CHAPTER 1

INTRODUCTION

1.1 Overview

Hemoglobin E is an abnormal hemoglobin with a mutation in the beta (β) globin gene causing substitution of glutamic acid by lysine at position 26 of the beta (β) globin chain [1]. The co-existence of hemoglobin E with β-thalassemia gene, a disease which can be life threatening, causes a reduce number of red blood cell in blood stream. This can affect transportation of oxygen to the body tissue, and causes red blood cells to be smaller than normal or contain less than the usual amount of hemoglobin. This disease can result in heart problem from the severe damage of red blood cells. Other symptoms such a big liver and spleen, slow growth rate may also occur [2]. The treatment processes normally includes repeated blood transfusion which is very expensive and mentally, physically and economically affect the patient and their societies. Therefore, screening and prevention the spreading of hemoglobin E are important, especially in the countries that have population with high percentage of hemoglobin E trait, high birth rate and low funding. Screening technique can help to identify carriers and cut down the cost of unnecessary extensive tests. Many hemoglobin E screening techniques have been reported. Examples are DCIP precipitation [3-5], HPLC [6-7], ion exchange microcolumn [8], electrophoresis [1] and red blood cell (RBC) indice determination including mean corpuscle volume (MCV) and mean corpuscle hemoglobin (MCH) [5]. One of the routine screening

methods commonly used in some hospitals in Thailand is dichlorophenolindophenol (DCIP) precipitation because it requires simple and low cost reagents. DCIP can oxidize hemoglobin E faster than any other types of hemoglobin at the optimum incubation temperature and time (37 °C, 1 h) [3]. Interpretation of results is done bare eyes based on the cloudiness of solution by experienced medical personnels to differentiate negative/positive samples. This process is prone to human error. In addition, fluctuation of incubated temperature and detection time easily cause imprecision of interpretation because other types of hemoglobin may also be precipitated if the incubation process were left too long. The main objectives of this work are to make the DCIP precipitation more precise and automated, to decrease the analysis time and to reduce risk of the operator from having direct contact with blood sample.

1.2 Hemoglobin

Hemoglobin is a protein in red blood cell [9] whose structure is shown in Figure 1.1. It carries oxygen from the lung to the tissues all over the body and facilitates the transportation of carbon dioxide from the tissues to the lungs [10]. The hemoglobin molecule is composed of two pairs of globin chains. Each globin chain has a heme group attached. Each heme can bind to one oxygen molecule. There are four heme groups per hemoglobin molecule, therefore, one hemoglobin molecule can bind four oxygen molecules [11, 12].

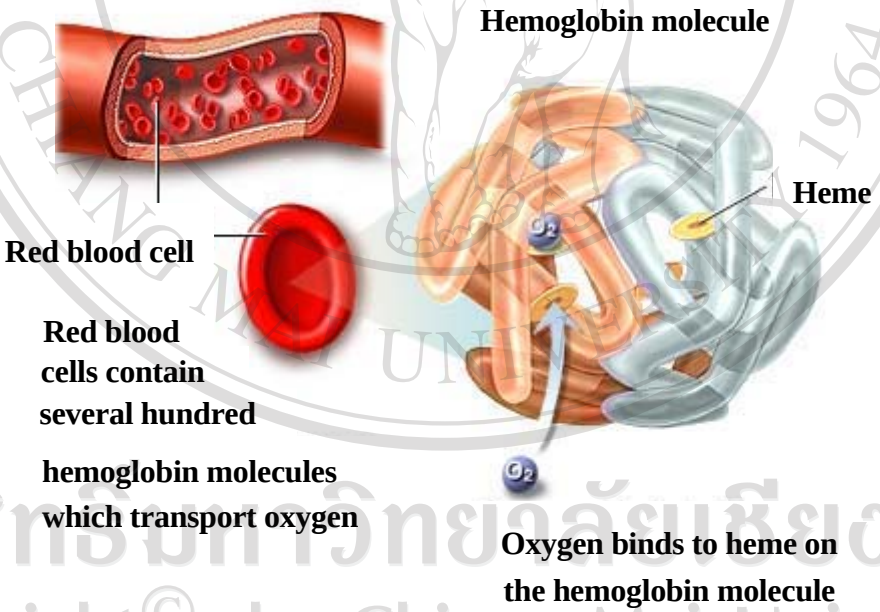


Figure 1.1 Structure of hemoglobin [12]

The globin gene are organized in a linear array on chromosomes 11 and 16 (Figure 1.2). The epsilon (ε), gamma (γ), delta (δ) and beta (β) genes are all linked on a

single piece of DNA on chromosome11. The delta (δ) and beta (β) genes are used in the production of adult hemoglobin. The two alpha(α) genes (α1 and α2) are linked on a single DNA fragment on chromosome 16 [10].

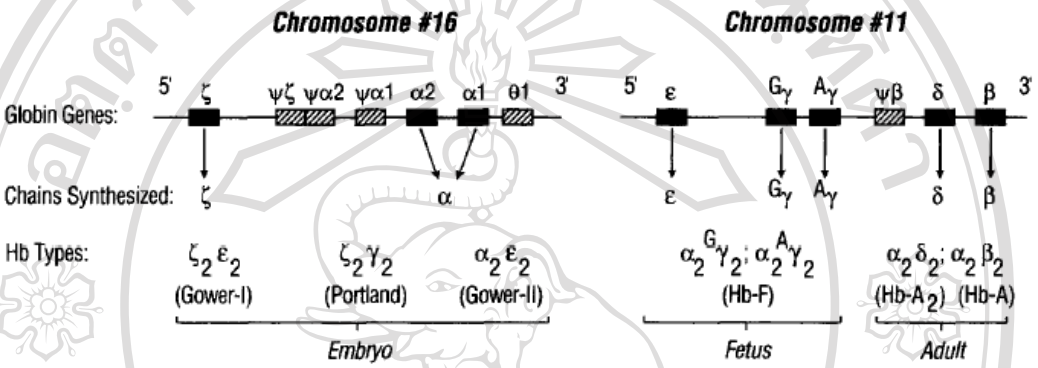


Figure 1.2 Organization of human globin gene on chromosome 11 and 16 [13]

Normal mammalian hemoglobins contain two pairs of chains: alpha (α) and non-alpha namely (beta (β); delta (δ); or gamma (γ)). The alpha (α) chains of the human hemoglobins after early embryogenesis are the same. The non-alpha (α) chains include the beta (β) chain of normal adult hemoglobin (HbA = $\alpha_2\beta_2$), the delta (δ) chain of hemoglobin A_2 ($HbA_2 = \alpha_2\delta_2$), and the gamma (γ) chain of fetal hemoglobin (HbF = $\alpha_2\gamma_2$) which is the minor component of the hemoglobin of normal adult. The most common hemoglobin type is hemoglobin A (95% of all hemoglobin) consisting of two alpha (α) and two beta (β) subunits non-covalently bound to each other. Each α (alpha) and β (beta) subunit is made of 141 and 146 amino acid residues, respectively [10, 14]. Another two minor hemoglobins are hemoglobin A_2 (2-3.5%) and hemoglobin F (less

than 2%). The relative amount of each type of hemoglobin at various ages is shown in Figure 1.3 [15].

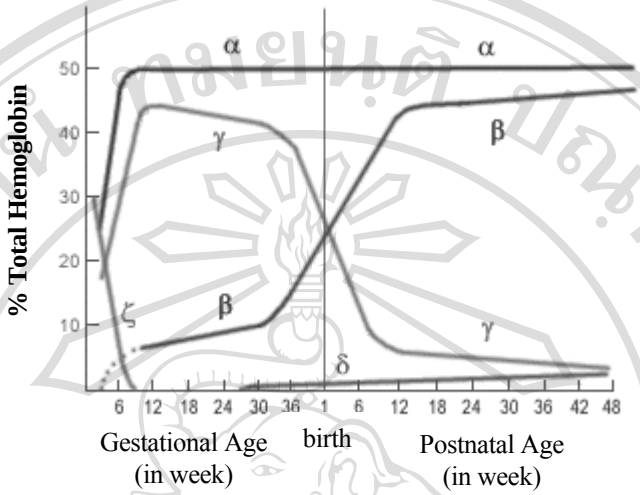


Figure 1.3 Developmental changes in hemoglobin production [15].

The alpha (α) globin molecule concentration is rather stable in fetal and adult life because it is needed for both fetal and adult hemoglobin production. The beta (β) globin appears in the early fetal life at low levels and rapidly increase after 30 week in gestational age and reaches the maximum level in about 30 week in postnatal age. The gamma (γ) globin molecule reaches high level in fetal life at about 6 week in gestational age and begins to decline at about 48 weeks in postnatal age. The delta (δ) globin appears at a low level at about 30 weeks in gestational age and maintains a low profile throughout life [15].

Mutation in the gene of the hemoglobin protein results in hemoglobin variants, some of which cause a group of hereditary disease known as hemoglobinopathies in humans for example hemoglobin E disease. A disease called thalassemia is slightly

different from hemoglobinopathies. It involves insufficient-production of normal and sometime abnormal hemogloins, due to problems and mutations in globin gene [14].

1.3 Thalassemia and hemoglobinopathies [10, 16-18]

A symptom of severe anemia associated with splenomegaly and bone changes was first reported by Cooley and Lee in 1925. The condition was named *thalassemia* which mean "the sea" due to the abundant of the disease found in the Mediterranean at that time. Thalassemia is not a single disease but is a group of disorders which are subsets of hemoglobinopathies. Hemoglobinopathies are resulted from inherited various abnormality globin productions. Thalassemia is a quantitative problem of insufficient globins synthesization, whereas a hemoglobinopathy is a qualitative problem of the synthesis of an abnormal globins. In other words, thalassemia results in under-production of normal globin proteins, due to mutations in regulatory genes, while hemoglobinopathies involve structural abnormalities in the globin proteins themselves. The two conditions may overlap, because some conditions which cause abnormalities in globin proteins (hemoglobinopathy) also affect their production (thalassemia). Thus, some thalassemias are hemoglobinopathies, but most are not. Either or both of these conditions may cause anemia. Thalassemia and hemoglobinopathies are the most common inherited disorders among humans and they represent a major public health problem in many areas of the world. There are many different gene mutations leading to various types of thalassemia and hemoglobinopathies. The two thalassemia types most commonly found are α-thalassemia and β-thalassemia.

1.3.1 α-thalassemia [10, 15, 19]

Since alpha (α) chains are present in both fetal and adult hemoglobins, see Figure 1.3, a deficiency of alpha (α) chain production affect hemoglobin synthesis throughout life span. Insufficient amount of alpha (α) chain results in an excess of beta (β) chain. Alpha thalassemia is caused by a deletion or mutation of one or more of the four alpha (α) globin gene located on chromosome 16, resulting in two main groups of α-thalassemia determinants; α^0 and α^+. The α^0 thalassemia has no production of alpha (α) chain from either alpha (α) globin locus on an affected chromosome. The α^+ thalassemia involves defective of one of the linked pair of alpha (α) globin gene. The two major clinical phenotypes of α-thalassemia are the Hb Barts hydrops syndrome which usually reflects the homozygous state for α^0 thalassemia, and the Hb H disease which usually results from the compound heterozygous state for α^0 and α^+ thalassemia. All the α-thalassemia is commonly found in Africa, the Middle East, India, Southeast Asia, Southern China and occasionally in the Mediterranean.

1.3.2 β-thalassemias [10, 15, 20, 21]

The β-thalassemia can be divided into two groups, β^0 and β^+. In β^0 thalasemia, the beta (β) chain production is totally absence while in β^+ thalassemia, the beta (β) chain production is partial. The thalassemia with some beta (β) chain production are often referred to as β^+ thalassemia when there is a significant deficiency of a beta (β) chain, and β^{++} thalassemia when the deficiency is milder. A less common class of β-thalassemia in which heterozygotes have normal levels of Hb A_2 is also found.

The β-thalassemia syndrome is much more diverse than the α-thalassemia syndromes due to the various mutations that produce the defects in the beta (β) globin

gene. Unlike the gene deletions that cause most of the α-thalassemia syndromes, β-thalassemia is caused by gene mutations on chromosome 11 that affect the whole process of beta (β) globin production: transcription, translation, and the stability of the beta (β) globin product. β-thalassemia is found in the Mediterranean, the Middle East, Africa, South Asia (Indian, Pakistan, etc.), Southeast Asia and Southern China.

1.3.3 Hemoglobin E

Hemoglobin E is the type of hemoglobinopathies that is probably the most common in the world or second in prevalence only to hemoglobin S [10, 22]. Hemoglobin E is commonly found in South-East Asia, where its prevalence was up to 60% in many regions of Thailand, Laos and Cambodia. Immigration causes hemoglobin E to spread over North America as well [23].

Hemoglobin E is an abnormal hemoglobin resulted from a single change in the amino acid at the position 26 of the beta (β) globin chain causing substitution of glutamic acid by lysine [1]. This unstable hemoglobin has free sulfhydryl group (-SH) which is easily oxidized and precipitated in certain condition [4]. This not only weakens the bond of hemoglobin tetramer, but also creates a new amino acid sequence. The beta (β) chain of hemoglobin E is synthesized at a reduced rate as compared to normal adult hemoglobin [1]. The formation of unstable hemoglobin causes the thalassemia-like nature of hemoglobin E trait and disease [4].

1.4 Hemoglobin E / β-thalassemia [24-25]

The most common hemoglobinopathies that co-exists with thalassemia is hemoglobin E. Hemoglobin E / β-thalassemia individual inherits one gene for hemoglobin E from one parent and one β-thalassemia gene from the other parent. This hemoglobin E / β-thalassemia disease can be life threatening. It is an autosomal recessive disease which is produced only when two copies of gene are present, as shown in Figure 1.4 [26].

If one has hemoglobin E trait and the partner has β-thalassemia trait, then there is 25% chance with each pregnancy that a child will inherit each condition from the parent.

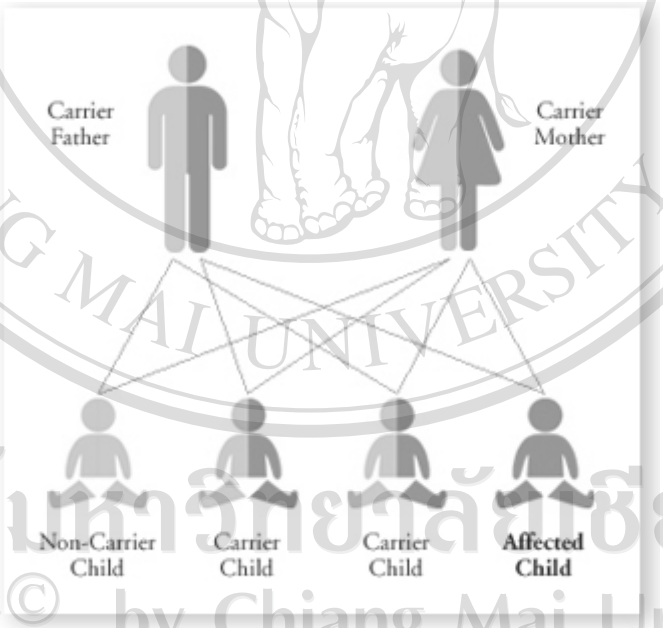
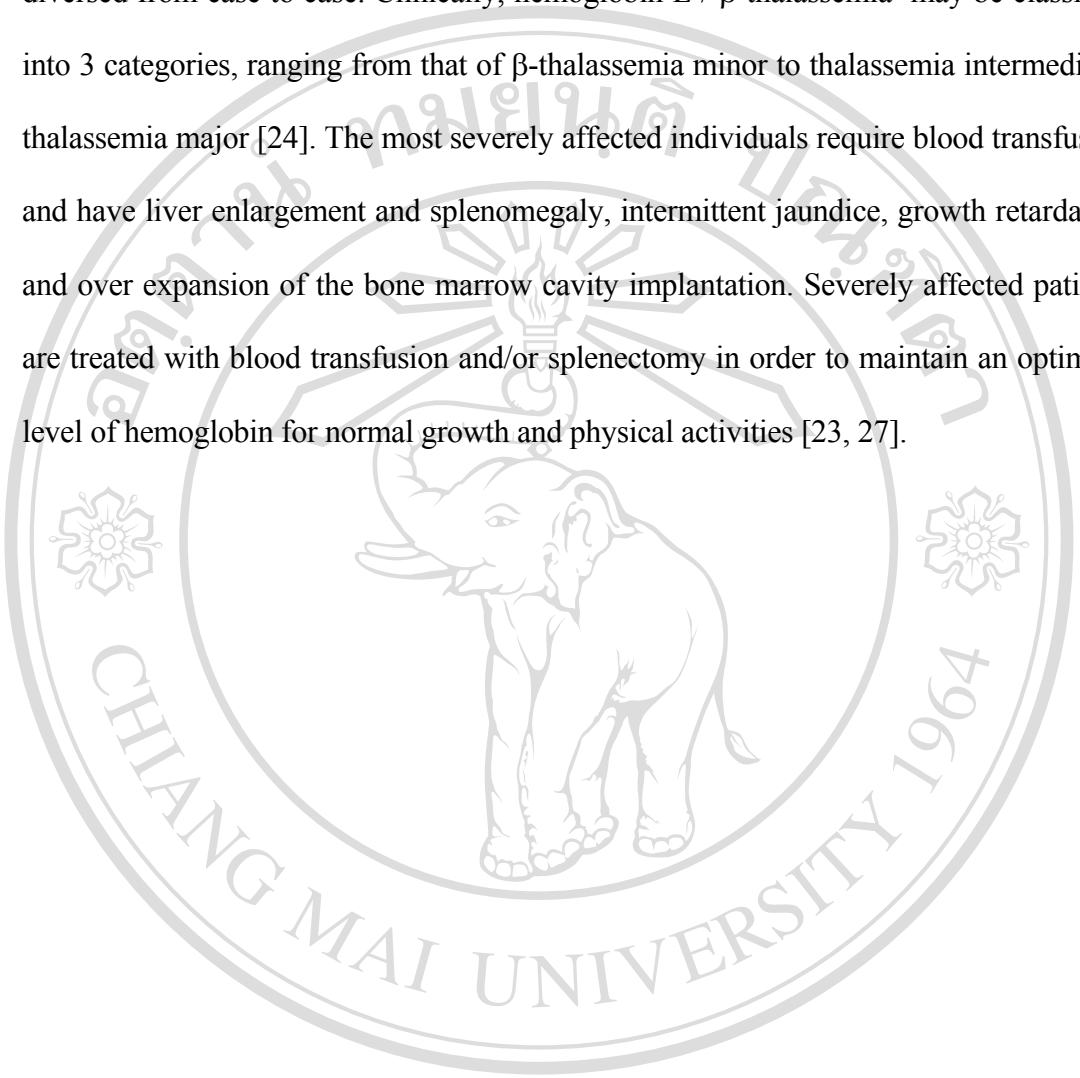


Figure 1.4 Diagram showing the chance with each pregnancy that a child will inherit from the parent [26].

The severity of compound heterozygotes for hemoglobin E and β-thalassemia is diversed from case to case. Clinically, hemoglobin E / β-thalassemia may be classified into 3 categories, ranging from that of β-thalassemia minor to thalassemia intermedia to thalassemia major [24]. The most severely affected individuals require blood transfusion and have liver enlargement and splenomegaly, intermittent jaundice, growth retardation and over expansion of the bone marrow cavity implantation. Severely affected patients are treated with blood transfusion and/or splenectomy in order to maintain an optimum level of hemoglobin for normal growth and physical activities [23, 27].



1.5 Screening method for hemoglobin E

As mentioned earlier, hemoglobin E is the second most common hemoglobinopathy found in the world [22] and can co-exist with other thalassemia especially β-thalassemia. Therefore, it is necessary to prevent and control hemoglobin E. Screening tests have become very important to help cut down the cost of unnecessary extensive tests, especially, in the countries that have populations with high percentage of hemoglobin E, high birth rate and low funding [28].

1.5.1 Methodology for hemoglobin E screening

1.5.1.1 Red blood cell indices (RBC) [29-32]

Red blood cell indices determination is usually carried out by an automated electronic cell counters. These counters produce many parameters of which only a few, such as the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and hemoglobin (Hb) concentration, are strictly relevant and useful for hemoglobinopathies screening. MCV and MCH are variably reduced in thalassemia carriers. MCH is more reliable than MCV, since the MCV does not remain stable due to a tendency for the red cells to increase in size over time. The value at which the normal range for a parameter changes to an abnormal range is called the cut-off value. The most widely used cut-off values of MCV and MCH for indicating thalassemia are 79 femtolitre (fl) per a red blood cell and 27 picogram (pg) per a red blood cell, respectively. Sometime, low MCV and MCH also give false positive results due to iron deficiency anemia.

1.5.1.2 Microcolumn chromatography

This method is used for determination of hemoglobin A_2 and the result can be used for diagnosis of hemoglobin E and β-thalassemia trait. The diethylaminoethyl DEAE anion exchanger was packed in a relatively cheap and small plastic syringe [8]. The blood sample is mixed with Tris-HCl buffer and introduced into the packed syringe. Then, working buffer is added to elute and separate different hemoglobins. Hemoglobin E is isolated before other hemoglobins by changing working buffer ionic strength. Because the weakest of anion charge of hemoglobin E as compared to other hemoglobins, it can be eluted from the column first when using optimum working buffer. If there was hemoglobin E in a blood sample (positive result), the eluted solution would be red. In contrast, if the sample contained no hemoglobin E (negative result), the eluted solution would remain colorless. However, if the separation process were left too long, eluate would show reddish color in both positive and negative results due to the elution of other types of hemoglobin. Therefore, it is necessary to control analysis time and volume of working solution. Another drawback of this technique is the long time consumption (1-2 day) for the preparation of DEAE sephadex column and the use of toxic agent, potassium cyanide, as working solution [29].

1.5.1.3 Dichlorophenolindophenol (DCIP) precipitation [4, 5, 16, 33]

Dichlorophenolindophenol (DCIP) precipitation test is one of the hemoglobin E screening techniques which is commonly employed in some hospitals in Thailand because it uses simple and low cost reagents. DCIP is a synthetic dye which has deep blue color in water. DCIP can oxidize and cause precipitation of unstable hemoglobins such as hemoglobin E faster than any other types of hemoglobin at the optimum incubation temperature and time [28].

Hemoglobin E has the sulfhydryl (-SH) exposed. These sulfhydryl groups are found on cysteine amino acid of the globin chain. The sulfhydryl (-SH) groups when oxidized will form S-S bond with other sulfhydryl (-SH) group as shown in Figure 1.5

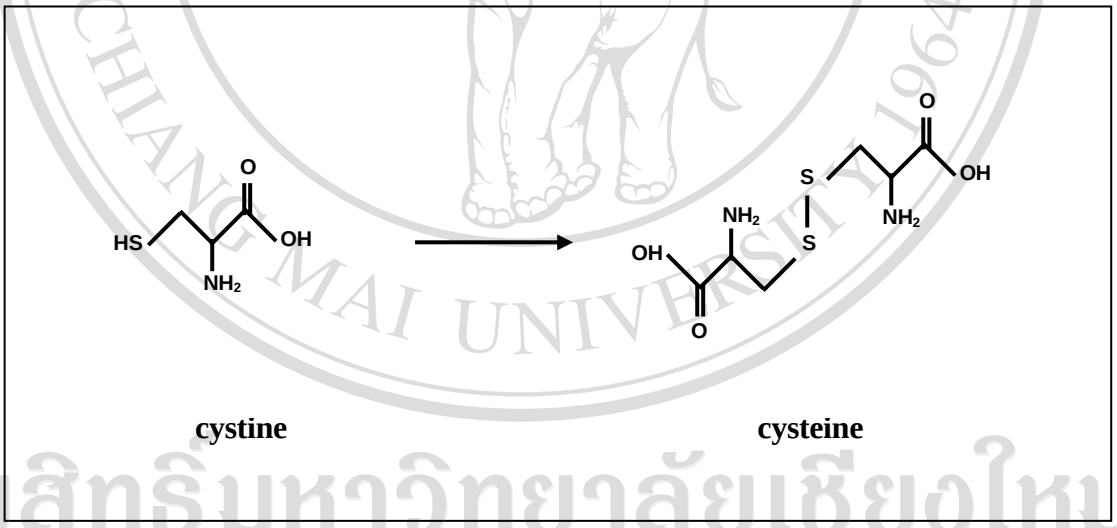


Figure 1.5 The formula of cystine formed from cysteine [34,35]

The blood sample containing hemoglobin E will be more cloudy due to precipitate as compared to a normal sample. From this reason, hemoglobin E people can be differentiated from normal people by placing a printed paper behind the test tubes. If the letters were visible through the solution, the test was considered negative (normal

people) and if the letters were not visible due to cloudiness from precipitation, it was considered as positive (hemoglobin E people).

Tongsong and his group [3] reported the procedure of this technique as follows; first, DCIP was incubated with blood sample at 37 °C for 1 h. The main property of DCIP is to lyse and precipitate unstable hemoglobin E. This was done in the dark blue color of DCIP solution which made it difficult to observe the turbidity. Precipitated hemoglobin can be visualized by naked eyes at the bottom of the tube (see Figure 1.6) [29]. In homozygous hemoglobin E, a heavy sediment will form at the bottom of the test tube. In hemoglobin E trait, and hemoglobin E / β-thalassemia**,** the precipitation of the hemoglobin produces a cloudiness or an evenly distributed particulate appearance.

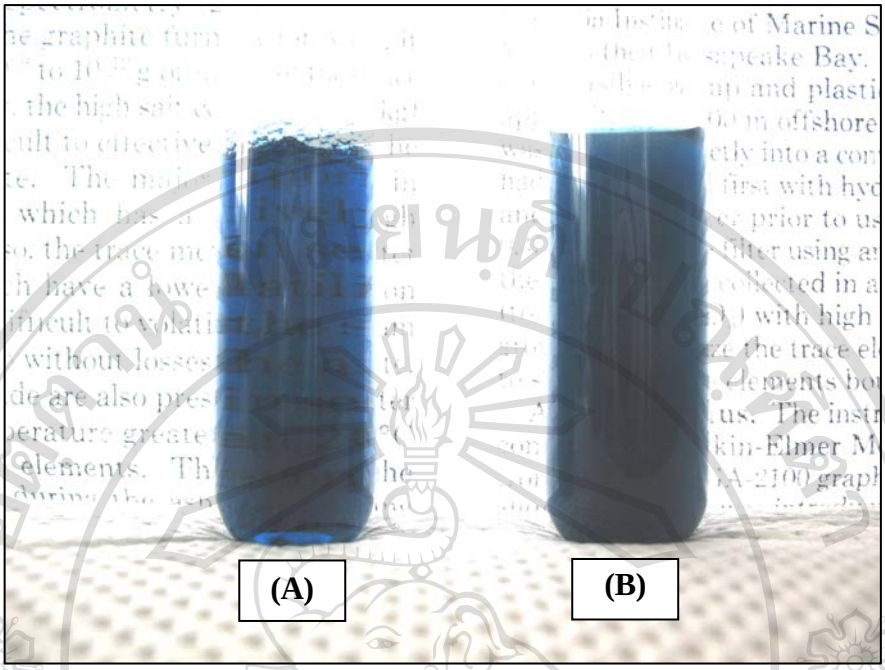


Figure 1.6 Naked eye DCIP precipitation showing slight difference between positive and negative samples in the dark blue color of the DCIP solution: tube (A) is negative sample and tube (B) is positive sample.

Addition of a reducing agent to stop the reaction and to convert DCIP solution (blue) into a reduced DCIP form (colorless) which is unable to oxidized hemoglobin E, can help to better translate the result. Normally, ascorbic acid solution was used as a stopping agent to reduce dark blue color of DCIP to a colorless form as shown in Figure 1.7.

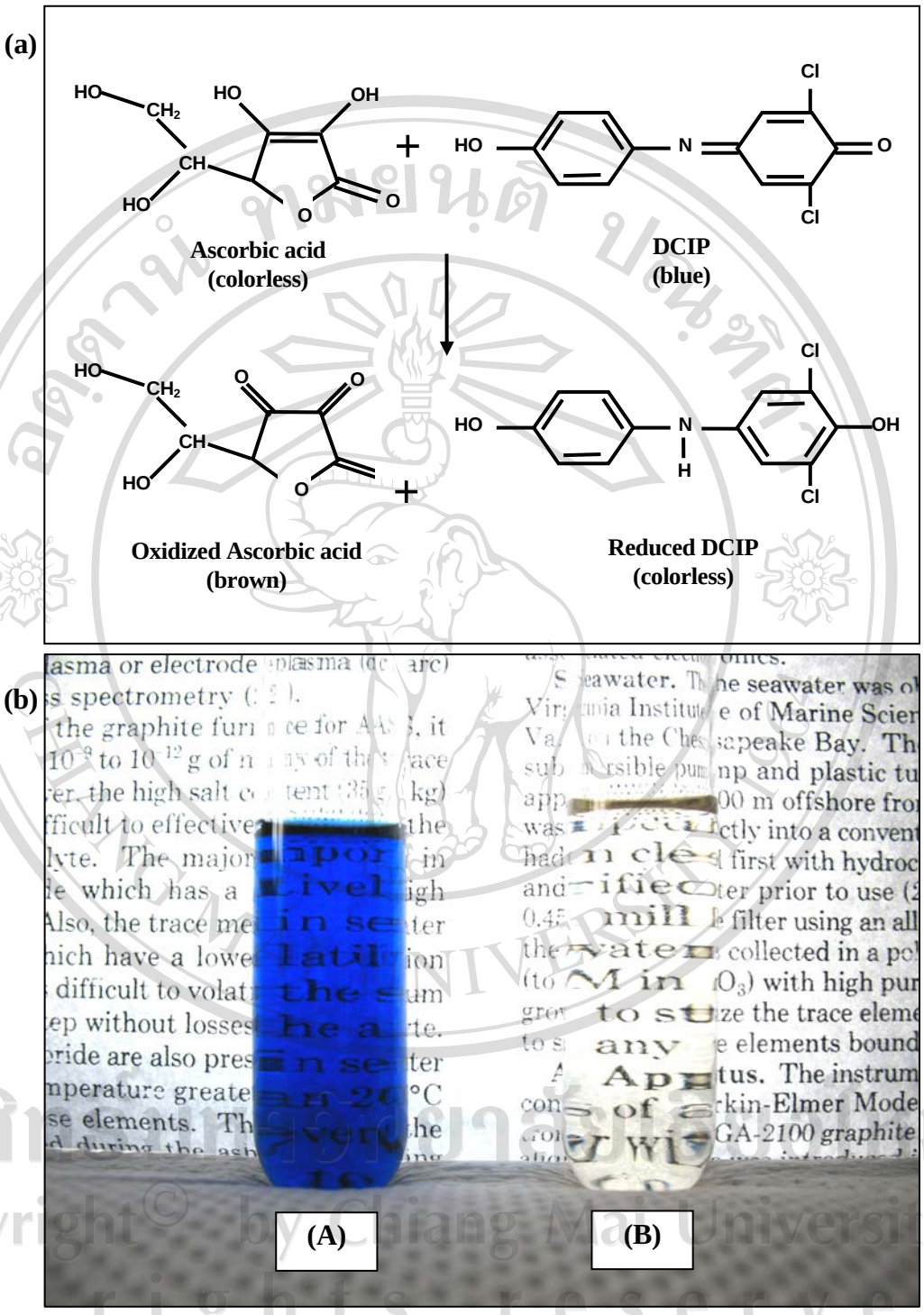


Figure 1.7 (a) Reducing of the oxidized form DCIP to the reduced form [36,37] and **(b)** The color of the oxidized form of DCIP solution (tube A) and the reduced form of DCIP solution (tube B) after adding ascorbic acid

In the present of red blood cells, after the DCIP was reduced the solution showed a tint of reddish color of red blood cells that is still easier to observe the turbidity as compared to the dark blue color.

Figure 1.8 shows that the result of this technique could be translated with bare eyes by placing a piece of newsprint behind the test tubes. If the letters were visible through the solution, the test was considered negative (normal people) and if the letters were not visible, it was considered as positive (hemoglobin E people).

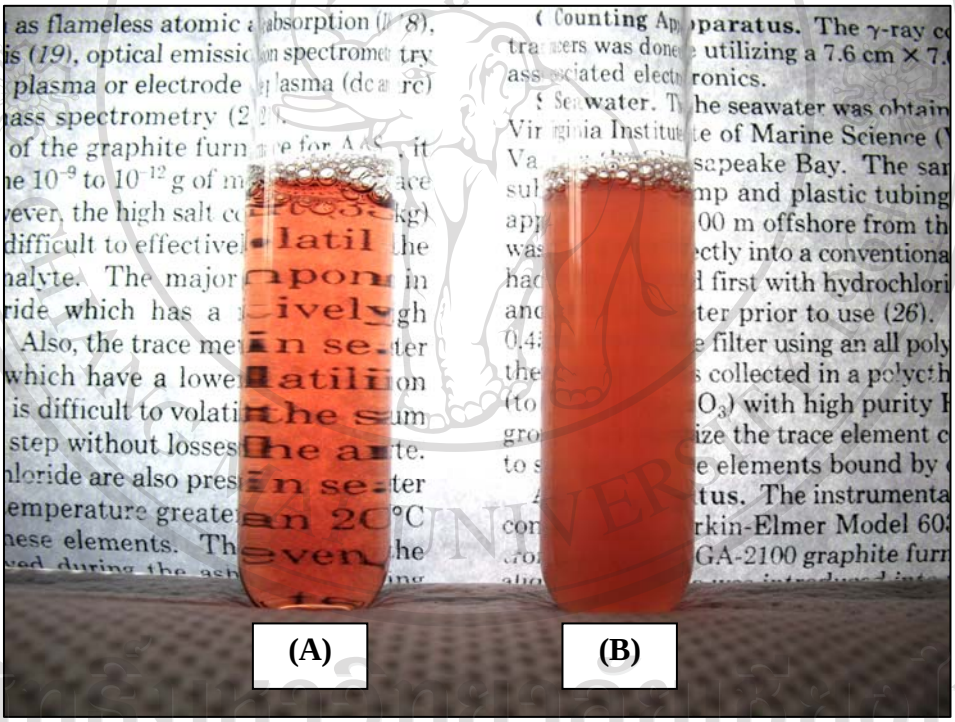


Figure 1.8 Naked eye DCIP precipitation showing positive and negative sample in different tubes: tube (A) is negative sample and tube (B) is positive sample

1.6 Flow Injection System and Hydrodynamic Injection

Many laboratories prefer not to perform DCIP test due to long analysis time and inconvenience in controlling temperature and incubation time. The proposes of this study are to differentiate negative samples from positive samples while improving the precision and reducimg analysis time.

The demands for rapid analysis in pharmaceutical, clinical, agricultural and industrial have been increased. The flow injection based system was developed to increase analytical efficiency, accuracy and precision, while reducing analysis time and cost. The principle of flow injection analysis is based on the injection of liquid sample into a moving carrier stream of a suitable liquid [38,39]. The injected sample and reagent form a zone, which is transported toward a detector that records the analytical signal. The signal is continuously changed due to the passage of the zone through the flow through cell. The simple flow injection systems consisted of a pump, which is used to propel the carrier stream through a narrow tube; an injection port, where a fixed volume of sample solution is injected into the carrier stream; and a micro reactor, in which the mixture of the sample zone disperses and reacts with the reagent or the carrier stream. The product formed is sensed by a flow-through detector which may be a spectrophotometric or electrochemical based detector. The typical recorder output is in the form of a peak as shown in figure 1.9, the height *H*, width *W*, or area *A* is related to the concentration of the analyte.

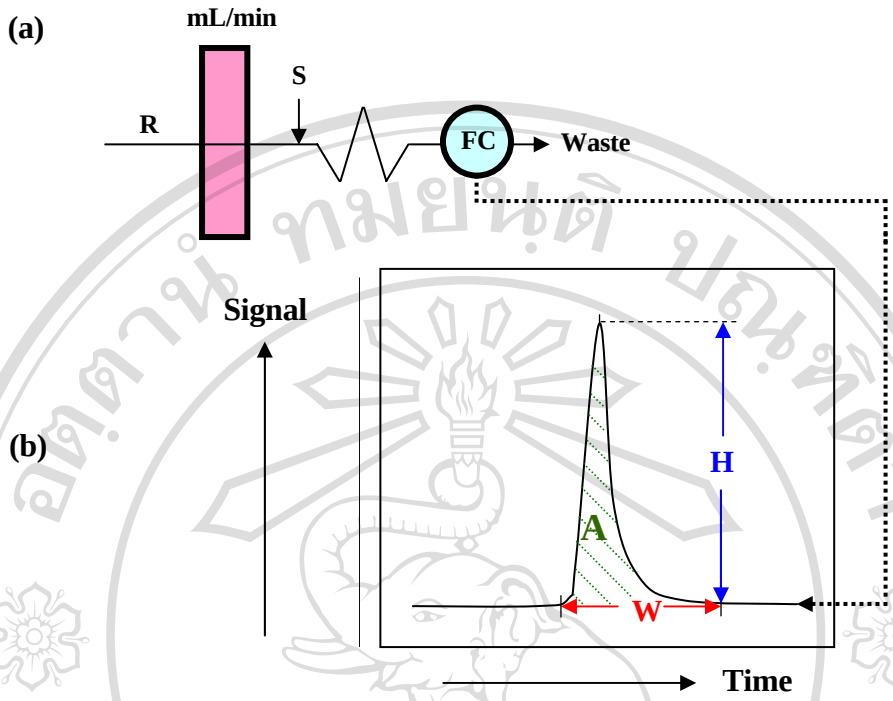


Figure 1.9 Single-line FIA manifold **(a)** with a typical recorder output **(b)** as obtained with a spectrophotometric flow-through cell. R carrier stream of a reagent; S, sample injection; FC, flow-through cell; *W*, peak width; *H*, peak height; and *A*, peak area [38].

Many reactions may need a period of time to form a product. In addition, the continuously flow system creates high dispersion of product and may yield too low sensitivity in the analytical readout. A simple stopped-flow system can be used to overcome these problems by increasing the resident time and thus the yield of the measured product which then serves as a base for the analytical readout [40]. In the stopped flow injection, the zone of sample and reagent mixture is stopped in the flow through cell for a period of time before flowing out of the system. In the stopped-flow system, the reaction coil can be kept short and the velocity of the carrier stream can be decreased.

In the part of injection port, hydrodynamic injection is one of the alternative sample injection systems. A solenoid valve is an electromechanical valve for use with liquid or gas. Opening/closing of the valve is controlled by running or stopping an electrical current through a coil of wire. Solenoid valves may have two or more ports, in this case three ways solenoid valve was used. The outflow is switched between the two outlet ports. Multiple solenoid valves can be placed together on a manifold [40]. Figure 1.10 shows a diagram of a three-way solenoid valve [41,42].

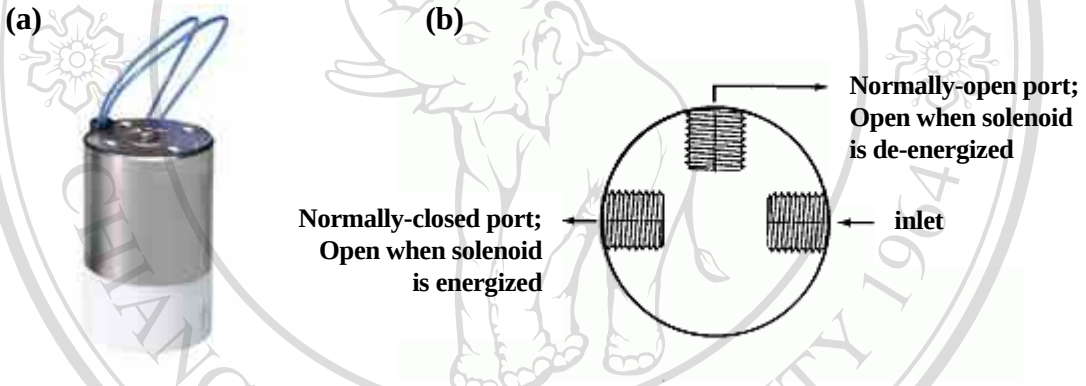


Figure 1.10 Three ways solenoid valve (a) solenoid valve from cole-parmer and (b) flow pattern (top view of valve shown) [41,42]

The principle of hydrodynamic injection is explained by referring to Figure 1.11. An A-B zone is a column of liquid whose length *L* and internal radius *R* can be exactly calculated. The, A-B zone is opened at all times to other channels. These columns of liquid exert a hydrostatic force, which serves as a lock while the sample volume is being filled. During sample loading step, the channels are filled by a stagnant liquid (sampling), see Figure 1.11 (a). Then, a well-defined sample zone can be inserted into

the carrier stream (injection) by switching solenoid valve to open the flow line, see Figure 1.11 (b). The manifold serving for this purpose is shown in figure 2.1 in chapter 2.

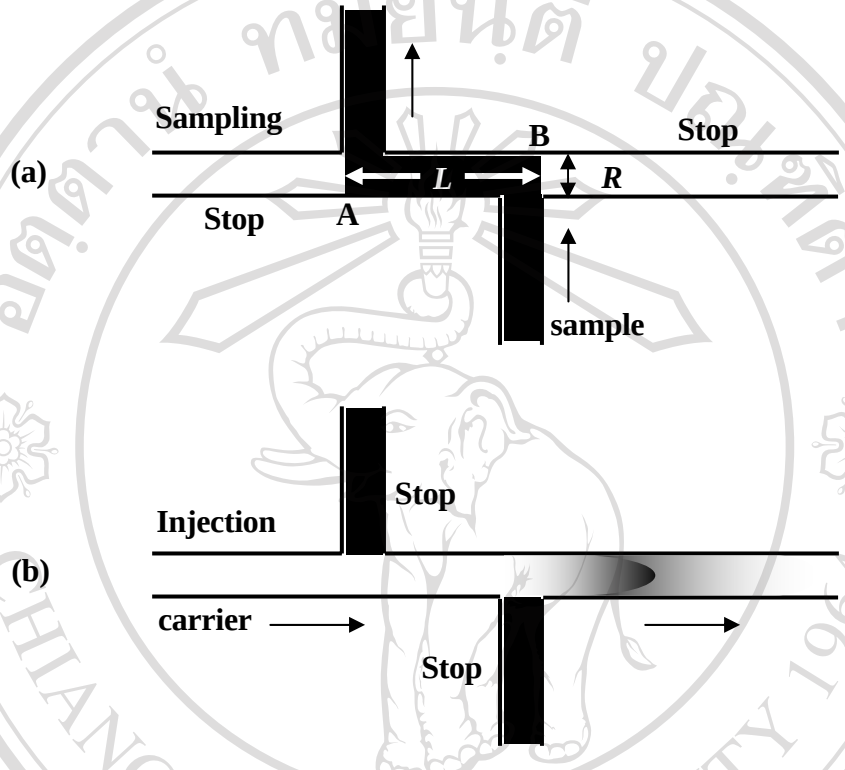


Figure 1.11 The principle of hydrodynamic injection. A fixed volume of sample solution (A-B zone) is metered into a conduit, of length *L* and internal radius *R* (**(a)**-Sampling), and this volume is subsequently propelled downstream by the carrier stream (**(b)**-Injection). During the sampling cycle, the carrier stream circuit is stopped. In the next injection cycle, the carrier stream circuit is flowed. When aspiration the next sample, the column of carrier stream solution is emptied to waste along with the excess solution.

1.7 Flow Injection Analysis for screening of hemoglobin E

Even though screening techniques cannot provide the information on the exact type of hemoglobinopathies, but it can help to cut down the cost of unnecessary extensive test. Therefore, effective hemoglobin E screening has become very important in the country that has population with high percentage of thalassemia carrier, high birth rate and low funding. However, the conventional batchwise DCIP precipitation has some limitations. The main problem involves fluctuation of temperature in water bath and imprecise timing which leads to inaccuracy and imprecision of the technique. In addition, it requires experienced medical personnel to translate the result which are normally observed with bare eyes. It also poses high risk of direct contact with blood samples.

Therefore, an automatic system for DCIP precipitation that can control incubation time with shorter incubation period would be useful. In this study, operation step of hemoglobin E precipitation with DCIP and detection step will be done online to increase precision of the test and reduce analysis time. The reagent and sample will be mixed in the mixing zone which is located in a water bath where the mixture of blood and DCIP will be incubated at optimum temperature for a short time. In this short period of incubation, problem of temperature fluctuation of the water bath would be diminished. Hydrodynamic injection using three-way solenoid valves will be designed to control injection step, incubation step and direction of solution [43]. In addition, the precision of the system can be further improved by using a spectrophotometer to measure turbidity based on transmittance. This system should make DCIP precipitation more precise, more automated and more rapid.

1.8 Research objective

To develop a more automated, rapid and precise system based on flow injection dichlorophenolindophenol precipitation (FI-DCIP) for screening of hemoglobin E.