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Laboratory Diagnosis of β -Thalassemia and HbE

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Abstract

β -Thalassemia and HbE, each, is a syndrome resulted from quantitative and qualitative defects of β -globin chain, respectively. In addition to history retrieve and physical examination, diagnosis of these disorders requires laboratory information. Laboratory tests that are conventionally performed to diagnose the β -thalassemia and HbE are classified into two groups, based on the purposes, including the screening tests and confirmatory tests. The screening tests are aimed to screen for carriers of the β -thalassemia and HbE, while confirmatory tests are the tests performed to definitely diagnose these disorders. This chapter will explain all of these tests, the information of which will be useful for those who are working and interested in the β -thalassemia and HbE.

Keywords: β -thalassemia, HbE, screening tests, confirmatory tests, thalassemia carrier, HbE carrier, β -thalassemia disease

1. Introduction

1.1 Thalassemia and hemoglobinopathies

Thalassemia is a type of anemia caused by reduction or absence of globin chain synthesis, which results in imbalanced-globin chain synthesis; the major pathogenesis of the disease. The unaffected globin chains continuing to be synthesized at the normal synthetic rate tend to form homotetramers and aggregation that can harm the red blood cells both at young and mature stages. The α -globin chain aggregation formed in β -thalassemia causes ineffective erythropoiesis due to oxidative stress. The γ - and β -globin chain homotetramer (γ_4, β_4) formed in α -thalassemia harm mature erythrocytes. The γ_4 or Hb Bart's has very high oxygen affinity and inhibits oxygen release from erythrocytes which, in turn, results in tissue anoxia. The β_4 or HbH is an unstable hemoglobin and precipitates easily under the stress condition in the body. Once precipitates, the erythrocytes are removed by the RE system resulting in anemia.

Severe thalassemia cases suffer from chronic and marked anemia with life relying solely on blood transfusion. Anemia causes expansion of bone marrow, leading to osteoporosis and changes of bone structure. Blood transfusion and hemolysis cause iron overloading state in the body which causes several complications such as heart disease, growth retardation, diabetes mellitus, and infection.

Thalassemia is considered the most common autosomal single-gene disorder worldwide. It can be found in more than 150 countries with an estimated carrier frequency of about 7%. The Mediterranean region, certain parts of North and West Africa, Middle East, Indian subcontinent, Southern Far East, and South East Asia have the highest prevalence of the disease [1].

In contrast to the thalassemia, hemoglobinopathy is an inherited disorder of hemoglobin productions characterized by production of abnormal hemoglobin or hemoglobin structural variants occurring from genetic alterations including point mutations, deletions or insertion of the normal globin genes. The well-known abnormal hemoglobins in the world are HbS, which is common in western countries as well as in the Middle East and HbE, which is common in Southeast Asia [2].

1.2 Types of thalassemia and hemoglobinopathies

Two major types of thalassemia are found across the world consisting of the α - and β -thalassemia. The α -thalassemia is further sub-divided into two types: α -thalassemia 1 or α^0 -thalassemia and α -thalassemia 2 or α^+ -thalassemia. Gene deletion is the leading cause of α -thalassemia. In α -thalassemia 1, two α -globin gene in-cis on chromosome 16 are deleted, while only one α -globin gene is absent in the α -thalassemia 2 [3]. In addition, there are two types of β -thalassemia; β^0 -thalassemia and β^+ -thalassemia. In contrast to α -thalassemia, mutations on the β -globin gene are found in the majority of the β -thalassemia patients [4].

Two broad types of hemoglobinopathies or structural variants are also found; α - and β -hemoglobinopathies. To date, approximately 1358 structural variants are described (<http://globin.bx.psu.edu>), around 90% of which are involved in the β , γ , and δ -globin chains and around 60% involves the α -globin chain. The most important β -globin hemoglobinopathies are HbS ($\alpha_2\beta_2^{6\text{Glu}-\text{Val}}$) and HbE ($\alpha_2\beta_2^{26\text{Glu}-\text{Lys}}$). Hb E is found around the world and accounts for approximately 13–17% on the population of Thailand, especially in the Thai-Laos-Cambodian boundary or “Hb E triangle” where more than 32–60% of the people carry HbE gene [5].

1.2.1 β -Thalassemia

The β -thalassemia is a diverse group of disorders of hemoglobin synthesis which is characterized by reduced or absent β -globin chain synthesis. There are two main types of β -thalassemia: β^0 -thalassemia in which no β -globin chain is produced and β^+ -thalassemia in which some β -globin is produced but less than normal.

β^0 -Thalassemia is severe β -thalassemia with no production of β -globin chain. It is mainly caused by point mutations in coding region (exon) or exon-intron junction of β -globin gene which lead to premature stop codon or generation of abnormal β -globin mRNA. The end results of these abnormalities are absence of the β -globin chain production [6]. In Thailand, at least three common mutations in the β -globin gene are of this category. They comprise A-T substitution at codon 17 (CD17: A-T) which creates premature stop codon, the TTCT-deletion at codons 41/42 (CD41/42: –TTCT) which causes reading frameshift and premature stop codon at codon 59 instead of codon 147, the G-T substitution at IVSI-nt1 which leads to abnormal splicing of immature β -mRNA and results in no production of normal β -mRNA. In general, thus, genotype of heterozygote is written as β^0/β^A and that for homozygote as β^0/β^0 [2, 7–9].

β^+ -Thalassemia is a milder form of β -thalassemia in which some β -globin chains are still produced. The majority of cases possess point mutations outside exons, especially in the promoter region. The mutations of β -globin gene leading to the β^+ -thalassemia include mutations at ATA box (nt-28, nt-29 or nt-30 from cap site), CACCC element (nt-86 to nt-90 from cap site), and mutations in introns or exons of gene to produce new splice site to race in RNA splicing process, as mutation in IVS2-nt654 (C-T) and mutation of IVS1-nt5 (G-C) [2, 6]. The genotypes were β^+/β^A and β^+/β^+ for heterozygote and homozygote consecutively.

1.2.2 β -Hemoglobinopathies

β -Hemoglobinopathies are characterized by the production of abnormal β -globin chains due to changes or mutations (missense mutations) on the β -globin gene. Two abnormal β -globin chains then assemble with two normal α -globin chains to form abnormal hemoglobin or β -structural variants. These abnormal hemoglobins generally have different electrophysical properties from their normal counterparts; that is, due to the molecular conformational alteration. In theory, synthetic rate of the abnormal β -globin chain should be normal. However, some are produced in reduced rate, thus producing a phenotype resembling the β^+ -thalassemia. To date, there are more than 737 β -globin structural variants reports across the world (<http://globin.cse.psu.edu/>).

1.3 Hemoglobin E

HbE is abnormal hemoglobin resulted from the G-A substitution at codon 26 of β -globin gene. This missense mutation partially activates a cryptic splice site toward the 3' end of exon 1, resulting in a proportion of abnormally splice mRNA. Thus, less β^E globin is synthesized, leading to a mild thalassemia phenotype. HbE is becoming the common β -globin structure variant across the world as a result of migration and inter racial marriage [2, 10]. It has been realized to be the hallmark of Southeast Asian region. In Thailand, HbE is very common accounting for approximately 8–70% of population [11].

1.4 Inheritance of genes for β -thalassemia and HbE

Gene for β -thalassemia and HbE is transmitted within the family from parents to descendants in an autosomal recessive fashion. Thus, those who are heterozygous for abnormal β -gene are clinically asymptomatic and called β -thalassemia carrier or β -thalassemia trait. Those who are heterozygote for HbE gene (β^E) are also clinically asymptomatic and called HbE carrier or HbE trait. However, homozygote or compound heterozygote of the β -thalassemia gene and/or HbE gene are clinically affected and suffer from chronic anemia with some life-threatening complication. Therefore, accurate diagnosis of carriers of the β -thalassemia and HbE as well as the disease is important.

1.5 Problem of β -thalassemia and HbE

The carriers of β -thalassemia and HbE do not have clinical burden as they are clinically normal and have normal quality of life. However, if the β -thalassemia carriers get married with the HbE carriers, they will have 25% chance of producing the HbE/ β -thalassemia babies. The HbE/ β -thalassemia or sometimes called the β -thalassemia/HbE disease is a thalassemia syndrome that presently is known to be clinically heterogeneous [10–12]. Some patients are very mild, while some are very severe. The severe cases always required regular blood transfusion which always ends up with iron overloading condition. Without proper management of this iron overloading, several fatal complications occur, leading to low quality of life and, finally, pre-death at young age.

1.6 Diagnosis of β -thalassemia and HbE

The diagnosis of β -thalassemia and HbE involves both clinical and laboratory investigations. Clinical data can only identify the affected patients, but cannot

definitely identify types of thalassemia the patients are suffering. Laboratory data thus help define specific types of thalassemia disease of those affected individuals. For the carriers, as they are clinically normal, clinical data are of no use. Only laboratory data can define β -thalassemia and HbE carriers.

Conventionally, the laboratory tests for diagnosis of β -thalassemia and HbE include screening tests and confirmatory tests. Initial screening tests are defined as techniques that are simple and relatively low cost, which can indicate the possibility of having thalassemia. These tests should involve the least sample pretreatment and rapid sample preparation and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. The screening tests, however, cannot provide the information on the exact type of thalassemia of the positive persons. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing.

The screening tests for β -thalassemia carriers comprise one-tube osmotic fragility test (OFT) and automated red blood cell indices (mean corpuscular volume; MCV, mean corpuscular hemoglobin; MCH, and red cell distribution width; RDW). Screening tests for HbE carriers are composed of all tests performed to screen for the β -thalassemia carrier plus the specifically established for HbE screen such as dichlorophenolindophenol precipitation (DCIP) test or HbE tube test or HbE test [13–16]. These screening tests, however, cannot provide the information on the exact type of thalassemia of the positive persons.

The purpose and methodologies of confirmatory tests for β -thalassemia and HbE are identical. The confirmatory tests must be highly specific in order to obtain the correct diagnosis of carriers of β -thalassemia and HbE as well as the disease state of HbE/ β -thalassemia and homozygote or compound heterozygote of the β -thalassemia gene. The confirmatory tests include;

- Hemoglobin studies: Tests for hemoglobin studies include cellulose acetate electrophoresis, microcolumn chromatography, alkaline denaturation test, cation-exchange high performance liquid chromatography (HPLC) [17–19], cation-exchange low pressure liquid chromatography (LPLC) [20–22], capillary zone electrophoresis (CZE) [23–27], sandwich enzyme linked immunosorbent assay (ELISA) for Hb F [28], Hb Bart's [29] and Hb A₂ [30], flow cytometric analysis of F cells [31], and immunochromatographic strip (IC strip) test for Hb Bart's [32].
- DNA analysis: Tests for analysis of β -globin gene mutations include multiplex allele-specific polymerase chain reaction (MAS-PCR) [29], amplification refractory mutation system (ARMS)-PCR [33], mutagenically separated (MS)-PCR [34–36], and high resolution melting curve (HRM) analysis [37–39].

2. Screening laboratory tests for β -thalassemia and HbE carriers

Two laboratory tests are usually performed for screening of the β -thalassemia carriers: automated red cell indices (mean corpuscular volume; MCV, mean corpuscular hemoglobin; MCH, and red cell distribution width; RDW) and one-tube osmotic fragility test (OFT). The screening tests for HbE carrier comprise those performed for the β -thalassemia screen plus the tests used for HbE screen. These tests include dichlorophenolindophenol precipitation (DCIP) test [40], HbE-tube test [15], and hemoglobin E test [16]. The results of these screening tests indicate chance that the blood samples are carriers of either β -thalassemia or HbE.

2.1 Red blood cell indices

Red blood cells or erythrocyte indices used for screening for β -thalassemia carriers and HbE carriers conventionally included mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). These red cell indices must be obtained by automated blood cell analyzers. Normal ranges of MCV and MCH are 85.5 ± 6.8 fL and 27.1 ± 3.1 pg, respectively. Cut-off points of MCV and MCH are 80 fL and 27 pg, respectively.

MCV and MCH in β -thalassemia carriers are 68.7 ± 5.4 fL and 20.6 ± 2.1 pg. In HbE carriers, MCV and MCH values are 76.3 ± 4.6 fL and 24.2 ± 1.5 pg [41]. We found that the MCV and MCH in normal individuals, β -thalassemia carriers, and HbE carriers were significantly different [41]. With the cut-off points of 80 fL, MCV has been shown to be effective in screening for the β -thalassemia carriers with sensitivity and specificity of 92.9 and 83.9%, respectively [42, 43]. At 26.5 pg cut-off point of MCH, Pranpanus and co-workers found 92.5% sensitivity and 83.2% specificity of MCH in screening for the β -thalassemia carriers. At the cut-off point of 80 fL for MCV and 27 pg for MCH, Karimi and co-workers found 98.5% sensitivity of MCH, which was more than that of MCV (97.6% sensitivity) in screening for β -thalassemia carriers. They concluded that the MCH was better than MCV [44].

MCV and MCH are not effective in screening for HbE carriers since mean levels in HbE carriers are just slightly lower than those in normal individuals, but the distribution overlaps substantially. Yeo et al. showed that the use of 80 fL cut-off point could miss cases of HbE carriers [45]. Ittarat et al. showed that 5% of HbE carriers would be missed in 80-fL cut-off points of MCV was used [46]. This group suggested the use of some discriminant functions, $F1 = 0.01 \times MCH \times (MCV)$ or $F2 = RDW \times MCH \times 2(MCV)/Hb \times 100$ or $F3 = MCV/RBC$, to increase effectiveness of using red cell indices for screening for HbE carriers. Our unpublished data showed that using 80-fL and 27 pg cut-off points of MCV and MCH, respectively, did not miss cases HbE carriers regardless of hemoglobin levels (4.2–15.1 g/dL).

2.2 One-tube osmotic fragility test (OFT)

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. In a hypotonic condition, the fixed concentration of salt on the outside is lower than that on the inside of red blood cell, resulting in net water movement into the red blood cell. Normal red blood cells are then lysed and the mixture then turns red-dish and clear. Red blood cells of the β -thalassemia and HbE carriers have higher osmotic resistance and thus have slower rupture rate, and the mixture remains turbid. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution such as 0.36% NaCl in distilled water (DW), 0.36% NaCl in phosphate buffer or buffered saline solution (BSS), and 0.45% NaCl in glycerine or glycerine saline solution (GSS). All of these solutions are normally based on the same concept of kinetic osmotic fragility.

2.2.1 0.36% NaCl-based and BSS-based one-tube osmotic fragility test

By mixing the 20 μ L of red blood cells of normal and β -thalassemia carriers in 2 mL of 0.36% NaCl in DW (0.36 g NaCl dissolved in 100 mL DW) or BSS (0.32 g NaCl, 0.05 g Na_2HPO_4 , 0.01 g NaH_2PO_4 dissolved in 100 mL DW) and leave for 5 min, the normal red blood cells will completely lyse and the mixture turns

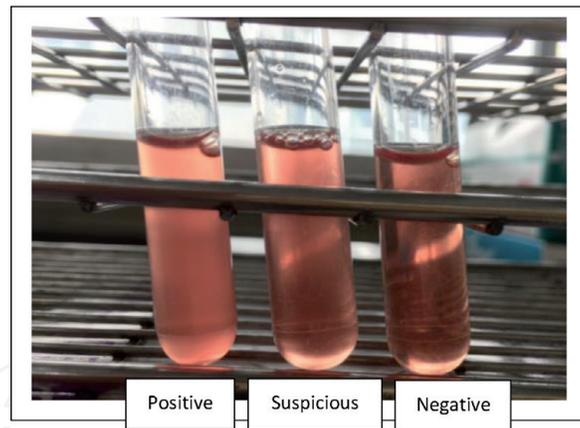


Figure 1.

0.36% NaCl-based and BSS-based one-tube osmotic fragility test for screening of β -thalassemia and HbE carriers. The β -thalassemia carriers all have the positive OFT results, while HbE carriers have either positive, negative, and suspicious OFT results (see the text for detail).

reddish-clear and reported as OFT-Negative. In contrast, the mixture of blood samples of β -thalassemia carriers and 0.36% NaCl remains turbid at 5 min and reported as OFT-Positive. In case that the appearance of the mixture is between positive and negative OFT-results, it is reported as OFT-suspicious (**Figure 1**). Chow et al. showed that this test has 95% sensitivity and 86% specificity for screening the β -thalassemia carrier [47]. Bobhate et al. demonstrated 97.1% sensitivity and 100% specificity of this test, which they called NESTROFT, in screening for β -thalassemia carriers [48], while Mamtani et al. showed 93.4% and 97.2% sensitivity and specificity of this test for screening of the β -thalassemia carriers [49]. For HbE carriers, Fucharoen et al. showed 37.7% false negative OFT result in HbE carriers [50], which was closed to our unpublished data that showed approximately 29.4% false negative results.

2.2.2 0.45% NaCl in glycerine or glycerine saline solution (GSS)-based one-tube osmotic fragility test

This test was established by Prof. Dr. Torpong Sa-nguansermisri of Thalassemia Unit, Department of Pediatrics, Faculty of Medicine, Chiang Mai University and named this test “Erythrocyte Osmotic Fragility Test” (EOFT) [51]. In this platform, the influx of water into the erythrocyte is slow and hemolysis can be measured at any points of time after mixing blood with 0.45% GSS [1.424 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.262 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.812 g NaCl, 19.27 g glycerine (87%), and DW to make 1000 mL]. Technically, 10 μL of EDTA blood is mixed with 10 mL of 0.45% GSS and read 620-nm absorbance at 15, 30, 45, 60, and 120 s, before calculating hemolysis rate at every time point.

The cut-off point of hemolysis rate is 60%. Positive blood samples have hemolysis rate $<60\%$, while the blood samples having hemolysis rate $\geq 60\%$ is judged as negative. The β -thalassemia carriers have hemolysis rate of $17.6 \pm 8.1\%$. Hemolysis rate of $32.6 \pm 13.2\%$ is observed in HbE carriers [52, 53].

2.2.3 0.45% NaCl in glycerine or glycerine saline solution (GSS)-based one-tube osmotic fragility test expressed in “hemolysis area”

The portable spectrophotometer was invented and capable of reading absorbance and transmission of red light through the red blood cell suspension inside

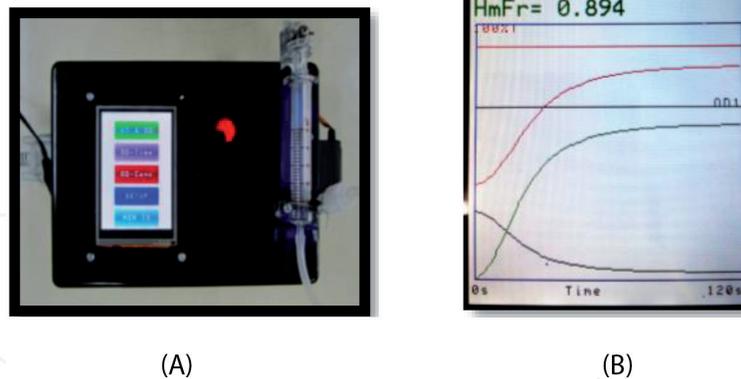


Figure 2.
Portable spectrophotometer (A) and on-screen GSS-based OFT result with the hemolysis area (HemeArea in the screen) shown in the screen (B).

cuvette [54]. To perform this test, 20 μ L of EDTA blood is mixed with 5 mL of GSS in 12 \times 75 polypropylene cuvette. Then place the cuvette inside the cuvette holder of the portable spectrophotometer (red dots in **Figure 2(A)**) and start the machine. Light transmission (red line in **Figure 2(B)**), light absorbance (black line in **Figure 2(B)**), and HemeArea or hemolysis area (green line in **Figure 2(B)**) are generated simultaneously in real-time manner. At 120 s, the numeric HemeArea (or hemolysis area) is shown in green alphabets (**Figure 2(B)**).

Cut-off point of the hemolysis area is 52.4 unit. Blood samples having the hemolysis area less than these cut-off values are judged as “positive samples,” while those samples having the hemolysis area \geq 52.4 units are classified as “negative sample.” The hemolysis area of normal individuals was 67.1 ± 12.6 units. In contrast, the hemolysis area of HbE carriers, β -thalassemia carriers were found to be 36.4 ± 13.9 , 18.6 ± 1.1 , respectively [54].

2.3 Dichlorophenolindophenol precipitation (DCIP) test

In dichlorophenolindophenol precipitation (DCIP) test, 2,6-dichlorophenol indophenol (DCIP) were oxidizing chemicals and used as indicator of ascorbic acid measurement. Hemoglobin E is resulted from amino acid change at codon 26 of β -globin chain from glutamic acid to lysine. This change makes contact of α -globin chain and β^E -globin chain less stable. Thus, in DCIP solution, molecule of HbE changes from tetramer to monomer, freeing sulfhydryl group of amino acid, oxidized by the DCIP, denatured, and precipitated [55].

The 500-mL DCIP reagent is composed of 4.36 g Trizma base, 2.68 g EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$, 0.0276 g of DCIP, and 0.05 g of saponin. The pH of reagent is adjusted to 7.5 by using 6 N HCl. To perform test, 20 μ L of EDTA blood is mixed with 5 mL DCIP reagent. The mixture is incubated in 37°C-water bath for 1 h before precipitation occurs in case of HbE carriers. To enhance visualization, 20 μ L of 6% (w/v) ascorbic acid is dropped into the mixture and the color of mixture turns from deep blue to pale red (**Figure 3**).

This test is now commercially available in Thailand. The commercial DCIP reagent has the same ingredient and incubation condition as the original test, except the volume of reagent is scaled down to 2 mL. The examples of commercially DCIP reagent presently distributed in Thailand are THALCONTM and KCU-DCIPTM reagent (**Figure 4**).

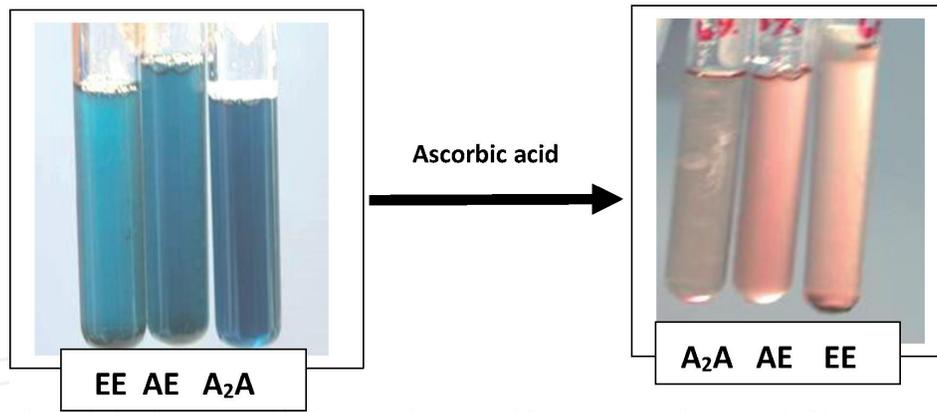


Figure 3. Dichlorophenolindophenol precipitation (DCIP) test. The DCIP results before adding ascorbic acid have deeply blue color, but after ascorbic acid addition, the color turns to pale red. The amount of precipitation is increased in homozygous HbE (EE), compared to HbE carrier (AE). No precipitation is seen for non-HbE (A₂A). Modified from Ref. [53].

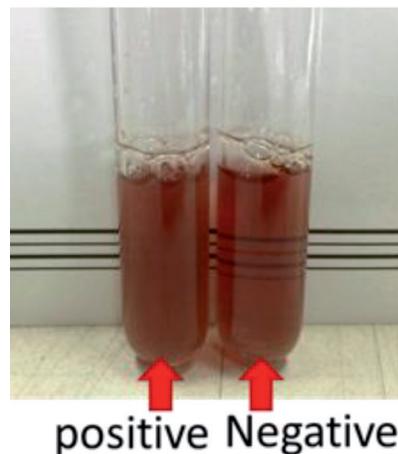


Figure 4. DCIP test results obtained by commercial DCIP kit. The black lines placed behind the tube aid the result reading. If we cannot see the black lines, the result is positive. If the black lines are clearly seen, the result is negative. DCIP test produces turbid solution.

This test has been validated and shown to be effective in screening for HbE carriers. Analysis in the author's laboratory showed that this test had 100% sensitivity and 98.4% specificity in screening for HbE carriers [56]. Wiwanitkit et al. found almost the same effectiveness of this test in HbE screen; 100% sensitivity and 97.2% specificity [14]. Chapple et al. re-evaluated the KKU-DCIP reagent kit and found 100% sensitivity and 92% specificity for screening for HbE carriers [55].

2.4 HbE-tube test

This test was invented by the author in 2012 [15]. It is based on the principle of anion-exchange liquid chromatography. The diethyl aminoethyl (DEAE)-cellulose having positive charge in buffer having fixed amount of NaCl is placed in the test tube. This condition allows all hemoglobins to bind DEAE-cellulose, except HbE and HbA₂. Then these two hemoglobins will still dissolve in the supernatant. However, since HbE quantity is more than 10% in HbE carriers, then in case of HbE carrier, the supernatant color is red. In contrast, in non HbE, that is, no HbE in blood, the supernatant is colorless (**Figure 5**). This test is simple, requiring no incubation and centrifugation. Standing the test tube after mixing the blood for about 10 min is enough for visualizing the supernatant color.

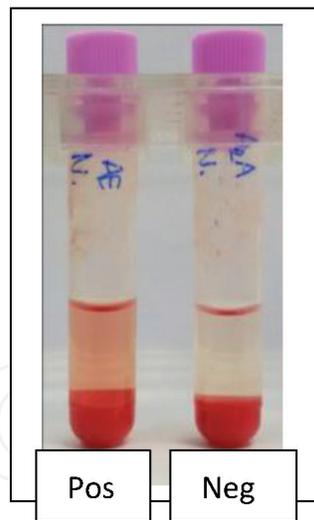


Figure 5.

HbE tube test for screening of HbE carriers. The positive tube (Pos) is the result of HbE carrier having Hb type AE and red supernatant is seen. The negative tube (Neg) is the result of normal individual having Hb type A₂A and colorless supernatant is seen.

To perform the test, 15 μ L packed red cell (PRC) is added to pre-prepared DEAE-cellulose suspension in the transparent test tube (0.5 mL DEAE-cellulose suspension in 1.0 mL glycine-NaCl buffer containing 0.2 M glycine–0.01% KCN–7.5 mM NaCl). The tube is shaken briefly, left for 5 min at room temperature, and spun at 3500 round per minute for 5 min before reading the results by visualizing the color of supernatant as shown in **Figure 5**.

This test was simple and effective in screening of HbE carriers. Its sensitivity and specificity for HbE screen are 100%. The reagent can be kept in the cold (2–8°C) for 5 months [15].

2.5 HbE screen test

This test was initially invented by Prof. Dr. Torpong Sa-nguansermisri of The Thalassemia Unit, Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Thailand [57] and adopted for HbE screen in pregnant women by Sirichotiyakul et al. [58]. This test was also established in author's laboratory (Unpublished data). This test works under the principle of anion-exchange column chromatography, modified from microcolumn chromatography for HbA₂ quantification [59, 60].

The test comprises small syringe packed inside with anion-coated resin such as diethylaminoethyl (DEAE)-Sephadex A50 suspended in Tris-HCl-KCN (THK) buffer (6.057 g tris hydroxymethyl aminomethane, 0.1 g KCN, 4 N HCl to adjust pH), pH 8.5. On passing hemolysate through this microcolumn, all hemoglobins bind to the resin. However, passing the eluting buffer (THK buffer, pH 8.2), only HbE and HbA₂ are eluted. If the patients have HbE, the color of entire length of the microcolumn will be red. If the patients do not have HbE, the color of this point will be colorless (**Figure 6**).

To perform this test invented by Prof. Dr. Torpong Sanguansermisri, 10 mL hemoglobin solution (40 μ L hemolysate mixed in 5 mL THK buffer pH 8.5) is dropped into microcolumn prepacked with DEAE-Sephadex A 50 to the height of 5.0 cm in pasture pipette. The solution is allowed to flow through the microcolumn which is subsequently equilibrated with 10 mL THK buffer pH 8.5. Finally, HbE and HbA₂ are eluted out of the microcolumn after poring 10 mL of THK buffer, pH 8.2. At the end, the red color of the column is observed. If almost the entire length of microcolumn is red,

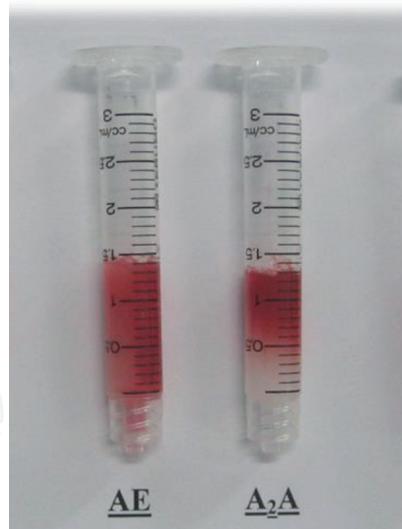


Figure 6. HbE screening test in a modified protocol so-called naked eye microcolumn for HbE screen (NEMES). The AE sample shows red color in the resin along entire length of the resin, but not for the A₂A sample.

the result is “Positive.” However, if the red color sticks to only at the top layer of the packed resin, the result is “Negative” [57]. This test was evaluated by Sirichotiyakul et al. and found to have 100% sensitivity and 100% specificity for HbE screen [58].

Alternatively, the test was modified in author’s laboratory. In this modification, DEAE-cellulose was used as the pre-packed resin, and glycine-NaCl buffer was equilibrating and eluting buffer. To prepare microcolumn, DEAE-cellulose resin suspended in equilibrating buffer (0.2 M glycine + 0.01% KCN) is pre-packed to the height of 2.5 cm in 3-mL plastic syringe with 1-cm diameter. To perform the test, 150 μ L hemolysate is dropped into the microcolumn and allowed to pass through the resin before eluting HbE with 4 mL eluting buffer (0.2 M glycine + 0.01% KCN + 0.005 M NaCl). The red color of the resin packed in column is observed in the way resemble that mentioned above (**Figure 6**). The protocol was named “Naked-Eye-Microcolumn-HbE-Screen or NEMES” [61]. This test was found to have 100% sensitivity and 100% specificity for screening of HbE carriers.

3. Interpretation of the screening tests

The β -thalassemia carriers always have positive OFT, MCV less than 80 fL, MCH less than 27 pg, and negative HbE. In contrast, HbE carriers may have MCV: more or less than 80 fL, MCH: more or less than 27 pg, OFT: positive or suspicious or negative, and hemolysis area: more or less than 52.4 unit. However, all cases of HbE carriers certainly have positive HbE screening tests performed by all mentioned tests.

The MCV, MCH, and OFT are all positive in β -thalassemia diseases such as homozygous β^0 -thalassemia, homozygous β^+ -thalassemia, compound heterozygous β^0/β^+ -thalassemia, compound heterozygous HbE/ β^0 -thalassemia, compound heterozygous HbE/ β^+ -thalassemia, and homozygous HbE. However, the DCIP test, HbE tube test, and HbE screen test are positive in all cases having HbE in blood.

4. Usefulness of screening tests

In financially burden countries that have considerably high prevalence of thalassemia and hemoglobinopathies, initial screen of the carriers in population is essential. This approach helps to decrease the number of cases seeking further for more

expensive confirmatory tests. Normally, the sophisticated laboratory tests for conforming the diagnosis of thalassemia and hemoglobinopathies are set up in big centers which are mostly located in the city. Thus, selected cases that are screened in for the definite diagnosis of thalassemia and hemoglobinopathies have to travel a long distance to the city. This would not consume much money for traveling and for laboratory tests.

4.1 Confirmatory method for thalassemia and hemoglobinopathies

Aims of confirmatory tests are to make the definite diagnosis of β -thalassemia and HbE. These tests generally performed only in blood samples having positive results of screening tests. Two sets of confirmatory tests are generally performed; hemoglobin studies and DNA analysis.

4.1.1 Hemoglobin studies by cellulose acetate electrophoresis (CAE) at alkaline condition

This test separates hemoglobins in blood by their negatively molecular net charge. Hemoglobins are allowed to dissolve in Tris-EDTA-Borate (TBE) buffer pH 8.6. This pH is more than isoelectric points (pI) of all hemoglobins (approximately 6.5–7.5) [62, 63]. At this pH, all hemoglobins have negative charge and migrate from cathodic part toward anodic end of the electrophoretic chamber.

In 1 L of TBE buffer, it is composed of 12.0 g tris-hydroxymethyl aminomethane (Tris), 1.22 g ethylenediaminetetraacetic acid (EDTA), and 1.5 g boric acid.

The electrophoresis is performed at a constant voltage of 250–300 volts for 15–20 minutes or until HbA and HbE bands are clearly separated. At the end of electrophoresis, the hemoglobin bands on cellulose acetate plate are stained for 5 minutes with Ponceau S stain (2 g of Ponceau S powder, 30 g of trichloroacetic acid, and 30 g of sulfosalicylic acid in 1 L of distilled water) and destained for 5 minutes in destaining solution (5% acetic acid in distilled water). Finally, the cellulose acetate plate is made clear for permanent record by immersing for 5 minutes in clearing solution (4 volume of methanol +1 volume of glacial acetic acid) and dried by using hair dryer. Hemoglobin pattern from cathodic to anodic ends is Constant Spring- A_2 /E/C/O-S/D/Lepore/G-F-A-Portland-Bart's-H [64] (**Figure 7**). Hemoglobin patterns on CAE at alkaline condition are shown in **Table 1**. In the past, densitometer was used to determine quantities of hemoglobins in blood samples. However, this technique is not conventionally performed presently because it may give falsely high levels of hemoglobins if the background is not completely cleared. However, CAE results can still give types of hemoglobins in blood samples. Thus, by

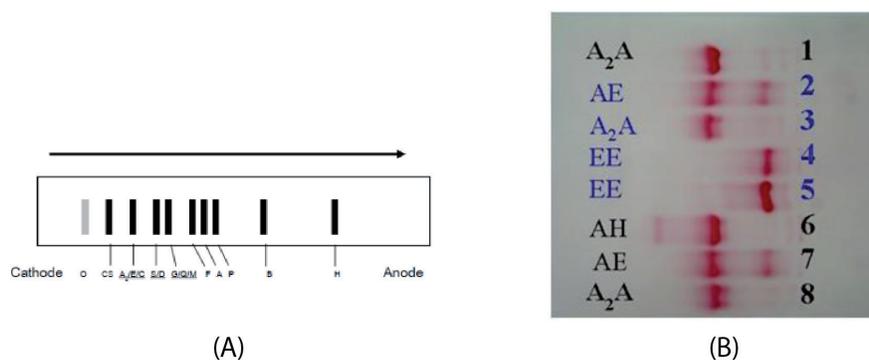


Figure 7. (A) Hemoglobin patterns on cellulose acetate electrophoresis (CAE) at pH 8.6. (B) Example of hemoglobin pattern on CAE at pH 8.6 stained with Ponceau S stain.

	Hb patterns on CAE at pH 8.6 of adult
HbE heterozygote (carriers)	EA
HbE homozygote	EE (only 1 band of HbE)
β^0 -thalassemia heterozygote (carriers)	A ₂ A
β^+ -thalassemia heterozygote (carriers)	A ₂ A
β^0 -thalassemia homozygote (β^0/β^0)	A ₂ F
β^+ -thalassemia homozygote (β^+/β^+)	A ₂ FA
β^0/β^+ -thalassemia (β^0/β^+)	A ₂ FA
HbE/ β^0 -thalassemia (β^E/β^0)	EF
HbE/ β^+ -thalassemia (β^E/β^+)	EFA

Table 1.
Hemoglobin pattern on CAE at pH 8.6 of β -thalassemia and HbE.

using this technique, further tests must be done in order to determine the level of HbA₂ and HbF. Raised level of HbA₂ beyond normal range is the diagnostic marker for β -thalassemia carriers, while elevated level of HbF helps identify the high HbF condition found in the hereditary persistence of fetal hemoglobin (HPFH).

4.1.2 Hemoglobin study by microcolumn chromatography

This test is preliminarily aimed to quantify HbA₂ levels that help diagnosis of the β -thalassemia carrier. However, HbE has the same pI as HbA₂, thus these two hemoglobins are co-eluted. Microcolumn chromatography is an anion-exchange chromatography-based method. Anion-resin such as DEAE-cellulose or DEAE-Sephadex A50 suspended in appropriate buffer is packed in the microcolumn. On passing hemolysate through the packed resin, negatively charged hemoglobins binds to the resin at different binding affinity. HbA₂ and HbE bind to the resin at the weakest strength (if there is no Hb Constant Spring) and are eluted out easily with small amount of external anion such as CL⁻.

There are two types of microcolumn chromatography, based on the anion-resin and buffers used. These include (1) DEAE-Sephadex A50 plus Tris-HCl-KCN buffer, and (2) DEAE-cellulose plus glycine-NaCl buffer.

4.1.2.1 Microcolumn chromatography using DEAE-Sephadex A50 resin

DEAE-Sephadex A 50 resin suspended in Tris-HCl-KCN buffer (0.05 M Tris plus 0.1 g KCN/1 L, adjust pH with 4 N HCl) pH 8.5 is packed in pasture pipette to the height of 8–9 cm [52]. Then, the microcolumn is applied with 100 μ L hemolysate, equilibrated with 10 mL THK buffer pH 8.5. Finally, 10 mL THK buffer pH 8.2 is applied to the microcolumn to elute HbA₂ and/or HbE before 10 mL eluate is collected for measuring light absorbance (A) or optical density (OD) at 415 nm. This is then called A₄₁₅-A₂ or OD₄₁₅-A₂. For measuring OD₄₁₅ of total hemoglobin or OD₄₁₅-Total Hb, 100 μ L hemolysate is mixed with 10 mL DW before measuring the absorbance. The level of HbA₂ or HbE is calculated by the Eq. 1 shown below.

$$\text{HbA}_2 \text{ or HbE (\%)} = [\text{OD}_{415} - \text{A}_2 / \text{OD}_{415} - \text{total Hb}] \times 100 \quad (1)$$

If percent is less than 10, it is HbA₂.

If percent is 10 up, it is HbE.

Normal range of HbA₂ by this protocol is $2.62 \pm 0.87\%$ [52].

4.1.2.2 Microcolumn chromatography using DEAE-cellulose

This protocol was described by Wood [64] and modified in the author's laboratory in 2007 [65].

In modified protocol, DEAE-cellulose resin suspended in equilibrating buffer (0.2 M glycine + 0.01% KCN) is packed to the height of 2 cm in plastic microcolumn with 1.0-cm diameter. Then the microcolumn is applied by 50 μ L hemolysate (prepared by mixing 1 part of PRC and 6 parts of 0.05% Triton X-100 as hemolysis buffer) and flushed with 5 mL eluting buffer (0.2 M glycine + 0.01% KCN + 0.005 M NaCl). The 5-mL eluate is then collected and measured for absorbance or optical density at 415 nm (A_{415-A_2} or OD_{415-A_2}). OD_{415} of total hemoglobin is measured in diluted hemolysate (50 μ L hemolysate mixed with DW to 15 mL) and called OD_{415} -Total.

Calculation of levels of HbA₂ or HbE must follow the Eq. 2 shown below.

$$\text{HbA}_2 \text{ or HbE (\%)} = [\text{OD}_{415} - A_2 / \text{OD}_{415}\text{-total} \times 3] \times 100 \quad (2)$$

If percent is less than 10, it is HbA₂.

If percent is 10 up, it is HbE.

Normal range of HbA₂ by this protocol is 1.3–3.7% (mean 2.5%) [64].

For both protocols, if HbA₂ level is less than 3.5%, the chance of β -thalassemia carrier is excluded. Instead, the cases may be either normal or α -thalassemia carriers. However, if HbA₂ level is between 3.5 and 10.0%, the case is definitely β -thalassemia carrier. In β -thalassemia carriers, mean HbA₂ is 4.8% with the range 3.7–7.0% [64].

4.1.2.3 Hemoglobin study by alkaline denaturation test of Betke

This test works under the principle that HbF is resistant to alkaline treatment, while other hemoglobins are not [66]. Therefore, if hemoglobin solution of normal adult is mixed with alkaline solution, HbA, HbA₂ is denatured, leaving only HbF dissolved in the solution. The dissolved HbF can be determined for its level by measuring optical density at 540 nm.

The reagents that are required for this test comprise Drabkin's solution (0.20 g of $K_3Fe(CN)_6$, 0.05 g of KCN, DW to 1 L), 1.2 N NaOH, and saturated ammonium sulphate.

To perform the test, 200 μ L hemolysate is mixed in 3.8 mL of Drabkin's solution to prepare cyanmethemoglobin. Thereafter, 2.8 mL of cyanmethemoglobin solution is mixed with 200 μ L of 1.2 N NaCl and shaken vigorously for 2 min exactly before adding 2.0 mL of saturated ammonium sulphate. Then, the precipitated hemoglobins are filtered out, and the OD_{540} of filtrate is measured and named OD_{540} -filtrate. OD_{540} of total hemoglobin is measured in a mixture of 400 μ L of hemolysate and 6.75 mL of Drabkin's solution and named OD_{540} -Total.

The percentage of HbF is calculated by the following Eq. 3:

$$\text{HbF or Alk F (\%)} = (\text{OD}_{540} - \text{filtrate} / \text{OD}_{540} - \text{total}) \times 10 \quad (3)$$

Since HbF is determined by alkaline treatment, its level is then named Alkaline F or, simply, Alk F. Besides HbF, Hb Bart's (γ_4) is also resistant to alkaline treatment. This protocol has maximum detection limit at only 50% of HbF. If the alkaline denaturation test is performed in fetal blood sample, the Alk F will not be more than 50%. Therefore, other techniques such as HPLC, CZE should be used to measure HbF in fetal blood.

Alk F is not diagnostic marker for both β -thalassemia carriers and HbE carriers. However, increased HbF level presently is considered advantageous in

β -thalassemia and β -hemoglobinopathies [6]. Patients with β -thalassemia disease who also inherit high HbF gene or quantitative trait loci (QTLs) will have mild clinical symptoms. Parents having high HbF gene can pass this gene to their β -thalassemia offspring. Thus, determining HbF in parents is useful in this way.

4.1.2.4 Hemoglobin study by cation-exchange high performance liquid chromatography (HPLC)

Cation-exchange HPLC has become the reference method for typing and quantitating hemoglobins in blood samples [19, 67, 68]. In this system, hemoglobins are dissolved in buffer having a pH of 6.4 that is less than pI of hemoglobins (6.5–7.5) and molecular net charge then is converted to be positive. Different hemoglobins then have different amount of positive charge which determines binding strength of hemoglobins to negatively charged resin. Hb Bart's has the weakest binding affinity, while Hb Constant Spring has the strongest binding affinity. Therefore, on passing external cation, the order of hemoglobins that are eluted fast to slowly should be as follows: Hb Bart's-HbH-HbF-HbA₀-HbA₂/E-Hb Constant Spring (**Figure 8**).

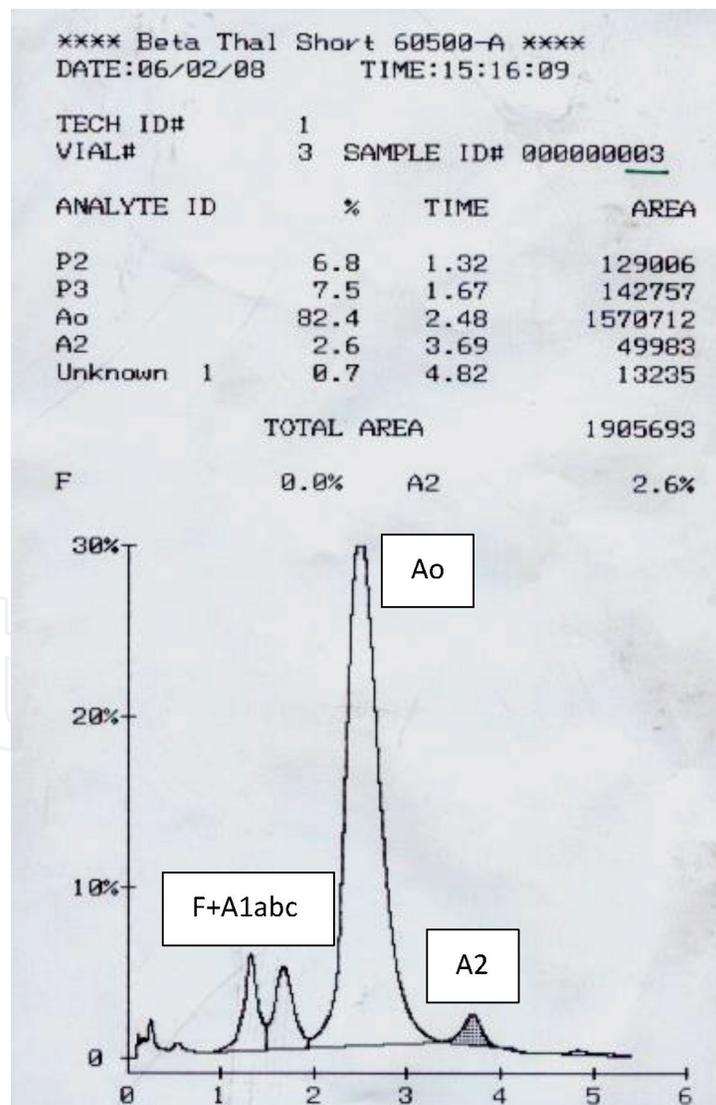


Figure 8.

Hemoglobin pattern of cation-exchange HPLC of normal human adult: A₂A. As shown in the figure, major hemoglobin is HbA₀ which accounts for 82.4%, while the minor HbA₂ accounts for 2.6%. Other minor hemoglobins are labeled P₂ and P₃, which are Hbs A_{1a}, A_{1b}, and A_{1c} mixture. HbF peak is negligible and reported as 0.0%. This HPLC result may be that of normal individuals or α -thalassemia carriers. It is noted that this kind of cation-exchange HPLC pattern may be also observed in carriers of α -thalassemia 1 and carriers of α -thalassemia 2.

HbA is normally derivatized to several fractions including the minor Hbs A1a, A1b, A1c, and major HbA_o. The minor Hbs A1a, A1b, and A1c are eluted just after HbF. For routine Hb typing work, only the major HbA_o is usually reported to clinicians. This makes sum of hemoglobin peaks does not equal to 100%.

HbE, which is common in Southeastern part of the world, is co-eluted with HbA₂. However, most of the manufacturers design program to read hemoglobin peak at the A₂ region as only HbA₂. Therefore, the operator must be aware that if the percentage of A₂-peak is more than 10, it is HbE plus HbA₂ and indicates that the sample has HbE. The operator must report HbE or HbE plus HbA₂, instead of reporting HbA₂ as reported by the machine.

There are several manufacturers producing the HPLC machine in the world and the operating procedures as well as quality control protocols are established specifically for each brand. Most importantly, all of these brands generate identical separation peaks of hemoglobins. **Figure 8** shows example of hemoglobin pattern obtained from Variant™ Hemoglobin Analysis System, the widely used HPLC machine in Thailand. In this protocol, the hemoglobin types in normal human adults are A₂A with HbA₂ of $2.6 \pm 0.38\%$ [19].

Contrast to the CAE at pH 8.6, hemoglobins separated by the cation-exchange HPLC are automatically calculated for their proportions in blood. Therefore, both types and quantities of hemoglobins are usually obtained when run in this platform.

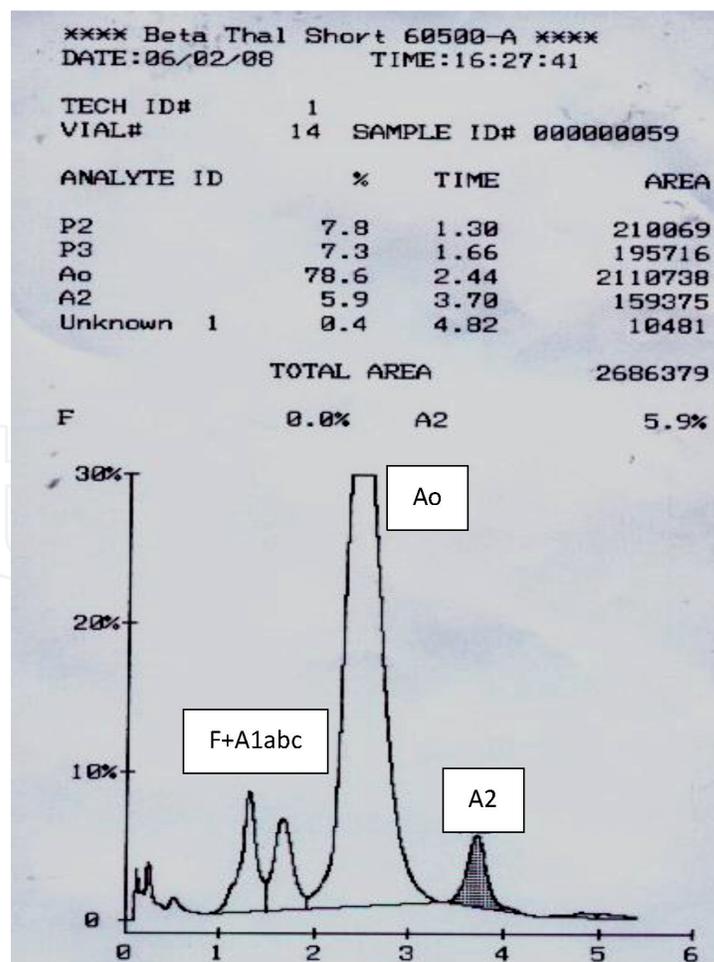


Figure 9. Hemoglobin pattern of cation-exchange HPLC of β -thalassemia carrier in human adult: A₂A. As shown in the figure, major hemoglobin is HbA_o which accounts for 78.6%, while the minor HbA₂ accounts for 5.9%. Other minor hemoglobins are labeled P2 and P3, which are Hbs A1a, A1b, and A1c mixture. HbF peak is negligible and reported as 0.0%.

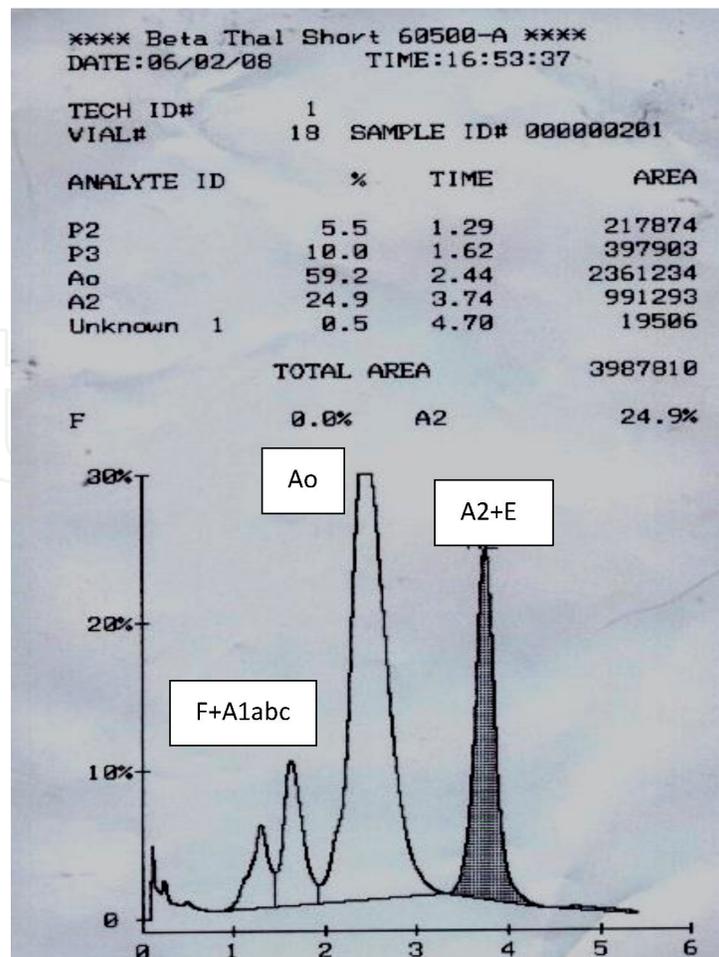


Figure 10.

Hemoglobin pattern of cation-exchange HPLC of HbE carrier in human adults: EA. As shown in the figure, major hemoglobin is HbAo which accounts for 59.2%, while the minor HbE (plus A₂) accounts for 24.9%. Other minor hemoglobins are labeled P2 and P3, which are Hbs A1a, A1b, and A1c mixture. HbF peak is negligible and reported as 0.0%.

4.2 Cation-exchange HPLC hemoglobin patterns of β -thalassemia carriers and HbE carriers in human adults

Hemoglobin patterns obtained from the cation-exchange HPLC of β -thalassemia and HbE are totally different. In carrier state, β -thalassemia carriers in adult life have normal Hb types for adult which is A₂A, but HbA₂ levels is increased to the levels of $5.9 \pm 1.35\%$ (Figure 9) [19]. HbE carriers in adult life have abnormal Hb typing by the cation-exchange HPLC which is AE with HbE (plus A₂) of $27 \pm 3.93\%$ [19], as shown in Figure 10.

4.3 Cation-exchange HPLC hemoglobin patterns of β -thalassemia diseases and HbE disease in human adults

Hemoglobin patterns by the cation-exchange HPLC of adult β -thalassemia disease consist of several patterns depending on the combination of the abnormal β -thalassemia mutations.

4.3.1 Homozygous β^0 -thalassemia

Individuals of homozygous β^0 -thalassemia (β^0/β^0) are usually affected by the severe thalassemia disease and require regular blood transfusion. This group of patients

is previously classified as thalassemia major, but now as transfusion dependent thalassemia [69]. Hence, hemoglobin patterns of homozygous β^0 -thalassemia in adult life of human should consist of HbF and HbA₂ with no HbA prior to blood transfusion, that is, A₂F (Figure 11). However, after recent blood transfusion, the hemoglobin types of A₂FA are shown. HbA is certainly from the transfused blood (Figure 12).

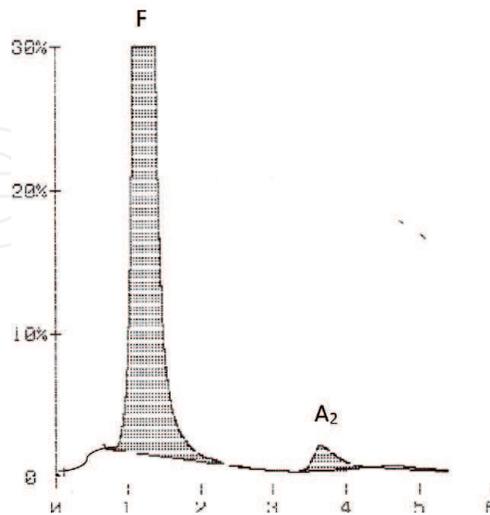


Figure 11. Cation-exchange HPLC pattern of homozygous β^0 -thalassemia prior to blood transfusion, which is read as A₂F (F: 97.8%, A₂: 2.2%) (credit to Eaktong Limveeraprajak of Sawan Pracharak Hospital).

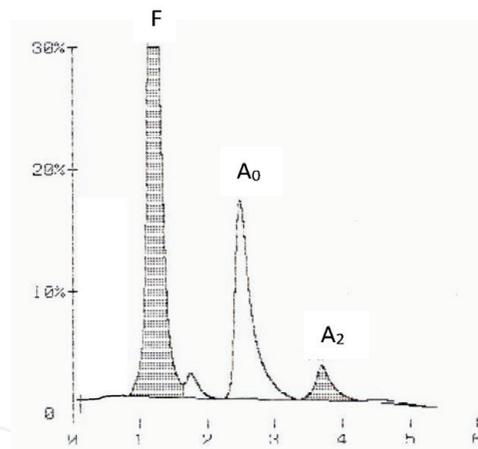


Figure 12. Cation-exchange HPLC hemoglobin pattern A₂FA seen in homozygous β^+ -thalassemia and compound heterozygous β^+/β^0 -thalassemia (HbA₀: 24.6%, HbF: 68.5%, HbA₂: 3.9%).

4.3.2 Homozygous β^+ -thalassemia

Individuals of homozygous β^+ -thalassemia always have mild clinical symptoms and previously are classified as β -thalassemia intermedia. The β -thalassemia intermedia cases usually require no blood transfusion. Thus, now this group of patients is newly classified as non-transfusion dependent thalassemia (NTDT) [69]. Hemoglobin pattern on cation-exchange HPLC of homozygous β^+ -thalassemia is quite resembling to that of transfused homozygous β^0 -thalassemia, that is, A₂FA. However, single population of red blood cells on blood smear is also revealed in this homozygous β^+ -thalassemia, in contrast for dimorphic population in case of transfused homozygous β^0 -thalassemia. Figure 12 shows hemoglobin pattern by cation-exchange HPLC of the homozygous β^+ -thalassemia.

4.3.3 Compound heterozygous β^0/β^+ -thalassemia

Patients with compound heterozygous β^0/β^+ -thalassemia always have severe disease and may require blood transfusion. Thus, they are classified as TDT. Hemoglobin patterns by cation-exchange HPLC of transfused and non-transfused cases is A_2FA , being similar to homozygous β^+ -thalassemia (**Figure 12**). However, the compound heterozygous β^0/β^+ -thalassemia has thalassemic red blood cell morphology like homozygous β^0 -thalassemia. In contrast, red blood cell morphology of homozygous β^+ -thalassemia is less abnormal than the other two β -thalassemia mentioned above.

Hb patterns by the cation-exchange HPLC of adult HbE disease also comprise several varieties depending on combination of β^E mutation.

4.3.4 Homozygous HbE

Homozygous HbE (β^E/β^E) is the mild form of β -thalassemia disease. The patients usually have good clinical symptom with only mild anemia with no need of blood transfusion. Thus, cation-exchange HPLC always shows HbE as major hemoglobin and HbF as the minor hemoglobin; that is, EF (**Figure 13**). Sometime, this hemoglobin type of EF may be confused with that of HbE/ β^0 -thalassemia as HbF in some cases of the later condition may be as low as 4.5% [70] and 2.1% [19]. This low level of HbF may overlap with that seen in homozygous HbE ($4.3 \pm 2.66\%$) [19]. Again, red blood cell morphology will help identify if the case is homozygous HbE or the HbE/ β^0 -thalassemia. Red blood cell morphology on

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**** Beta Thal Short 00405-A ****
DATE:25/05/10      TIME:13:29:32

TECH ID#          1
VIAL#             11
SAMPLE ID# 000000000000001440183

ANALYTE ID        %      TIME      AREA
F                  1.7    1.12    48731
P3                 5.9    1.72   160241
Ao                 4.4    2.32   118335
A2                 77.3    3.72  2059359

TOTAL AREA        2386666
F                  1.7%   A2        77.3%

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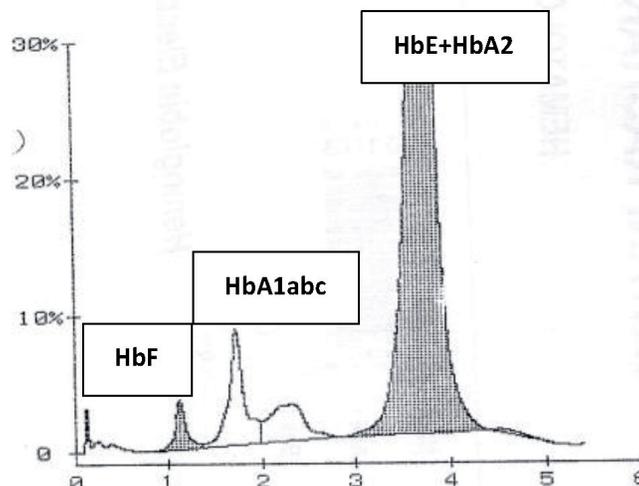


Figure 13.

Hemoglobin pattern by cation-exchange HPLC of homozygous HbE in adults. The major peak contains HbE plus HbA₂, but it is labeled A₂ by software. Thus, level of HbE plus HbA₂ in this case is 77.3, and that of HbF is 1.7%. This case has no HbA₁, but the software mislabeled the HbA₁ fraction as Ao.

blood smear stained with Wright-Giemsa stain is totally different between homozygous HbE and HbE/ β^0 -thalassemia. In homozygous HbE, mild change of red blood cell morphology with considerable amount of target cells is usually observed. In contrast, thalassemia type of red blood cell morphology is typical for the HbE/ β^0 -thalassemia.

4.3.5 Compound heterozygous β^E and β^0 -thalassemia

Individuals with compound heterozygote of β^E and β^0 -thalassemia are always affected by the thalassemia disease and some require blood transfusion. Therefore, the hemoglobin patterns by cation-exchange HPLC of this case will be EF (61.2 \pm 13.6% HbE, 31.1 \pm 14.5% HbF) prior to blood transfusion [19] and EFA after recent blood transfusion (**Figure 14**).

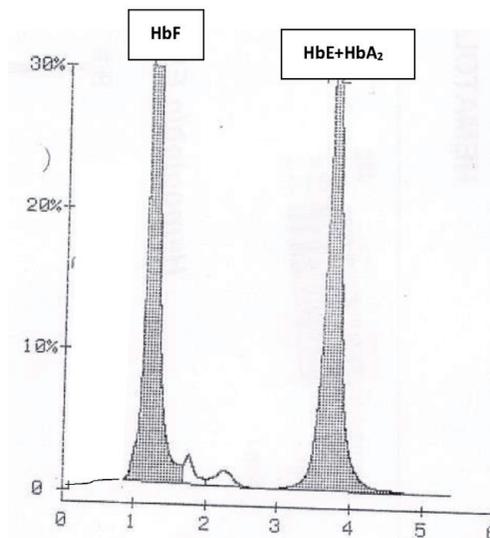


Figure 14. Hemoglobin pattern in cation-exchange HPLC of cases with HbE/ β^0 -thalassemia (F 53.1%, E 35.8%).

4.3.6 Compound heterozygous β^E and β^+ -thalassemia

Individuals with compound heterozygote of β^E and β^+ -thalassemia have mild clinical symptoms and classified as NTDT. Therefore, interference of transfused blood is not possible. The cation-exchange HPLC pattern of hemoglobin in this case should be EFA. This is because some β -globin chains are still produced.

4.3.7 Double form of HbE carrier and HbH disease

This thalassemia syndrome is conventionally termed AEBart's disease. This is a mild form of α -thalassemia syndrome, and blood transfusion is not required. Thus, transfused blood would not also interfere result reading in this situation. Hemoglobins A, E (with A₂), Bart's are always seen under the cation-exchange HPLC (**Figure 15**). This is why it is called AEBart's disease.

4.3.8 Double form of HbE homozygote and HbH disease

This is also a mild form of α -thalassemia disease that shows hemoglobins E, F, and Bart's in the cation-exchange HPLC. It is thus called EFBart's disease. By running the cation-exchange HPLC, Hbs E, F, and Bart's are always seen in the chromatogram.

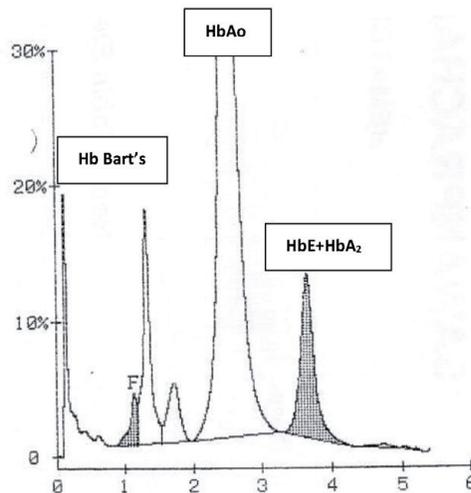


Figure 15.

Cation-exchange HPLC of hemoglobin component in AEBart's disease. HbE: 13.5%, HbA₀: 71.8%, Hb Bart's (no numeric proportion as the analysis software was not designed for Hb Bart's quantification).

4.3.9 Double form of HbE/ β^0 -thalassemia and HbH disease

This is a rare form of thalassemia syndrome. On running in the cation-exchange HPLC, EF Bart's pattern of hemoglobin is also seen, being similar to the double form of homozygous HbE and HbH disease. Red blood cell morphology on blood smear may help differentiate these two conditions, but skillful personnels are needed to examine red blood cell morphology. However, DNA analysis is the only technique that can correctly differentiate this EF Bart's syndrome.

5. Hemoglobin study by capillary zone electrophoresis (CZE)

Capillary zone electrophoresis (CZE) has been introduced for use as a tool for analysis of hemoglobin variants [23–27, 71]. Conventionally, separation of hemoglobin is performed in alkaline condition, in which HbH has the maximum molecular negative charge, followed, respectively, by Hb Bart's, HbA, HbF, HbA₂/E, and Hb Constant Spring. The separation is based on high voltage (7500 V) and electro-endo-osmotic force (EOF). Hemoglobins are forced in the system to move from anode to cathode with the cuvette placed at the cathodic end. Once hemoglobin band moves into the cuvette, the 415-nm absorbance is measured and the light signals are converted by the software to electropherogram. Each hemoglobin has its own location or zone in the electropherogram, HbCS-zone 2: Z(C), HbA₂-zone 3: Z(A₂), HbE-zone 4: Z(E), HbF-zone 7: Z(F), HbA-zone 9: Z(A), Hb Bart's – zone 12, and Hb H – zone 15. HbE and HbA₂ are clearly separated by this system (Figures 16 and 17).

5.1 The CZE pattern of β -thalassemia carriers and HbE carriers

In β -thalassemia carrier at adult life, the CZE pattern of hemoglobin is similar to that obtained from cation-exchange HPLC. The normal hemoglobin typing result of A₂A or A₂FA with HbA₂ levels of more than 3.5% ($5.4 \pm 0.5\%$) and HbF levels of less than 2% ($0.9 \pm 1.4\%$) are always observed [27] (Figure 18).

In HbE carrier of adult life, the hemoglobin pattern of CZE is different from that of cation-exchange HPLC. HbE and HbA₂ co-eluted in the cation-exchange HPLC. In CZE, HbE moves behind HbA₂. Thus, hemoglobin pattern of HbE carrier

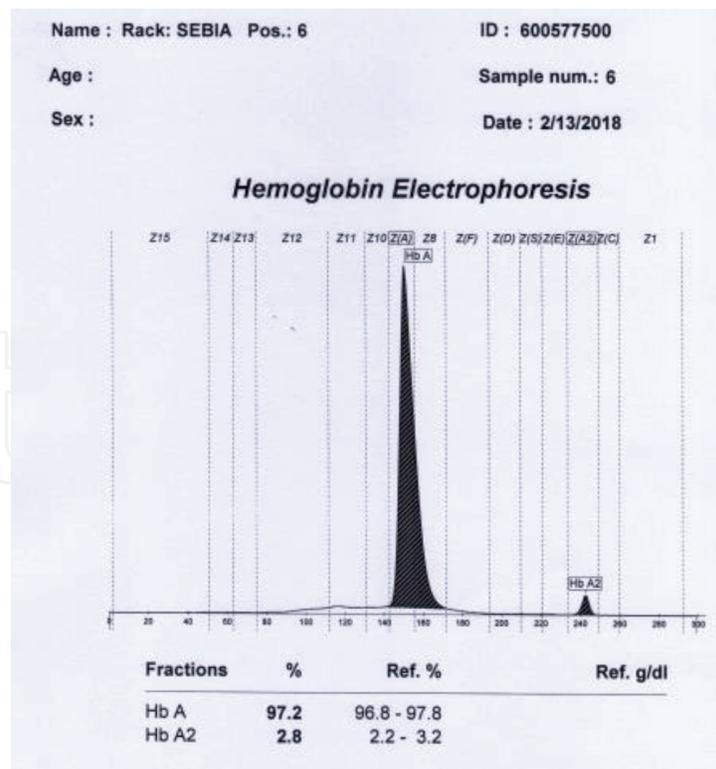


Figure 16.
 CZE electropherogram of normal human hemoglobins. HbA: 94.3%, HbF: 2.4%, HbA₂: 3.3%.

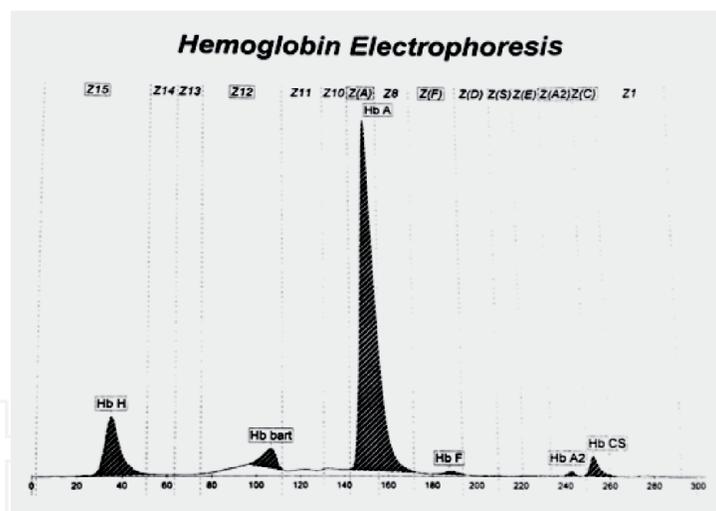


Figure 17.
 CZE pattern of hemoglobins from zone (Z) 1 to zone (Z) 15.

in CZE should be A₂EFA. The percentage of these hemoglobins is as follows: HbA₂: 3.5 ± 0.4%, HbE: 25.6 ± 1.4%, and HbF: 0.4 ± 0.8% [27]. HbE level in the CZE system is usually lower than that obtained from cation-exchange HPLC (27.8 ± 7.5%). This is due to the fact that the level of HbE from HPLC is the sum of HbE and HbA₂ that are co-eluted, while only HbE is reported in the CZE system. Thus, performers must be careful in reporting HbE. HbA₂ level is slightly elevated. This confirms that HbE carrier also acts as mild β -thalassemia carrier (**Figure 19**).

5.2 The CZE pattern in β -thalassemia disease and HbE disease

CZE pattern of hemoglobins in β -thalassemia disease in adults depends on types of the disease. Although, principles of separation are different, the patterns of hemoglobin

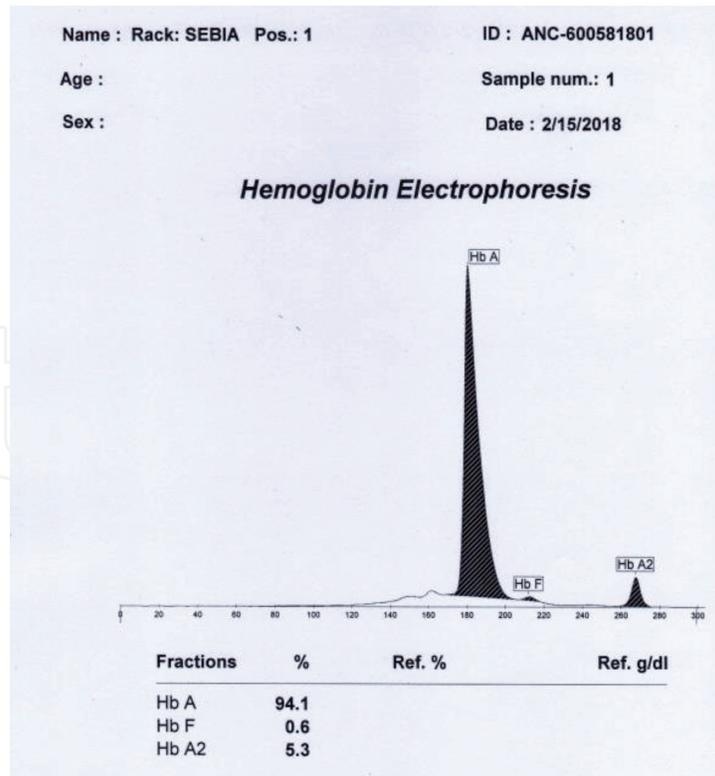


Figure 18.
 CZE pattern of hemoglobins of β -thalassemia carrier.

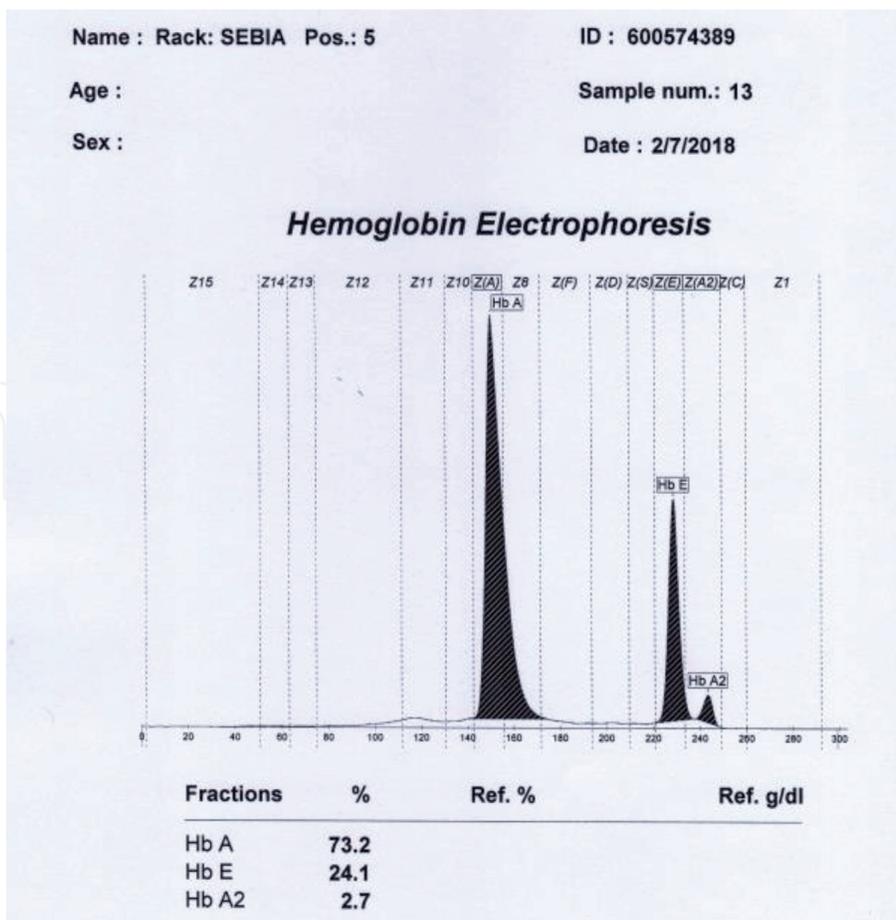


Figure 19.
 CZE pattern of hemoglobin of HbE carrier.

Name : Rack: SEBIA Pos.: 8

ID : 600950249

Age :

Sample num.: 8

Sex :

Date : 7/25/2018

Hemoglobin Electrophoresis

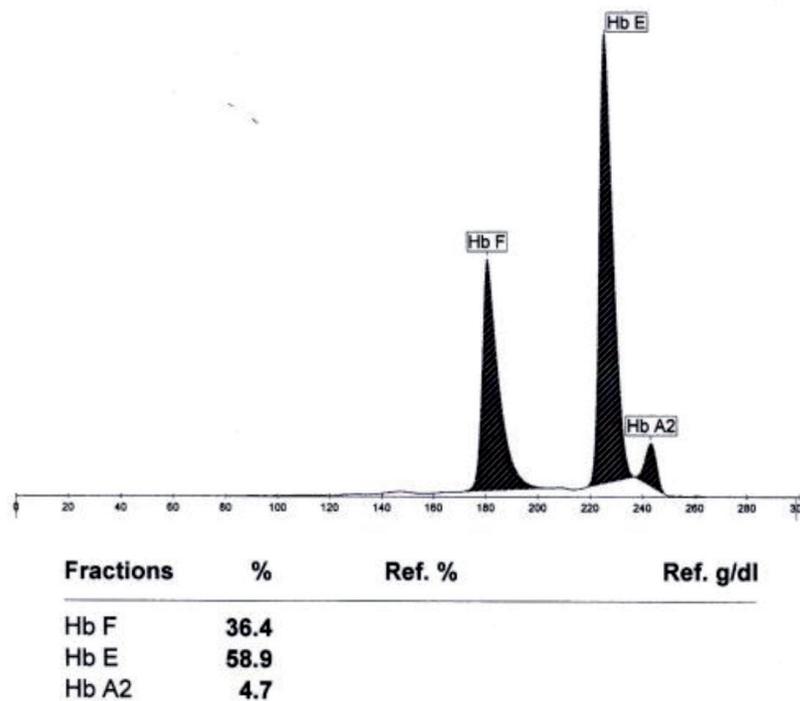


Figure 20.

CZE pattern of hemoglobins of HbE/ β^0 -thalassemia, composing of A_2EF with 4.7% HbA₂, 58.9% HbE, and 36.4% HbF.

in β -thalassemia disease and HbE disease obtained from CZE are quite similar to those obtained from cation-exchange HPLC. For homozygous β^0 -thalassemia, A_2F is the typical hemoglobin typing results. For homozygous β^+ -thalassemia and compound heterozygous β^+/β^0 -thalassemia, the A_2FA is generally seen in CZE platform.

CZE patterns of hemoglobins in HbE disease in adults also depend on types of the diseases. However, as HbE and HbA₂ are clearly separated in the CZE platform, these two hemoglobins must be separately reported. For example, in HbE/ β^0 -thalassemia, for example, A_2EF must be reported together with the proportion of each hemoglobin (Figure 20).

6. Hemoglobin study by monoclonal antibody

Thalassemia and hemoglobinopathies can be identified accurately by using monoclonal antibodies (mAbs) against human hemoglobins [72, 73]. Application of mAb-based protocols aims primarily to identify the carriers of thalassemia and hemoglobinopathies. For instance, α -thalassemia carrier can be detected by using mAbs against Hb Bart's [29] and HbH [74]. Immunochromatographic strip test utilizing mAb to Hb Bart's was produced and successfully applied for screening α -thalassemia carriers [32, 75].

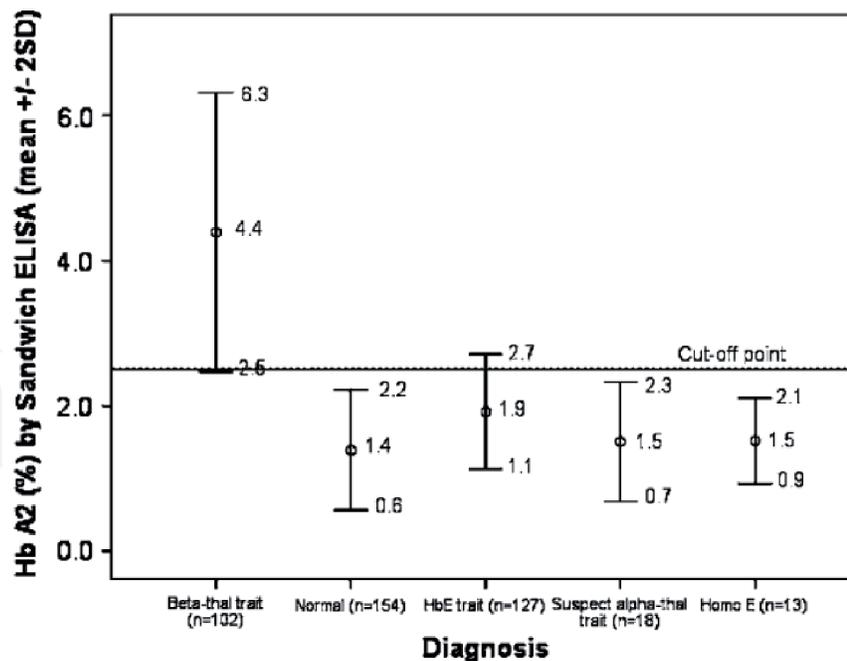


Figure 21.

HbA₂ levels determined by sandwich ELISA set up in the author's laboratory. Note that HbA₂ levels in β -thalassemia trait are higher than those in normal, HbE trait, homozygous HbE, and suspected α -thalassemia trait (modified from [30]).

The β -thalassemia carrier can also be identified by using antigen-antibody reaction. Since elevated HbA₂ level has been shown to be diagnostic marker of the β -thalassemia carrier, mAbs against δ -globin chain of HbA₂ were produced and ELISA set up to quantify HbA₂ levels by Shyamala et al. [76]. Using this ELISA, Shyamala found mean value of HbA₂ in normal and β -thalassemia carrier to be 2.5 and 5.4%, respectively. The mAb against HbA₂ was also produced and sandwich ELISA developed in the author's laboratory [30]. Under this developed sandwich ELISA, Kuntaruk found that the levels of HbA₂ between normal and β -thalassemia carrier were also significantly different (**Figure 21**). Thus, the β -thalassemia carrier can be identified by the sandwich ELISA to quantify HbA₂ level.

7. DNA analysis for β -thalassemia and HbE

Analysis of mutations in β -globin gene to identify β -thalassemia and HbE is now performed routinely in most laboratories. The finding of the causative point mutations in the β -globin gene provides definite diagnosis of these disorders. More than 900 point mutations have been reported for β -thalassemia and β -hemoglobinopathies (Globin Gene Server: <http://globin.cse.psu.edu/>). Certain ethnic groups have their own pattern of point mutations of β -globin gene [2, 4, 77, 78].

There are several allele-specific PCR protocols for detecting both carrier and disease state of β -thalassemia and HbE. These include mutagenically separated (MS)-PCR [35] and amplification refractory mutation system (ARMS)-PCR [79]. These two protocols were modified and adapted in author's laboratory. Another allele-specific PCR protocol was established in the author's laboratory and named "Multiplex Allele-Specific (MAS)-PCR" [29].

7.1 Identification of β -thalassemia and HbE by MS-PCR

The MS-PCR was used to detect β -globin gene mutations by several centers. In author's laboratory, this PCR protocol was modified and adapted to identify

the β -globin gene mutations commonly found in Thai individuals. These included TTCT deletion or 4 bp-deletion at codons 41/42 ($\beta^{41/42}$) and A > T substitution at codon 17 (β^{17}) of β -globin gene [80]. These two mutations have been shown to account for approximately 67.5% in Thais by author's survey [29] and 83.9% by others' studies [81]. The MS-PCR was performed in the author's laboratory under the protocol described previously.

7.1.1 MS-PCR for $\beta^{41/42}$ mutation

Procedure: The 25- μ L PCR is performed containing 250 ng genomic DNA, 200 μ M of each dNTP; 0.5 units Taq DNA polymerase, 100 ng of "Common primer" for $\beta^{41/42}$; 5'-TCA TTC GTC TGT CCA TTC TAA AC-3', 150 ng of "Normal primer" for $\beta^{41/42}$; 5'-TTC CCA CCA TTA GGC TGC TGG TGG TCT ACC CTT GGA CCC AGA GGT TCT T-3', 250 ng of "Mutant primer" for $\beta^{41/42}$; 5'-ACC CTT GGA CCC AGA GGT TGA G-3', 10 mM Tris pH 8.8; 50 mM KCl and 1.5 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 324 and 351 bp indicate presence and absence of the $\beta^{41/42}$ mutation, respectively (**Figure 22**).

Interpretation: Samples having only 324-bp amplified fragments are homozygote for $\beta^{41/42}$ with genotype $\beta^{41/42}/\beta^{41/42}$. Samples having only 351-bp fragments are negative for the $\beta^{41/42}$ with genotype β^A/β^A or β^T/β^T (A represents HbA; T represents other types of β -globin gene mutation). Samples having both 324 and 351-bp amplified products are heterozygote for the $\beta^{41/42}$ with genotype of either $\beta^{41/42}/\beta^A$ or $\beta^{41/42}/\beta^T$ (A represents HbA; T represents other types of β -globin gene mutation).

7.1.2 MS-PCR for β^{17} mutation

Procedure: The 25- μ L PCR is performed containing 250 ng genomic DNA, 200 μ M of each dNTP; 0.5 units Taq DNA polymerase, 100 ng of "Common primer" for β^{17} ; 5'-GGC AGA GAG AGT CAG TGC CTA-3', 150 ng of "Normal

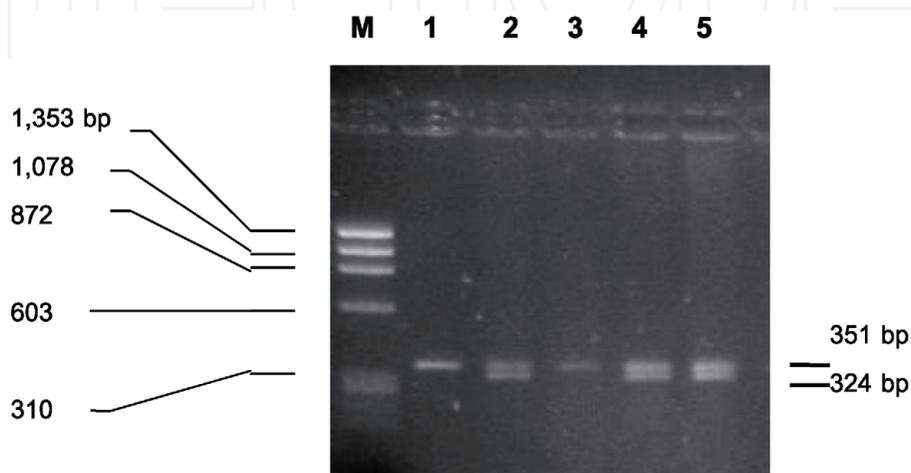


Figure 22. MS-PCR for detecting $\beta^{41/42}$ mutation. Lanes 1 and 3 are negative for the $\beta^{41/42}$ mutations as only 351-bp amplified products are seen. Lanes 2, 4, and 5 are heterozygote for the $\beta^{41/42}$ mutation as both 351 and 324-bp amplified products are seen.

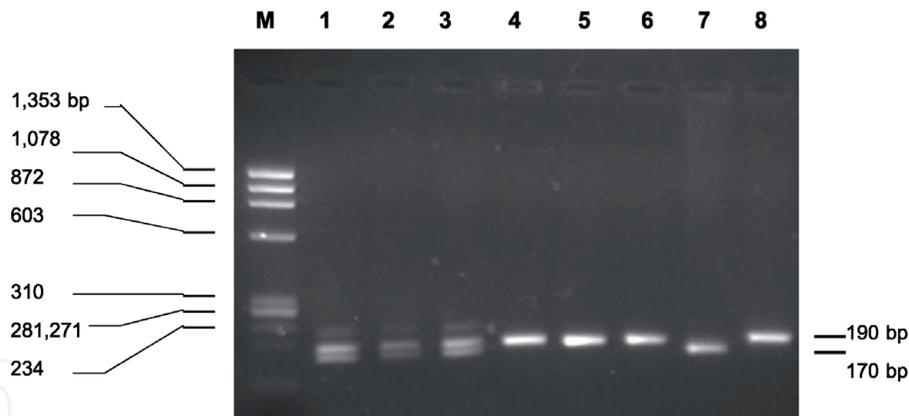


Figure 23.

MS-PCR for identifying β^{17} mutation. Lanes 4, 5, 6, and 8 are negative for the β^{17} mutation as only 190-bp amplified products are seen. Lanes 7 is homozygote for the β^{17} mutation as only 170-bp amplified products is seen. Lanes 1, 2, and 3 are heterozygote for the β^{17} mutation since both 170 and 190-bp amplified products are seen.

primer” for β^{17} ; 5'-ACC TGA CTC CTG AGG AGA AGA CTG CCG TTA CTG CCC TGT GGG ACA-3', 100 ng of “Mutant primer” for β^{17} ; 5'-TCT GCC GTT ACT GCC CTG TGG CAC-3', 10 mM Tris pH 8.8; 50 mM KCl and 1.5 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min, and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 170 and 190 bp indicate presence and absence of the β^{17} mutation, respectively (**Figure 23**).

Interpretation: Samples having only 170-bp amplified fragments are homozygote for β^{17} with genotype β^{17}/β^{17} . Samples having only 190-bp fragments are negative for the β^{17} with genotype β^A/β^A or β^T/β^T (A represents HbA; T represents other types of β -globin gene mutation). Samples having both 170 and 190-bp amplified products are heterozygote for the β^{17} with genotype of either β^{17}/β^A or β^{17}/β^T (A represents HbA; T represents other types of β -globin gene mutation).

7.1.3 MS-PCR for β^E mutation or HbE

Procedure: The 25- μ L PCR is performed containing 250 ng genomic DNA, 200 μ M of each dNTP; 0.5 units Taq DNA polymerase, 100 ng of “Common primer” for β^E ; 5'-GGC AGA GAG AGT CAG TGC CTA-3', 100 ng of “Normal primer” for β^E ; 5'-CGT GGA TGA AGT TGG TGG AG-3', 150 ng of “Mutant primer” for β^E ; 5'-CTG CCC TGT GGG CAA GGT GAA CGT GGA TGA AGT TGG TGG AA-3', 10 mM Tris pH 8.8; 50 mM KCl and 1.25 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 4 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 160 and 138 bp indicate presence and absence of the β^E mutation, respectively (**Figure 24**).

Interpretation: Samples having only 160-bp amplified fragments are homozygote for β^E with genotype β^E/β^E . Samples having only 138-bp fragments are negative for the β^E with genotype β^A/β^A or β^T/β^T (A represents HbA; T represents other types

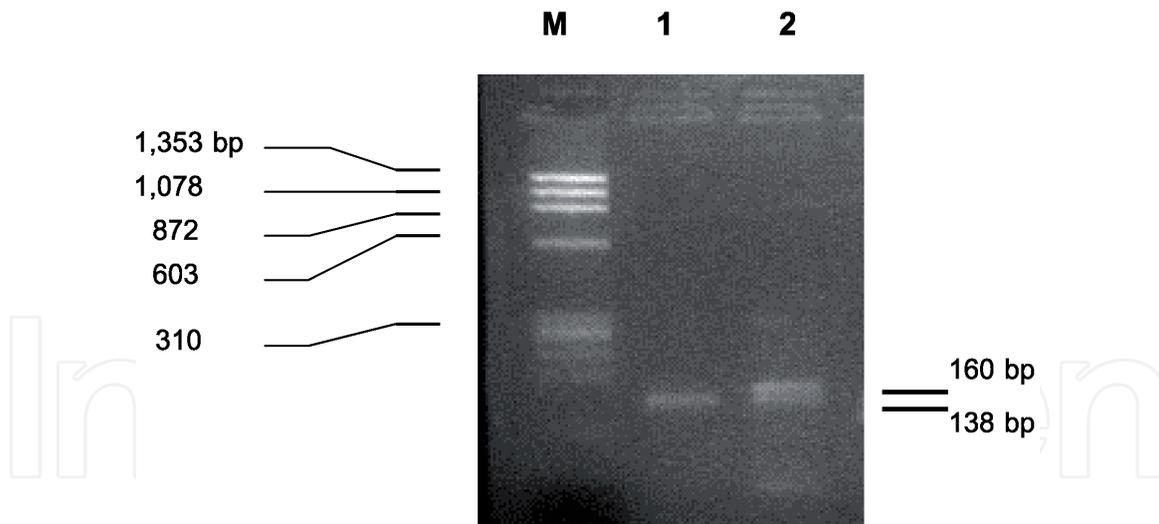


Figure 24.

MS-PCR for identifying β^E mutation. Lane 1 is negative for the β^E mutation as only 138-bp amplified products is seen. Lane 2 is heterozygote for β^E mutation as both 138-bp and 160-bp amplified products are seen.

of β -globin gene mutation). Samples having both 138 and 160-bp amplified products are heterozygote for the β^E with genotype of either β^E/β^A or β^E/β^T (A represents HbA; T represents other types of β -globin gene mutation).

8. Identification of β -thalassemia and HbE by ARMS-PCR

ARMS-PCR was established by Old et al. [79]. This technique also uses three oligonucleotide primers. However, the length of normal and mutant primers is similar. Therefore, size of normal and mutant amplified products is the same and cannot be separated in the agarose gel electrophoresis. Thus, two PCRs must be performed in the ARMS-PCR. Both PCRs have the same ingredients, except normal and mutant oligonucleotide primers are added in separated reaction tubes (M and N-tube). In addition, a pair of oligonucleotide primers specific to other gene must also be added into both PCRs. The amplified products obtained by this pair of primers are the internal control for the ARMS-PCR.

8.1 ARMS-PCR for $\beta^{41/42}$

Procedure: Two 25- μ L reactions are performed; M-reaction and N-reaction. Both M and N-reactions contain 150 ng genomic DNA, 200 μ M of each dNTP; 0.6 units Taq DNA polymerase, 0.2 μ M of “S-primer”; 5'-ACC TCA CCC TGT GGA GCC AC-3', 0.15 μ M of “M41/42 primer”; 5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3' (for M-reaction only), 0.15 μ M of “N41/42 primer”; 5'-GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA-3' (for N-reaction only), 0.2 μ M of “P1 primer”; 5'-GCG ATC TGG GCT CTG TGT TCT-3', 0.2 μ M of “P2 primer”; 5'-GTT CCC TGA GCC CCG ACA CG-3', 10 mM Tris pH 8.8; 50 mM KCl and 1.5 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 5 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 439 bp is the specific amplified products, and the PCR products sizing 314 bp are the control products (Figure 25).

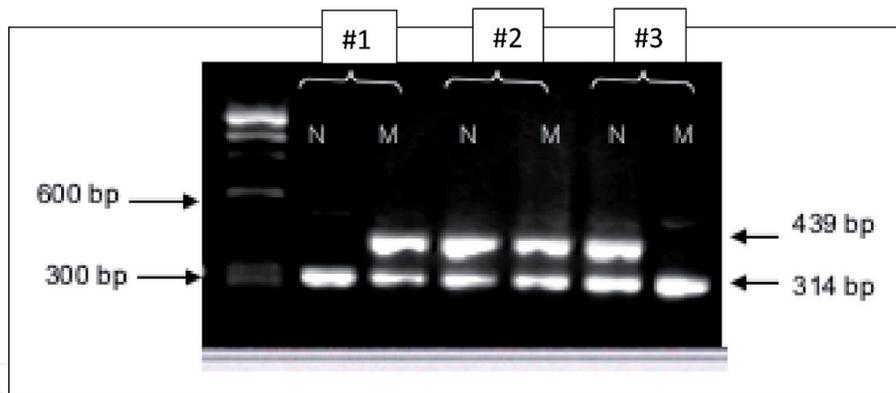


Figure 25.

ARMS-PCR for identifying the $\beta^{41/42}$ mutation. The 314-bp amplified products are internal control. The 439-bp amplified products are the $\beta^{41/42}$ specific products. Sample #1 is homozygote for $\beta^{41/42}$ mutation as the 439-bp amplified products are seen in only "M-reaction." Sample #3 is negative for the $\beta^{41/42}$ mutation since the 439-bp amplified products are seen in only "N-reaction." Sample #2 is heterozygote for the $\beta^{41/42}$ mutation since the 439-bp amplified products are seen in both "M-reaction" and "N-reaction."

Interpretation: Both M and N-reactions must have the 314-bp control products and the results can be read. If both M and N-reactions have 439-bp PCR products, the samples are heterozygote for $\beta^{41/42}$ with genotype of either $\beta^{41/42}/\beta^A$ or $\beta^{41/42}/\beta^T$ (A represents HbA; T represents other types of β -globin gene mutation). If the 439-bp PCR products are seen in only M-reaction, the samples are homozygote for $\beta^{41/42}$ with genotype $\beta^{41/42}/\beta^{41/42}$. If samples have the 439-bp PCR products in only N-reaction, the samples are negative for the $\beta^{41/42}$ with genotypes of either β^A/β^A or β^T/β^T (A represents HbA; T represents other types of β -globin gene mutation) (**Figure 25**).

8.2 ARMS-PCR for β^{17}

Procedure: Two 25- μ L reactions are performed; M-reaction and N-reaction. Both M and N-reactions contain 150 ng genomic DNA, 200 μ M of each dNTP; 0.6 units Taq DNA polymerase, 0.2 μ M of "S-primer"; 5'-ACC TCA CCC TGT GGA GCC AC-3', 0.15 μ M of "M17 primer"; 5'-CTC ACC ACC AAC TCA GCC ACG TTC AGC ATA-3' (for M-reaction only), 0.15 μ M of "N17 primer"; 5'-CTC ACC ACC AAC TTC ATC CAC GTT CAC ATT-3' (for N-reaction only), 0.2 μ M of "P1 primer"; 5'-GCG ATC TGG GCT CTG TGT TCT-3', 0.2 μ M of "P2 primer"; 5'-GTT CCC TGA GCC CCG ACA CG-3', 10 mM Tris pH 8.8; 50 mM KCl and 1.5 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 5 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 239 bp is the specific amplified products, and the PCR products sizing 314 bp are the control products (**Figure 26**).

Interpretation: Both M and N-reactions must have the 314-bp control products and the results can be read. If both M- and N-reactions have 239-bp PCR products, the samples are heterozygote for β^{17} with genotype of either β^{17}/β^A or β^{17}/β^T (A represents HbA; T represents other types of β -globin gene mutation). If the 239-bp PCR products are seen in only M-reaction, the samples are homozygote for β^{17} with genotype β^{17}/β^{17} . If samples have the 239-bp PCR products in only N-reaction, the samples are negative for the β^{17} with genotypes of either β^A/β^A or β^T/β^T (A represents HbA; T represents other types of β -globin gene mutation) (**Figure 26**).

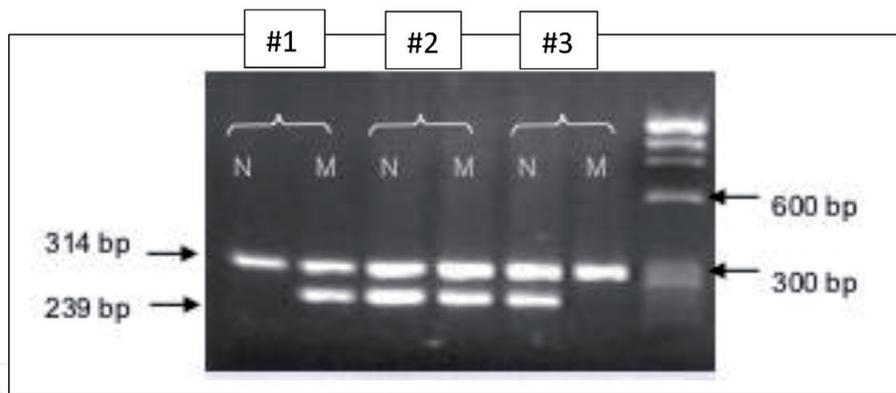


Figure 26. ARMS-PCR for identifying the $\beta^{\beta 7}$ mutation. The 314-bp amplified products are internal control. The 239-bp amplified products are the $\beta^{\beta 7}$ specific products. Sample #1 is homozygote for $\beta^{\beta 7}$ mutation as the 239-bp amplified products are seen in only “M-reaction.” Sample #3 is negative for the $\beta^{\beta 7}$ mutation since the 239-bp amplified products are seen in only “N-reaction.” Sample #2 is heterozygote for the $\beta^{\beta 7}$ mutation since the 239-bp amplified products are seen in both “M-reaction” and “N-reaction.”

8.3 ARMS-PCR for β^E

Procedure: Two 25- μ L reactions are performed; M-reaction and N-reaction. Both M and N-reactions contain 150 ng genomic DNA, 200 μ M of each dNTP; 0.6 units Taq DNA polymerase, 0.2 μ M of “S-primer”; 5’-ACC TCA CCC TGT GGA GCC AC-3’, 0.15 μ M of “HbE-M primer”; 5’-TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT-3’ (for M-reaction only), 0.15 μ M of “HbE-N primer”; 5’-TAA CCT TGA TAC CAA CCT GCC CAG GGC GTC-3’ (for N-reaction only), 0.2 μ M of “P1 primer”; 5’-GCG ATC TGG GCT CTG TGT TCT-3’, 0.2 μ M of “P2 primer”; 5’-GTT CCC TGA GCC CCG ACA CG-3’, 10 mM Tris pH 8.8; 50 mM KCl and 1.5 mM of $MgCl_2$.

Thermal cycles: A total of 35 thermal cycles was carried out with each cycle comprising DNA denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min and primer extension at 72°C for 1 min; the initial denaturation was extended to 5 min while the final extension was prolonged to 5 min.

Detection of amplified products: The amplified products were separated in 2.5% agarose gel electrophoresis at 120 V for 15–20 min before visualizing with a UV-transilluminator. The fragments sizing 260 bp is the specific amplified products, and the PCR products sizing 314 bp are the control products (Figure 27).

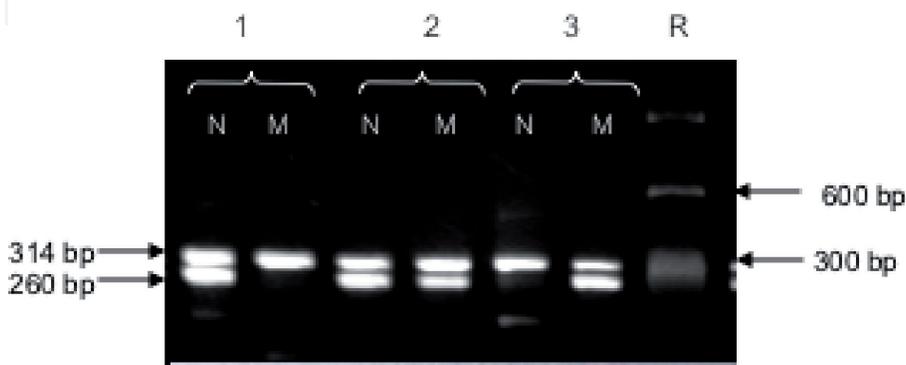


Figure 27. ARMS-PCR for identifying β^E mutation. The 314-bp amplified products are internal control. The 260-bp amplified products are the β^E specific products. Sample #3 is homozygote for β^E mutation as the 260-bp amplified products are seen in only “M-reaction.” Sample #1 is negative for the β^E mutation since the 260-bp amplified products are seen in only “N-reaction.” Sample #2 is heterozygote for the β^E mutation since the 260-bp amplified products are seen in both “M-reaction” and “N-reaction.”

Interpretation: Both M and N-reactions must have the 314-bp control products and the results can be read. If both M- and N-reactions have 260-bp PCR products, the samples are heterozygote for β^E with genotype of either β^E/β^A or β^E/β^T (A represents HbA; T represents other types of β -globin gene mutation). If the 260-bp PCR products are seen in only M-reaction, the samples are homozygote for β^E with genotype β^E/β^E . If samples have the 260-bp PCR products in only N-reaction, the samples are negative for the β^E with genotypes of either β^A/β^A or β^T/β^T (A represents HbA; T represents other types of β -globin gene mutation) (**Figure 27**).

9. Identification of β -thalassemia and HbE by MAS-PCR

This PCR technique was established by author and named multiplex allele specific (MAS)-PCR [29]. MAS was intended to be used for identifying $\beta^{\text{Thalassemia}}$ and β^E -mutations in samples that have already been diagnosed to be β -thalassemia carrier, HbE carrier, and SEA- α thalassemia 1 carrier. Therefore, only mutant primers are put together in this MAS-PCR. However, the internal control of this PCR protocol is the amplified products generated by the normal pair of primers for α -globin gene cluster.

Procedure: The PCR was performed in a total volume of 25 μL containing 1.1–1.4 ng genomic DNA, 140 μM dNTPs, 0.1 unit/ μL of DNA polymerase, 0.25 ng/ μL “Beta-common-multiplex” primer (5'-AAG AGC CAA GGA CAG GTA CGG CTG T-3'), 0.125 ng/ μL “Beta-17-multiplex” primer (5'-CCA ACT TCA TCC ACG TTC ACG TA-3'), 0.125 ng/ μL “Beta cds-41/42-multiplex” primer (5'-AGA TCC CCA AAG GAC TCA ACC T-3'), 0.125 ng/ μL “Beta-E-multiplex” primer (5'-CGT ACC AAC CTG CCC AGG GCC AT-3'), 25 ng/ μL “SEA-1-multiplex” primer (5'-TGA CTC CAA TAA ATG GAT GAG GA-3'), 0.25 ng/ μL “SEA-2-multiplex” primer (5'-GCC TGC GCC GGG GAA CGT AAC CA-3') and 0.5 ng/ μL “SEA-3-multiplex” primer (5'-CGC CAA AGA TGG CTA CTC GGA GA-3') in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.0% DMSO and 2.0 mM MgCl_2 .

Thermal cycles: A total of 37 thermal cycles were carried out with each cycle comprising denaturation at 95°C for 1 min, primer annealing at 62°C for 30 s, and primer extension at 72°C for 30 s. Initial denaturation was extended to 5 min and final extension was prolonged to 7 min.

Detection of amplified products: The PCR products were separated via 2.0% agarose gel electrophoresis and visualized by the UV-transilluminator (**Figure 28**).

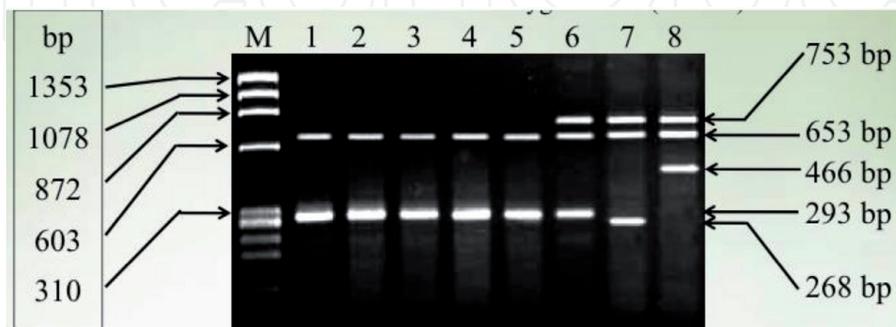


Figure 28.

MAS-PCR for identifying β -thalassemia mutation, β^E -mutation, and SEA- α thalassemia 1 mutation. The 653-bp amplified products are the internal control generated by the α -globin gene cluster specific oligonucleotide primers. Lanes 1–5 are positive for β^E mutation as the 293-bp amplified products are seen. Lane 6 is positive for both SEA- α thalassemia 1 and β^E mutations as both 753-bp amplified products and 293-bp amplified products are seen. Lane 7 is positive for both SEA- α thalassemia 1 and β^{E7} mutation as both 753-bp amplified products and 268-bp amplified products are seen. Finally, lane 8 is positive for SEA- α thalassemia 1 and $\beta^{41/42}$ mutation as both 753-bp amplified products and 466-bp amplified products are seen (modified from [82]).

Interpretation: All reactions must have the 653-bp control products. Presence of 753-bp product indicates presence of the SEA- α thalassemia 1. Presence of the 466-bp, 293-bp, and 268-bp products indicates presence of $\beta^{41/42}$, β^E , and β^{17} , respectively (**Figure 28**).

10. Pitfalls in laboratory diagnosis of β -thalassemia and HbE

There are several pitfalls to be concerned when using laboratory data in diagnosis of β -thalassemia and HbE. The pitfalls are in both screening and confirmatory steps of laboratory diagnosis.

HbE tube test relies on concentration of NaCl. Therefore, exact amount of NaCl in the reagent must be prepared following the suggested ingredient. False positive results will be obtained if NaCl concentration is too high, and *vice versa*.

HbE screen test relies on pH-based microcolumn chromatography. Therefore, exact pH of buffer in the test kit must also be prepared. Too high pH causes falsely negative results and *vice versa*.

10.1 Pitfalls in screening tests for β -thalassemia and HbE carriers

- 1. Pitfalls in one-tube osmotic fragility test (OFT).** The concentration of reagent must be exactly 0.36 and 0.45%, otherwise false positive results will be obtained if concentration is over 0.36 or over 0.45%. In contrast, false negative results will be obtained if concentration is less than 0.36 or 0.45%. Anemic blood samples will also give positive results. Therefore, Hb/Hct must also be checked if the results are positive. Not all HbE carriers have positive OFT results. Therefore, blood samples having negative OFT results must also be sent for HbE screen.
- 2. Pitfalls in MCV and MCH evaluation.** MCV/MCH are the numeric data that must be obtained from automated blood cell counters that have good quality control. MCV must be obtained using fresh blood, but MCH may be obtained using 1 week-old blood. MCV is directly measured in automated blood counter, while MCH is generated by calculation. Thus, if blood samples have high degree of variation of red blood cell sizes or anisocytosis, false MCV values may be obtained. This situation may be found in β -thalassemia carriers or HbE carriers with co-existence iron deficiency anemia during treatment. Not all HbE carriers have MCV/MCH values below cutoff points. Therefore, all blood samples sent for MCV/MCH determination must also be sent for HbE screen. Most importantly, some blood samples may have discordant MCV-OFT results. It is then highly recommended to perform both OFT and MCV/MCH for screening for β -thalassemia and HbE carriers.
- 3. Pitfalls in HbE screening test.** DCIP test is based on using the oxidizing reagent; dichlorophenolindophenol. This reagent can be reduced over long storage and the screening results will be falsely negative. Therefore, if color of the DCIP reagent turns deeply blue, it should not be used. HbH also denatured in the DCIP reagent. Therefore, positive samples must be checked if they are HbH disease.

10.2 Pitfalls in confirmatory tests for β -thalassemia and HbE

Normal hemoglobin type depends on age of the patients. Thus, reading and interpreting the hemoglobin typing results by all methodologies, age of the patients must be taken into account. In addition, transfused blood interferes the real

hemoglobin typing results of the patients. If the patients of β -thalassemia disease (β^0/β^0) have just received blood transfusion, their hemoglobin types will be A_2AF , instead of A_2F which is the real hemoglobin type of the β^0/β^0 . If patients of HbE/ β^0 -thalassemia (β^E/β^0) have recent blood transfusion, their hemoglobin types will be EFA , instead of EF which is the real hemoglobin type of β^0/β^E . Therefore, recent blood transfusion should always be taken into account when reading and interpreting the hemoglobin typing results.

Co-existence of α -thalassemia 1 in β -thalassemia carrier and in HbE carrier can be found some regions. This is called double α -thalassemia 1/ β -thalassemia carriers and double α -thalassemia 1 and HbE carriers. Results of one-tube osmotic fragility test are always positive in double α -thalassemia 1/ β -thalassemia carriers, being the same as that of the single β -thalassemia carrier. However, MCV/MCH in double α -thalassemia 1/ β -thalassemia carriers are slightly higher than those in the single β -thalassemia carriers (70.7 ± 2.6 vs. 68.4 ± 2.7 for MCV, 21.0 ± 1.2 vs. 20.7 ± 0.9 for MCH) [83]. HbA₂ level in double α -thalassemia 1/ β -thalassemia carriers is lower than that in single β -thalassemia carrier ($5.27 \pm 0.77\%$ vs. $5.65 \pm 0.78\%$) [29]. In contrast, the results of one-tube osmotic fragility test of double α -thalassemia 1/HbE carrier are usually positive, being different from those of single HbE which can be either positive or negative. The MCV/MCH in double α -thalassemia 1/HbE carrier are lower than those of the single HbE carrier (68.7 ± 8.9 fL vs. 75.7 ± 7.5 fL for MCV, 22.5 ± 3.1 pg vs. 24.9 ± 2.8 pg for MCH). HbE level in double α -thalassemia 1/HbE carrier are lower than those of the single HbE carrier ($22.8 \pm 4.4\%$ vs. $27.6 \pm 3.3\%$) [84]. Therefore, if HbE carriers have low HbE levels as well as MCV/MCH, further identification of α -thalassemia 1 genotype must be done.

11. Conclusions

Diagnosis of β -thalassemia and HbE requires laboratory investigations. Screening tests are used to search for carriers, while confirmatory tests are carried out in those samples positive for screening tests with the aim to make the definite diagnosis. There are several pitfalls in these laboratory tests, both in screening tests and confirmatory tests that must not be overlooked, otherwise misdiagnosis will occur.

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Conflict of interest

The authors declare no conflict of interest.

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