very efficiently using an automated DNA-sequencing machine utilizing fluorescence detection technology. Direct DNA sequencing becomes the primary method of mutation detection such as to characterized alpha gobin genes mutation [48].

The amplification of DNA by ARMS analysis, gap PCR and restriction enzyme analysis before gel electrophoresis provides a simple, quick and cheap approach to the direct detection of mutations, enabling developing countries to introduce prenatal diagnosis services for the hemoglobin disorders [49]. Fluorescence-based automatic DNA sequencing will become the method of choice for characterization of unknown mutations and even diagnosis as cheaper, second-generation machines are developed using capillary electrophoresis to separate amplified DNA fragments.

1.3 Flow Injection Analysis (FIA) [50-51]

Batch process chemistry is currently being replaced by flow injection analysis (FIA), which is computer compatible and allows automated handling of sample and reagent solutions with a strict control of reaction conditions. Ruzicka and Hansen first described FIA in 1975 [50].

FIA relies on the injection of a precisely measured sample solution into an unsegmented carrier stream. The chemical reagents for reacting with sample may be a carrier solution itself or can be added downstream by merging with the carrier. During its transportation through the FIA-manifold, the injected sample zone is dispersed within the carrier in a highly controlled manner. A well-defined concentration is formed and at the same time the sample solution can react with the reagent(s) to form a product, which subsequently is monitored by a suitable detector for readout. Thus, FIA is based on three important principles, which are sample injection, controlled dispersion of the injected sample zone and reproducible timing, that is, the time elapsed between the point of injection and the point of detection. The injected sample form a zone, which is then transported toward a detector that continuously records the absorbance, electrode potential, or other physical parameter as it continuously changes due to the passage of sample material through the flow cell [50].

FIA is a common method that has been applied for online and laboratory processing of wastewater, soil testing, and other environmental studies and also in clinical chemistry. Because of the small amount of reagents required, FIA method

produces only small amount of waste. It offers short analysis and the highly reproducible. The scope of the method had grown from serial assay of samples to a tool for enhancement of performance of spectroscopic and electrochemical instruments. Most recently FI was been applied in biological studies of living cells by fluorescence microscopy and flow cytometry [50]. Other fields of application include real time monitoring of chemical process [51] and immunoassays [52].

1.4 Diethylaminoethyl (DEAE) – sephadex A50 ion exchange chromatography [23,51]

In ion exchange chromatography, substances are separated based on their charges by the stationary phase (bead or resin) with a fixed charge. If the fix charge is negative, then the process is called cation-exchange chromatography, and if fix charge is positive, then it is anion-exchange chromatography. Two common resins are carboxymethyl (CM) cellulose (cation exchanger) and diethylamino-ethyl (DEAE) cellulose (anion exchanger).

Fixed charges on the beads will interact with substances of opposite charge in the buffer solution (mobile phase). For example, the fixed negative charge of a cation exchange resin will always be associated with a positively charged species in the buffer solution (**Figure 1.12 a)**). If a protein of positive charge is passed through the column, it may bind to the matrix (**Figure 1.12 b)**), and it can subsequently be eluted by increasing the anion concentration of the mobile phase. The elution can be looked on as a competition for binding sites on the matrix. As the ionic strength of the buffer increases, it becomes more likely that a salt ion from the buffer will take the site on the resin and

the substances previously bind to the resin will be eluted from the column. The types of matrix and functional groups of the ion exchangers are shown in **Table 1.6.**

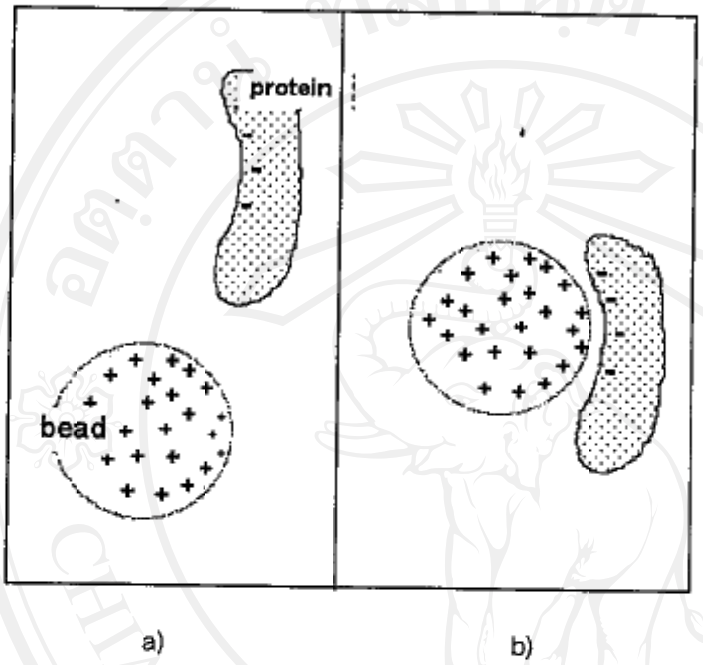

Figure 1.12 Protein binding to an ion exchange bead [51]

a) Positively-charged bead associates with negatively-charged counter ions from buffer solution. The negatively-charged protein associates with positively charged counter ions it.

b) When the protein binds to the bead, some of the counter ions are displaced from both the bead and the protein.

Table 1.6 Types of matrix and functional group

Matrix	functional group
Anion exchangers	
Aminoethyl (AE-)	$-OCH_2CH_2NH_3^+$
Diethylaminoehtyl (DEAE-)	$-OCH_2CH_2N^+H(CH_2CH_3)_2$
Quarternary aminoethyl (QAE-)	$-OCH_2CH_2N^+H(C_2H_5)_2\ CH_2CH(OH)CH_3$
Cation exchangers	
Carboxymethyl (CM-)	$-OCH_2COO^-$
Phospho	$-PO_4H_2^-$
Sulphopropyl (SP-)	$-CH_2CH_2CH_2\ SO_3^-$

The matrices that have been used for stationary phase can be classified into 3 groups:

1. Resin (eg. Polystyrene)
2. Cellulose
3. Cross linked polyacrylamide or polydextran gels

In this study, sephadex was used as ion exchanger. It is a polydextran and a hydrophilic, highly stable in chemical, non-soluble in any solvent, stable under high pressure and temperature (121 °C, in neutral pH longer than 30 minutes) [52].

DEAE-Sephadex bead has diethylaminoethyl ($-OCH_2CH_2N^+H(CH_2CH_3)_2$) functional group that interacts with anionic groups on hemoglobin and thus can retain all

types of hemoglobin. Different types of hemoglobin contain different amount of net negative charge and therefore can be separated with the pH gradient elution. Upon elution using the more acidic buffer, containing more HCl, hemoglobin becomes less negative and thus anionic groups of hemoglobin captured by DEAE-bead can be exchanged with Cl^-. The order of hemoglobin eluted from a DEAE-Sephadex column is HbA_2 co-eluted with HbE, then HbA and finally HbF.

1.5 Flow-based technique for thalassemia screening

Techniques commonly used in the hospital around the world to indicate the existence of thalassemia in patients are cellulose electrophoresis, micro-column chromatography and HPLC [53-54]. Electrophoresis can qualitate but cannot conveniently quantitate for different types of hemoglobin. It is used to find out the exact type of thalassemia after the indication of having thalassemia was found. On the other hand, HPLC technique can be used to qualitatively and quantitatively analyze hemoglobin but it requires an expensive instrumentation. In many countries including Thailand, separation of hemoglobin using DEAE-Sephadex column is well established and it is normally done to screen for thalassemia before performing further examinations. However, the conventional column technique involves analyzing many fractions of eluate collected batch-wise leading to long time, high amount of reagents and sample consumption per analysis run. This work attempts to develop a simple, low cost and fast system to perform hemoglobin typing.

In the proposed system, flow injection analysis coupled with a reduced volume chromatographic column for hemoglobin separation has been developed. Hemoglobin typing is achieved using a reduced volume micro-DEAE-Sephadex ion exchange column. It was found that the flow based-system significantly decreased the analysis time per run. Very small volume of diluted blood was used which in turn generated only small amount of biological hazardous waste. Its closed system also reduces the possibility of sample contamination. The absorbance of the hemoglobin was spectrophotometrically monitored at 415 nm and relative amount of each Hb type was estimated. The technique is used to screen for some types of thalassemia based on abnormally high ratio of HbA_2 and HbE relatively compared to total amount of hemoglobin as percentage. Comparison of the results with those obtained from the larger conventional column technique suggests the possibility of applying the proposed system to screen for thalassemia.