Laboratory Diagnosis of Thalassaemia and Abnormal Haemoglobins

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Disorders of Hemoglobin Synthesis

1. Decreased or absence of globin chain synthesis of hemoglobin: quantitative disorder
   - Thalassemia

2. Synthesis of an abnormal globin chain (amino acid change): qualitative disorder
   - Abnormal Hb (Hb Variant)

3. Synthesis of an abnormal globin chain with reduced synthesis of the Hb variant: quantitative & qualitative disorder
   - Thalassemia-hemoglobinopathy

4. Persistence in the synthesis of γ-globin chain
   - Hereditary Persistence of Fetal Hemoglobin (HPFH)

LABORATORY DIAGNOSIS OF THALASSEMA

- RBC Indices
- RBC Osmotic Fragility
- RBC Morphology
- Intraerythrocytic Inclusion Bodies
- Hb Typing
- Hb A2 Quantitation
- Hb F Quantitation
- Acid Elution Staining
- Serum Ferritin
- Globin Chain Synthesis
- DNA Analysis
- Family Study

SCREENING FOR THALASSEMA

- One tube osmotic fragility (OF) test or MCV/MCH
- Dichlorophenolindophenol (DCIP) precipitation test
- Detection of Hb Bart’s

RED CELL OSMOTIC FRAGILITY TEST

Normal vs. β-thalassemia/HbE disease

SIMPLE OSMOTIC FRAGILITY TESTS
Red Blood Cell Indices

- Mean Corpuscular Volume (MCV) 87±5 fL
- Mean Corpuscular Hemoglobin (MCH) 29±2 pg
- Mean Corpuscular Hemoglobin Concentration (MCHC) 34±2 %

Mean Corpuscular Volume (MCV)

<table>
<thead>
<tr>
<th></th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>88±6</td>
<td>29±2</td>
</tr>
<tr>
<td>α-Thal 2 trait</td>
<td>83±7</td>
<td>27±3</td>
</tr>
<tr>
<td>α-Thal 1 trait</td>
<td>68±5</td>
<td>23±5</td>
</tr>
<tr>
<td>Homozygous α-Thal 2</td>
<td>74±3</td>
<td>22±1</td>
</tr>
<tr>
<td>β-Thal trait</td>
<td>64±5</td>
<td>20±3</td>
</tr>
<tr>
<td>Hb E trait</td>
<td>81±5</td>
<td>26±2</td>
</tr>
<tr>
<td>Hb E with α-Thal 1 trait</td>
<td>66±10</td>
<td>23±9</td>
</tr>
<tr>
<td>Homozygous HbE</td>
<td>65±3</td>
<td>20±1</td>
</tr>
</tbody>
</table>

Dichlorophenolindophenol (DCIP) precipitation test

Hb E (α₂β₂(Glu→Lys)) เป็นฮีโมโกลบินผิดปกติที่เกิดจากกรดอะมิโนต่างจาก 26 ในสาย β-globin เปลี่ยนจาก glutamic เป็น lysine ทำให้การรวมตัวของสาย α-globin และ β-globin ไม่เสถียรและแพร่รูปรูป sulphydryl (-SH) อิสระออกมา จึงถูก oxidized ด้วยสาร DCIP ได้ง่ายและเร็วกว่าฮีโมโกลบินชนิดอื่นและตกตะกอนลงมา

Immunodetection of Hb Bart’s

Immunodetection of Hb Bart’s

Screening for α-thalassemia

- Positive for: α-thalassemia 1 heterozygote, α-thalassemia 2 homozygote, Hb H disease, EA Bart’s disease and Hb Bart’s hydrops fetalis
**Routine Laboratory Diagnosis of Thalassemia**

- Hb Concentration
- RBC Indices and Morphology
- Hb Typing
- Hb F and A2 Quantitation
- RBC Inclusion Bodies

**MCV or One tube osmotic fragility**

<table>
<thead>
<tr>
<th>Normal</th>
<th>Decrease</th>
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</thead>
<tbody>
<tr>
<td>DCIP</td>
<td>DCIP</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dx : Normal</td>
<td>α-Thal 1 trait</td>
</tr>
<tr>
<td>α-Thal 2 trait</td>
<td>Hb Bart’s</td>
</tr>
<tr>
<td>Hb CS trait</td>
<td>Fe deficiency</td>
</tr>
</tbody>
</table>

**Repeat study after iron supplementation x 3 mo.**

**Hemoglobin Type by Starch Gel Electrophoresis**

**Cellulose acetate electrophoresis**

- B/E disease
- homo E
- E trait
- homo E
- B trait
- E trait
- B/E disease

**Hb analysis and quantification**

- Cellulose acetate electrophoresis and elution
- Microcolumn chromatography
- Automated HPLC, LPLC
- Capillary electrophoresis
Variant Hb testing system

- Automated high performance liquid chromatography system (HPLC) improves resolution quality (sharp peaks)
- Cation exchange analytical cartridge (30 mm, ID 4.6mm) / particle size ~ 6-9 microns
- 5 μL of blood only. Blood stable for testing ~30 days
- Fast analysis intervals of 6.5 minutes

Principle: HPLC as a Hb testing system

1. Hemoglobins in the hemolysate are adsorbed on a resin of anionic or cationic particles column (stationary phase).
2. Phosphate buffer at different concentration (mobile phase) pass under pressure (either high or low) through the ionic exchange column.
3. Due to the buffer gradient of increasing ionic strength and pH, Hbs are separated according to their ionic interaction with the stationary phase.
4. Separated Hbs pass through the flow cell of photometer where changes in the absorbance at 415 nm are measured.
5. The assays provide quantitative results for percent HbA2 and HbF, while detecting the most commonly occurring abnormal hemoglobins.

RT (retention time) values

Retention time is measured from the time of sample injection to maximum point of each elution peak.

Windows are the time ranges in which Hbs are eluted.
- Common Hbs:  F, A, A2
- Abnormal Hbs:  S, C, D

Interpretation of results: RT

Hb variants have specific RT values:

- F: 1.15 (1.00-1.30) mins
- F2: 1.45 (1.30-1.66) mins
- F3: 1.70 (1.60-1.80) mins
- A0: 2.60 (2.20-3.30) mins
- A2 (E): 3.83 (3.68-3.98) mins
- D-window: 4.99 (4.39-4.42) mins
- S-window: 4.27 (4.12-4.42) mins
- C-window: 5.03 (4.88-5.18) mins
Capillary Electrophoresis Principle

The Capillary® system uses the principle of capillary electrophoresis in free solution, charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH.

- Separation occurs according to the electrolyte pH and electro-osmotic flow, from cathode to anode.
- Hemoglobins were separated within 4 minutes, according to isoelectric point, by migration in an electric field in the liquid phase. Direct detection of the hemoglobin is made at an absorbance wavelength of 415 nm at their cathodic end.

**α-Thalassemia**

<table>
<thead>
<tr>
<th>Normal Hb Type</th>
<th>α-Thalassemia 2</th>
<th>α-Thalassemia 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A=α₂β₂</td>
<td>HbA₂=α₂δ₂</td>
<td>Hb A=α₂β₂</td>
</tr>
<tr>
<td>Hb F=α₂γ₂</td>
<td>HbF=α₂γ₂</td>
<td>Hb F=α₂γ₂</td>
</tr>
</tbody>
</table>

**α-Globin Genotype**

- Normal α-Globin Genotype
- α-Thalassemia 2
- α-Thalassemia 1

**α-Globin Gene Deletions**

- Normal
- α-Thalassemia 2
- α-Thalassemia 1
**α - Thalassemia 1 Trait**
- Normal Hb level
- Low MCV (~70 fl)
- Hb type A₂+A
- Normal or slightly decreased Hb A₂
- DNA analysis
- Obligatory cases of Hb Bart’s hydrops fetalis

**β - Thalassemia**

<table>
<thead>
<tr>
<th>Hb Type</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A²=HbA</td>
<td>Normal</td>
</tr>
<tr>
<td>β²=HbH</td>
<td>Increased Hb A₂ (4-6%)</td>
</tr>
<tr>
<td>γ²=HbBart’s</td>
<td></td>
</tr>
</tbody>
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**Beta-Thalassemia Trait**

- **β-Thalassemia trait**
  - Normal Hb level
  - Low MCV (~65 fl)
  - Hb type A₂+A
  - Increased Hb A₂ (~4-5%)

**Hb E heterozygote**

**Homozygous Hb E**
Comparison of hemoglobins A₂ and E levels among capillary electrophoresis, automated HPLC and elution techniques

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<td>Hb E trait</td>
<td>3.5±0.4</td>
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<td>27.8±7.5</td>
<td>29.4±2.3</td>
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<td>4.0±0.3</td>
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<td>21.9±6.6</td>
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<td>β-Thal/Hb E</td>
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Hemoglobin H Disease

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Hb H –Cs Disease

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Intraerythrocytic Inclusion Bodies

Hb Q (α 74 Asp- His)–H Disease

- Hb: 9.5 g/dl
- RBC: 4.83×10¹²/L
- HCT: 30.5 %
- MCV: 63 fl
- MCH: 19.6 pg
- MCHC: 31 g/dl
  - Hb A₂: 1.8%
  - Hb Q (RT 4.66): 96.4%
  - Hb Bart’s: positive
  - DNA studies

Hb Q (α 74 Asp- His) trait

- Hb: 11.9 g/dl
- RBC: 4.99×10¹²/L
- HCT: 35.5 %
- MCV: 71 fl
- MCH: 23.8 pg
- MCHC: 33 g/dl
  - Hb A₂: 2.5%
  - Hb Q (RT 4.65): 30.9%
  - Hb A₂: 2.5%
  - DNA studies: α⁻²⁴αα
β-Thalassemia / Hb E

- Hb: 4-10 g/dl
- RBC indices: MCV: 75 fl, MCH: 25 pg, MCHC: < 30 g/dl
- RDW: increased
- Reticulocyte: increased
- S.ferritin: normal or increased
- Dichlorphenol- indophenol (DCIP) test: positive
- Hb E: 30-70%

Hb CS E A Bart’s disease

Effects of α-thalassemia to Hb E levels

- Hb E trait: 25-30%
- Hb E α-thal 2: 25-30%
- Hb E α-thal 1: 19-23%
- Hb E + Hb H: 13-18% (EA Bart’s disease)

Comparison of hemoglobins A₂ and E levels among capillary electrophoresis, automated HPLC and elution techniques

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<tr>
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<th>Hb A₂-Hb E/Eletion</th>
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<td>21.3±0.6</td>
<td>20.7±1.2</td>
</tr>
<tr>
<td>EA Bart’s</td>
<td>3.7±0.2</td>
<td>11.8±0.7</td>
<td>14.9±1.6</td>
<td>13.0±2.1</td>
</tr>
<tr>
<td>CS EA Bart’s</td>
<td>2.2±0.2</td>
<td>12.6±0.8</td>
<td>16.8±0.8</td>
<td>13.9±1.8</td>
</tr>
<tr>
<td>Hb E homozygote</td>
<td>4.1±0.8</td>
<td>92.9±3.3</td>
<td>98.2±4.9</td>
<td>87.7±5.9</td>
</tr>
<tr>
<td>β-Thal/Hb E</td>
<td>4.9±1.6</td>
<td>50.3±13.8</td>
<td>59.4±12.9</td>
<td>56.3±13.6</td>
</tr>
</tbody>
</table>

Problem Case

CS EF Bart’s disease or Homozygous Hb E or β-thal/Hb E disease

- Clinical data
- Rbc morphology
- Family study
- DNA analysis
**Problem Case**

- Hb 4-5 g/dl
  - β-thal/HbE
  - blood transfusion

- Hb 8-10 g/dl
  - no transfusion
  - β-thal/HbE
  - Hb F ~ 3-10%
  - Hb E ~ 60%

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**Hb E/β+-thalassemia vs Hb E/Hb Malay**

- Hb: 10 g/dl
- RBC: 5.3X10^12/L
- Hct: 31%
- MCV: 62 fl
- MCH: 19 pg
- MCHC: 32 g/dl
- Hb E+ Hb A2: 58.9%
- Hb F: 5.4%
- Hb A (Hb Malay): 26.5%
- Parents: Hb Malay and Hb E trait
- DNA studies

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**Hb E/Hb Dhonburi (β126 Val-Gly)**

- Hb: 12.1 g/dl
- RBC: 5.8X10^12/L
- Hct: 44%
- MCV: 77 fl
- MCH: 21 pg
- MCHC: 27 g/dl

Hb type
- E +“A”

- Hb E+ Hb A2: 38.3%
- Hb F: 0.8%
- Hb Dhonburi (“A”) 42.6%

DNA studies

- Triton xylene gel: the abnormal fraction migrates more rapidly than β+-chain

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**Increased hemoglobin F**

* Acquired:
  - Physiology: pregnancy (HbF ≥ 3-7%)
  - Recovery from marrow hypoplasia: after BM transplant, chemotherapy
  - Fetal erythropoiesis: juvenile chronic myeloid leukemia
  - Miscellaneous: aplastic anemia, PNH, leukemia, hepatoma

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**Hemoglobin F**

Normal adult: Hb F ≤ 1%

Increased Hemoglobin F

* Genetic: Thalassemia
  - Hereditary persistence of fetal hemoglobin (HPFH)
  - Pancellular
  - Heterocellular
Hereditary Persistence of Fetal Hemoglobin

Hb F-like Variants (by LPLC): Hb Hope

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age (yrs)</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>Hb typing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband</td>
<td>30</td>
<td>11.3</td>
<td>32.4</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>Mother</td>
<td>10.0</td>
<td>50.0</td>
<td>88</td>
<td>24.6</td>
<td>0</td>
</tr>
</tbody>
</table>

HPFH? Negative for F-cell analysis by Flow cytometry and alkaline F = 1.1%

F - Cell Staining by Acid Elution Technique

Red Cell Indices

- **Normocyte**
  - Hb Analysis
  - A₂ + A
  - A-α-thal 2
  - Normal
  - Hb Constraint
  - E ~ 25-30%
  - Hb E trait
  - α-thal 2/Hb E
  - E < 10 g/dL
  - Hb E with Fe deficiency
  - Hb Bart's

- **Microcyte**
  - Hb Analysis
  - A₂ + A
  - A₂ + CS
  - A₂ + E
  - EE
  - Thalassemic disease (specified by Hb types)
  - Hb E Determination
  - E 21% or less
  - A₂ < 3.5%
  - Hb Bart's
  - Hb Bart's
  - Hb > 10 g/dL
  - Hb > 10 g/dL
  - Hb > 10 g/dL
  - Hb Bart's
  - α-thal 1 trait
  - Fe deficiency or Fe deficiency on top of thalassemia trait
  - Homo α-thal 2

F - Cell Staining by Acid Elution Technique

F-cell

A-cell
THALASSEMIA DIAGNOSIS

- **PRENATAL**
  Blood or fetal tissues
- **NEONATAL**
  Cord blood
- **POSTNATAL**
  Peripheral blood

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Normal Cord Blood

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Cord blood: Hb E trait

- **Homozygous Hb E**
- **β-Thalassemia/Hb E**

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Hb E in Cord Blood

<table>
<thead>
<tr>
<th></th>
<th>%Hb E</th>
<th>%Hb A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb E trait</td>
<td>4.5±1.56</td>
<td>10.1±3.52</td>
</tr>
<tr>
<td>Homozygous E</td>
<td>8.0±3.55</td>
<td>0</td>
</tr>
<tr>
<td>β-Thal/Hb E</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

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Hb Bart’s Hydrops Fetalis
**HEMOGLOBINOPATHIES IN THAILAND (1)**

**1. HEMOGLOBIN VARIANTS**

**1.1 COMMON**
- Hb E: β 26 Glu-Lys
- Hb Constant Spring: elongated α-chain

**1.2 RARE ALPHA MUTANTS**
- Anantharaj: α 11 Lys-Glu
- Queens: α 34 Leu-Arg
- J-Buda: α 61 Lys-Asn
- Suan Dok: α 109 Leu-Arg

**HEMOGLOBINOPATHIES IN THAILAND (2)**

**1.3 RARE BETA MUTANTS**
- Hb C: β 6 Glu-Lys
- Malay: β 19 Asn-Ser
- J-Bangkok: β 56 Gly-Asp
- New York: β 113 Val-Glu
- Dhonburi: β 126 Val-Gly
- Srinakarin: β 117 Val-Glu
- Siriraj: α 7 Glu-Lys
- G-Hsin Chu: β 22 Glu-Ala
- Pyrgos: β 83 Gly-Asp
- D-Punjab: β 121 Glu-Gln
- Tak: elongated β-chain
### RT Values for Hb Variants

- **pH 8.5**
  - A2 (3.6)
  - Tak (4.3)
  - D Punjab (4.2)
  - Montgomery (4.6)
  - Q-Malcolm (4.6)
  - C (5.0)
  - O-Arab (4.8)
  - D-Iran (3.6)
  - Hb Malay (2.4)
  - Hb Dhonburi (1.9)
  - Hb Tak (1.8)
  - Hb Hope (1.38)
  - Pyrgos (1.38)
  - New York (1.4)
  - J Bangkok (1.9)
  - Bart’s (0.2)

### Abnormal Hbs

- **Hb G-Coushatta**
  - $\alpha_2$ Glu-Ala

- **Hb J-Buda**
  - $\alpha_1$ Lys-Asn

- **Hb Siam**
  - $\alpha_1$ Gly-Arg

- **Hb Hope**
  - $\beta_136$ Gly->Asp

- **Abnormal Hb at P2**

- **Hb D/Hb E**

- **Hb Tak / Hb E**

### Hb Tak (β147 +AC) trait
- Hb: 16.6 g/dl
- RBC: 5.6x10^12/L
- Hct: 48%
- MCV: 87 fl
- MCH: 30 pg
- MCHC: 34 g/dl
- RDW: 12.3
- Hb F: 64.4% RT 1.11
- Hb A2: 55.7% 2.46
- Hb A: 4.0% 3.6
- Hb Tak: 34.4% 4.23

### Hb E/Hb Tak
- Hb: 13.7 g/dl
- RBC: 5.7x10^12/L
- Hct: 48%
- MCV: 87 fl
- MCH: 30 pg
- MCHC: 34 g/dl
- RDW: 15.8%
- Hb E: Hb A2: 47.3%
- Hb F: 1.9%
- Hb Tak: 46.4%
- DNA studies
**β₀-thalassemia**
- Hb: 20.4 g/dl
- RBC: 8.2 X 10¹²/L
- Hct: 59%
- MCV: 72 fl
- MCH: 25 pg
- MCHC: 35 g/dl
- MCV: 72 fl
- Hb A₂: 5.2%
- Hb F: 5.5%
- Hb Tak: 85.1%
- DNA studies

**Erythrocytosis**

**Problem:**

**Hb E vs Hb Lepore or Hb D-Iran**

HPLC: Hb Lepore has same RT value as Hb A₂ and Hb E, Hb D-Iran

In heterozygote:
- Hb Lepore + Hb A₂: 8-15%
- Hb E α-thalassemia: Hb E + Hb A₂ <25%
- Hb E + Hb A₂: 25-30%
- Hb D-Iran + Hb A₂: 35-45%

**Hb Lepore trait**
- Hb: 14.0 g/dl
- RBC: 6.48 X 10¹²/L
- Hct: 39%
- MCV: 72 fl
- MCH: 22 pg
- MCHC: 30 g/dl
- RDW: 13.7%
- Mild hypochromia, microcytosis, target cells
- Phenotype: thalassemia trait

- Analyte  %  Time
  - F  2.2  1.11
  - A₂  75.4  2.49
  - Hb Lepore + A₂  13.2  3.6

**Homozygous Hb Lepore**

- Hb: 8.0 g/dl
- RBC: 3.05 X 10¹²/L
- MCV: 78 fl
- MCH: 26 pg
- MCHC: 33 g/dl
- RDW: 29
- Hb F: 86%
- Hb Lepore: 10%
- No Hb A

**Hb E/β₀-thalassemia vs Hb E/Hb Lepore**
- Hb: 11.4 g/dl
- RBC: 4.91 X 10¹²/L
- Hct: 34%
- MCV: 70 fl
- MCH: 23 pg
- MCHC: 33 g/dl
- Reticulocyte: 2.4%
- HPLC Hb E + Lepore: 67.6
  - Hb E: 53%
  - Hb A₂: 53%
  - Hb F: 30%
  - Hb Lepore: 12.7%
- Parents: Hb E and Hb Lepore trait
- DNA studies