# CHAPTER 2

# RESEARCH DESIGN AND METHODS

## 2.1 Research design for detection of α-thalassemia 2 : rightward type ($-\alpha^{3.7}$)

According that α- thalassemia 2 : rightward type involves a deletion of 3.7 kb of DNA between the alpha 1 and alpha 2 globin gene and some part of both gene. The method of polymerase chain reaction (PCR) was applied to amplify on this area. The location of the amplification primers covers the promoter of α 2 to the UTR of α 1 for 3.7 kb deletion, another pair of primers was designed to cover the complete α 2 gene only to show the normal α 2 gene ( no deletion ). The PCR product for 3.7 kb deletion and normal allele are 1.76 kb . The hematological characteristics ; MCV, hemoglobin concentration, osmotic fragility, hemoglobin F inclusion body and Hb typing were also analyzed in this study.

The method of each procedure was given in detail later in this chapter. The diagram below shows the whole procedure orderly.

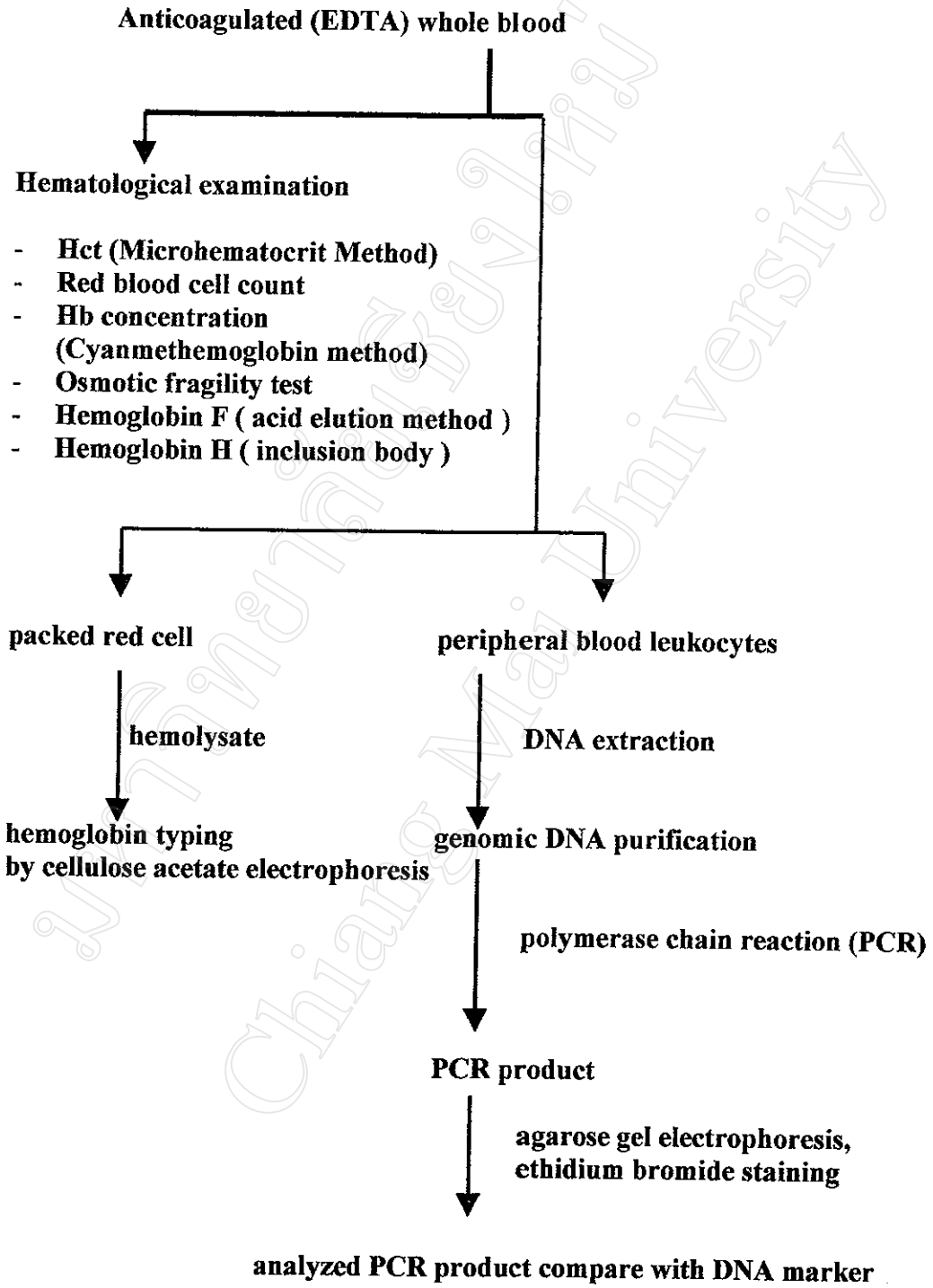


**Figure 8.** Schematic representation of the procedure in this research

## 2.2 Methods

### 2.2.1 Samples

### Blood samples

The peripheral venous blood were collected from 200 blood donors of Blood Bank , 100 samples from pregnant women from Maharaj Nakorn Chiang Mai Hospital, and 100 samples from Chiang Mai university's students. All subjects lived in Northern part of Thailand. There were 218 samples from Chiang Mai, 35 from Lampoon, 31 Lampang, 22 from Chiang Rai, 18 from Phitsanulok, 17 from Naan, 15 from Phare, 14 from Nakorn Sawan, 10 from Payao, 8 from Uttraradit, 5 from Khampangpech, 2 from Tak, and one each from Sukhothai and Uthaitanee. The 6 ml of whole blood were mixed with ethylenediaminetetra-acetic acid (EDTA) by inverting the tube four or five times then kept in refrigerator until used.

### Genomic DNA Preparation

Genomic DNA preparation was modified from the method described by Miller et al.(1988). The first step was to separate nucleated cells from 6 ml. of the whole blood. White blood cell, the major nucleated cells in blood, were kept from the buffy coat between plasma and packed red cells after centrifugation the whole blood at 3,000 rpm for 5 minutes. Then they were washed with 12 ml of Red Cell Lysis Buffer in order to get rid of the intact red blood cells. The mixture was subsequently centrifuged at 1,000 rpm for 10 minutes and the supernatant was discarded. The step was repeated three times to ensure the purity of the cells. The pellet was resuspending in 8 ml Nuclei Lysis Buffer then the white blood cells were lysed. After Added with 0.5 ml of 10 % SDS, the mixture become viscous then added with 0.5 ml of proteinase K solution and incubated in shaking water bath at 55° C overnight. The mixture was then taken another step of protein separation. The digested protein was separated by salting out with 5 M. NaCl at

the ratio of 1 to 4 volumes of the mixture. To complete protein precipitation, the mixture was kept at 4° C for 20 minutes. The mixture was added two volume of ice - cold absolute ethanol. Invert the tube immediately, DNA was then hooked out, rinsed with 70 % ethanol to wash the excess salt, and kept dry at room temperature for 60 minutes. Finally, the DNA was dissolved in 0.5 ml Tris - EDTA (TE) buffer and the concentration was determined.

The concentration of the complete dissolved DNA was determined by measuring the optical density. An OD at wavelength of 260 nm corresponds to approximately 50 ug / ml for double - stranded DNA. The obtained DNA was diluted to 1 % solution in TE buffer before measuring. The equation for calculate the DNA concentration shows as follow.

**DNA concentration ( μg / ml ) = OD 260 x 50 x dilution factor**

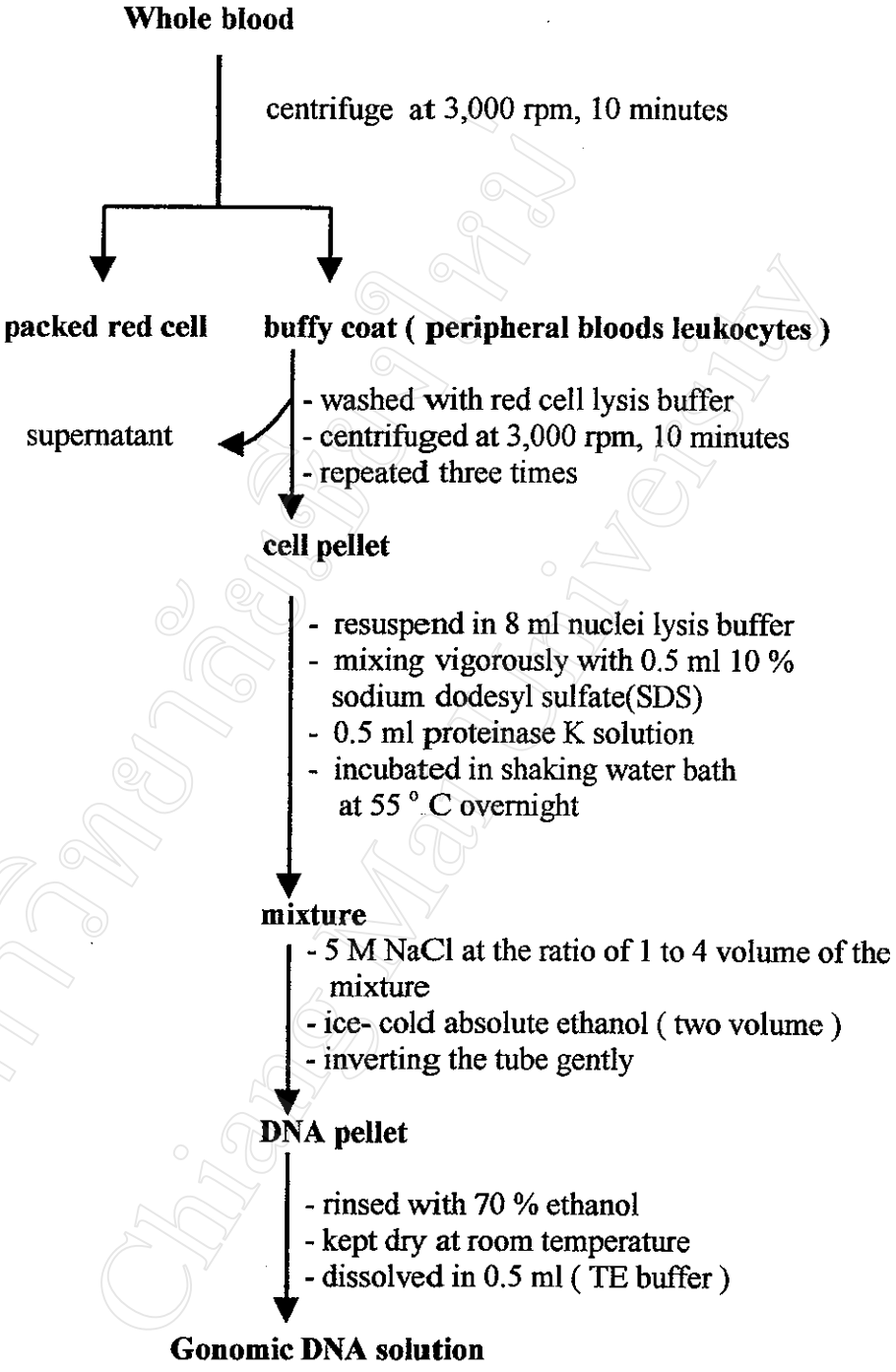


**Figure 9. Schematic representation of genomic DNA preparation.**

## 2.2.3 Polymerase chain reaction

### 2.2.3.1 Primer selection

Specific primers were designed to allow the selective amplification of the α 1 and α2 genes ( Smetanina, 1996 ). The primers that amplified normal α2 gene ( A + B ) are amplified between promotor to untranslated region behind the α2 gene. Another pair of primers ( A + C ) for the 3.7 kb deletion are amplified between promotor of α2 gene. Because of the PCR products from both pair of primers are 1.76 kb for normal α2 gene and 3.7 kb deletion, the two separate reactions are run simultaneously for each DNA sample.

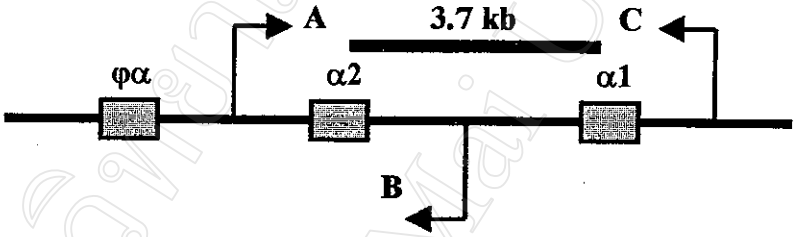


Figure 10. The locations of amplification for the specific primers

**A** = common forward primer ( position + 5671 to +5695 in the α2 promoter)
**Sequence = 5 '- CCC TCC CCC TCG CCA AGT CCA CCC C – 3'**

**B** = normal reverse primer ( positions + 7431 to + 7409 in the 3' UTR of α2 gene )
**Sequence = 5' – GGG AGG CCC ATC GGG CAG GAG GAA C – 3 '**

**C** = mutant reverse primer ( positions +11254 to +11231 in the 3' UTR of the α1 gene)
**Sequence = 5'- GGG GGG AGG CCC AAG GGG CAA GAA – 3'**

The nucleotide sequences between the promotor of α 2 - globin gene to the 3' untranslated region of the α 1 - globin gene. The sequences obtained from Gene Bank Locus HUMHBA 4.

A

5641 GATGCACCCA CTGGACTCCT GGACCTCCCA CCCTCCCCCT CGCCAAGTCC ACCCCTTCCT

5701 TCCTCACCCC ACATCCCCTC ACCTACATTC TGCAACACAG GGGCCTTCTC TCCCCTGTCC

5761 TTTCCCTACC CAGAGCCAGG TTTGTTTATC TGTTTACAAC CAGTATTTAC CTAGCAAGTC

5821 TTCCATCAGA TAGCATTTGG AGAGCTGGGG GTGTCACAGT GAACCACGAC CTCTAGGCCA

5881 GTGGGAGAGT CAGTCACACA AACTGTGAGT CCATGACTTG GGGCTTAGCC AGTACCCACC

5941 ACCCCACGCG CCACCCCACA ACCCCGGGTA GAGGAGTCTG AATCTGGAGC CGCCCCCACC

6001 CCAGCCCCGT GCTTTTTGCG TCCTGGTGTT TGTTCCTTCC CGGTGCCTGT CACTCAAGCA

6061 CACTAGTGAC TATCGCCAGA GGGAAAGGGA GCTGCAGGAA GCGAGGCTGG AGAGCAGGAG

6121 GGGCTCTGCG CAGAAATTCT TTTGAGTTCC TATGGGCCAG GGCGTCCGGG TGCGCGCATT

6181 CCTCTCCGCC CCAGGATTGG GCGAAGCCCT CCGGCTCGCA CTCGCTCGCC CGTGTGTTCC

6241 CCGATCCCGC TGGAGTCGAT GCGCGTCCGG CGCGTGCCAG GCCGGGGCGG GGGTGCGGGC

6301 TGACTTTCTC CCTCGCTAGG GACGCTCCGG CGCCCGAAAG GAAAGGGTGG CGCTGCGCTC

6361 CGGGGTGCAC GAGCCGACAG CGCCCGACCC CAACGGGCCG GCCCCGCCAG CGCCGCTACC

6421 GCCCTGCCCG GGCGAGCGGG ATGGGCGGGA GTGGAGTGGC GGGTGGAGGG TGGAGACGTC

6481 CTGGCCCCCG CCCCGCGTGC ACCCCCAGGG GAGGCCGAGC CCGCCGCCCG GCCCCGCGCA

6541 GGCCCCGCCC GGGACTCCCC TGCGGTCCAG GCCGCGCCCC GGGCTCCGCG CCAGCCAATG

6601 AGCGCCGCCC GGCCGGGCGT GCCCCCGCGC CCCAAGCATA CCATGGTGCT CGCTCGCGGC

6661 CCGGCACTCT ACGGCTCTGC ACAGACTCAG AGAGAACCCA CGCACGCTGG GTCTCCTGCC

6721 GACAAGACCA TGGCGCACGT CGCCTGGGGT AAGGTCGGCG CGCACGCTGG CGAGTATGGT

6781 GCGGAGGCCC AGCTTCGGGT AGGCTCCCTC CCCTGCTCCG ACCCGGGCTC CTCGCCCGCC

6841 CGGACCCACA AGGGGCGAGA AACCGTCCTG GCCCCGGACC CAAACCCCAC CCCTCACTCT

6901 GCTTCTCCCC CGCAGGCGGC CCTGTCCTTC CCCACCACCA AGACCTACTT CCCGCACTTC

6961 GACCTGAGCC CACTGCCTGC CCAGGTTAAG GGCCACGGCA AGAAGGTGGC CGACGCCCTG

7021 ACCAACGCCG GCCTCCCTGG GGACGACATG CCCAACGCGC TGTCCGCCCT GAGCGACCTG

7081 CACGCGCACA GCTGGAGCCT GGACCCGGTC AACTTCAAGG TGAGCGGCGG GCCGGGAGCG

7141 ATCTGGGTCG TCCTTGCACC TGGCGCCTTC CTCTCAGGGC AGAGGATCAC GCGGGTTGCG

7201 GGAGGTGTAG GCCTGGGTTC GGCTGCGGGC CTGGGCCGCA CTCCCGCCCG TCTCTGCACA

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7261  GCTCCTAAGC AAGGACCTCT TGGTGACCCT GGCCGCCCAC CTCCCCGCCG AGTTCACCCC
7321  TGCGGTGCAC GAAGCTGAGG ACAAGTTCCT GGCTTCTGTG AGCACCGTGC TGACCTCCAA
7381  ATACCGTTAA CAGGCCTGCA CGGTAGCCGT TCCTCCTGCC CGATGGGCCT CCCAACGGGC
7441  CCTCCTCCCC GGGAGTCCCA GGCCCTTCCT GGTCTTTGAA TAAAGTCTGA GTGGGCGGCA
7501  GCCTGTGTGT GCTCTCCATC TCTCTGTCCC GGAATGTGCC AACAATGGAG GTGTTTACCT
7561  GTCTCAGACC AGTGCAGGTG CTGCAGCTGC ATGGGGCTGG GGAGGGAGAA CTGCAGGGAG
7621  TATGGGAGGG GGAGTGAGGG TGGGCCTGCT CAAGAGAAGG TGCTGAACCA TCCCCTGTCC
7681  TGAGAGGTGC CAGGCCTGCA GGCAGTGGCT CAGAAGCTGG GGAGGAGAGA GGCATCCAGG
7741  GTTCTACTCA GGGAGTCCCA GCATCGCCAC CCTCCTTTGA AATCTCCCTG GTTGAACCCA
7801  GTTAACATAC GCTCTCCATC AAAACAAAAC GAAACAAAAC AAACTAGCAA AATAGGCTGT
7861  CCCCAGTGCA AGTGCAGGTG CCAGAACATT TCTCTCATTC CCACCCCTTC CTGCCAGAGG
7921  GTAGGTGGCT GGAGTGAGGG TGCTGGCCCT ACTCACACTT CCTGTGTCAC GGTGACCCTC
7981  TGAGAGCAGC CCAGTCAGTG GGGAAGGAGG AAGGGGCTGG GATGCTCACA GCCGGCAGCC
8041  CACACCTAGG GAGACTCTTC AGCAGAGCAC CTTGCGGCCT TACTCCTGCA CGTCTCCTGC
8101  AGTTTGTAAG GTGCATTCAG AACTCACTGT GTGCCCAGCC CTGAGCTCCC AGCTAATTGC
8161  CCCACCCAGG GCCTCTGGGA CCTCCTGGTG CTTCTGCTTC CTGTGCTGCC AGCAACTTCT
8221  GGAAACGTCC CTGTCCCCGG TGCTGAAGTC CTGGAATCCA TGCTGGGAAG TTGCACAGCC
8281  CATCTGGCTC TCAGCCAGCC TAGGAACATG AGCAGCACTT CCAACCCAGT CCCTGCCCCA
8341  CAGCAAGCCT CCCCCTCCAC ACTCACAGTA CTGGATTGAG CTTTGGGGAG GGTGGAGAGG
8401  ACCCTGTCAC CGCTTTCCTT CTGGACATGG ACCTCTCTGA ATTGTTGGGG AGTTCCCTCC
8461  CCCTCTCCAC CACCCGCTCT TCCTGCGCCT CACAGCCCAG AGCATTGTTA TTTCAGCAGA
8521  AACACTTTAA AAAATAAACT AAAATCCGAC AGGCACGGTG GCTCACGCCT GTAATCCCAG
8581  CACTTTGGGA CGCCGAGGTG GGAGGATCAC CTGAGGTCGG GAGTTTGAGA CCACCCTGAT
8641  CAACATGTAG AAACCCCATC TATACTAAAA ATACAAAATC AGCCGGGCAT GGTGGCCCAT
8701  GCCTGTAAAC CCACCTACTC CGGAGGCTGA GGCAGGAGAA TCATTTTAAC CAAGGAGGCA
8761  GAGGTTGCAG TGAGCTAAGA TCACACCATT GCACTCCAGC CTGGAAAACA ACAGCGAAAC
8821  TCCGCCTCAA AAAAAAAAAA GCCCCCACAT CTTATCTTTT TTTTTTCCTT CAGGCTGTGG
8881  GCAGAGTCAG AAAGTCAGAA GAGGGTGGCA GACAGGGAGG GGAAATGAGA AGATCCAACG
8941  GGGGAAGCAT TGCTAAGCTG GTCGGAGCTA CTTCCTTCTC TGCCCAAGGC AGCTTACCCT
9001  GGCTTGCTCC TGGACACCCA GGGACGGGCC TGAGTAAGGG CCTGGGGAGA CAGGGCAGGG
9061  AGCAGGCTGA AGGGTGCTGA CCTGATGCAC TCCTCAAAGC AGATCTTCTG CCAGACCCCC
9121  AGGAAATGAC TTATCAGTGA TTTCTCAGGC TGTTTTCTCC TCAGTACCAT CCCCCCAAAA
9121  AACATCACTT TTCATGCACA GGGATGCACC CACTGGCACT CCTGCACCTC CCACCCTTCC
9181  CCAGAAGTCC ACCCCTTCCT TCCTCACCCT GCAGGAGCTG GCCAGCCTCA TCACCCCAAC
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B

9241 CCAGAAGTCC ACCCCTTCCT TCCTCACCCT GCAGGAGCTG GCCAGCCTCA TCACCCCAAC

9301 ATCTCCCCAC CTCCATTCTC CAACCACAGG GCCCTTGTCT CCTCTGTCCT TTCCCCTCCC

9361 CGAGCCAAGC CTCCTCCCTC CTCCACCTCC TCCACCTAAT ACATATCCTT AAGTCTCACC

9421 TCCTCCAGGA AGCCCTCAGA CTAACCCTGG TCCCCTTGAA TGCCTCATCC ACACCTCCAG

9481 ACTTCCTCAG GGCCTGTGAT GAGGTCTGCA CCTCTGTGTG TACTTGTGTG ATGGTTAGAG

9541 GACTGCCTAC CTCCCAGAGG AGGTTGAATG CTCCAGCCGG TTCCAGCTAT TGCTTTCTTT

9601 ACCTGTTTAA CCAGTATTTA CCTAGCAAGT CTTCCATCAG ATAGCATTTG GAGAGCTGGG

9661 GGTGTCACAG TGAACCACGA CCTCTAGGCC AGTGGAGAG TCAGTCACAC AAACTGTGAG

9721 TCCATGACTT GGGGCTTAGC CAGCACCCAC CACCCCACGC GCCACCCCAC AACCCCGGGT

9781 AGAGGAGTCT GAATCTGGAG CCGCCCCCAG CCCAGCCCCG TGCTTTTTGC GTCCTGGTGT

9841 TTATTCCTTC CCGGTGCCTG TCACTCAAGC ACACTAGTGA CTCTCGCCAG AGGGAAAGGG

9901 AGCTGCAGGA AGCGAGGCTG GAGAGCAGGA GGGGCTCTGC GCAGAAATTC TTTTGAGTTC

9961 CTATGGGCCA GGGCGTCCGG GTGCGCGCAT TCCTCTCCGC CCCAGGATTG GGCGAAGCCC

10021 TCCGGCTCGC ACTCGCTCGC CCGTGTGTTC CCCGATCCCG CTGGAGTCGA TGCGCGTCCA

10081 GCGCGTGCCA GGCCGGGGCG GGGGTGCGGG CTGACTTTCT CCCTCGCTAG GGACGCTCCG

10141 GCGCCCGAAA GGAAAGGGTG GCGCTGCGCT CCGGGGTGCA CGAGCCGACA GCGCCCGACC

10201 CCAACGGGCC GGCCCCGCCA GCGCCGCTAC CGCCCTGCCC GGGCGAGCGG GATGGGCGGG

10261 AGTGGAGTGG CGGGTGGAGG GTGGAGACGT CCTGGCCCCC GCCCCGCGTG CACCCCCAGG

10321 GGAGGCCGAG CCCGCCGCCC GGCCCCGCGC AGGCCCCGCC CGGGACTCCC CTGCGGTCCA

10381 GGCCGCGCCC CGGGCTCCGC GCCAGCAAT GAGCGCCGCC CGGCCGGGCG TGCCCCCGCG

10441 CCCCAAGCAT AAACCCTGGC GCGCTCGCGG CCCGGCACTC TTCTGGTCCC CACAGACTCA

10501 GAGAGAACCC ACCATGGTGC TGTCTCCTGC CGACAAGACC AACGTCAAGG CCGCCTGGGG

10561 TAAGGTCGGC GCGCACGCTG GCGAGTATGG TGCGGAGGCC CTGGAGAGGT GAGGCTCCCT

10621 CCCCTGCTCC GACCCGGGCT CCTCGCCCGC CCGGACCCAC AGGCCACCCT CAACCGTCCT

10681 GGCCCCGGAC CCAAACCCCA CCCCTCACTC TGCTTCTCCC CGCAGGATGT TCCTGTCCTT

10741 CCCCACCACC AAGACCTACT TCCCGCACTT CGACCTGAGC CACGGCTCTG CCCAGGTTAA

10801 GGGCCACGGC AAGAAGGTGG CCGACGCCCT GACCAACGCC GTGGCGCACG TGGACGACAT

10861 GCCCAACGCG CTGTCCGCCC TGAGCGACCT GCACGCGCAC AAGCTTCGGG TGGACCCGGT

10921 CAACTTCAAG GTGAGCGGCG GGCCGGGAGC GATCTGGGTC GAGGGGCGAG ATGGCGCCTT

10981 CCTCGCAGGG CAGAGGATCA CGCGGGTTGC GGGAGGTGTA GCGCAGGCGG CGGCTGCGGG

11041 CCTGGGCCCT CGGCCCCACT GACCCTCTTC TCTGCACAGC TCCTAAGCCA CTGCCTGCTG

11101 GTGACCCTGG CCGCCCACCT CCCCGCCGAG TTCACCCCTG CGGTGCACGC CTCCCTGGAC

11161 AAGTTCCTGG CTTCTGTGAG CACCGTGCTG ACCTCCAAAT ACCGTTAAGC TGGAGCCTCG

11221 GTGGCCATGC TTCTTGCCCC TTGGGCCTCC CCCCAGCCCC TCCTCCCCTT CCTGCACCCG

C

### 2.2.3.2 Components of the polymerase chain reaction

#### Primer

Each of primer was used at the concentration of 50 pmole in 25 µl reaction mixture.

#### Deoxynucleotide triphosphates ( dNTPs )

dNTPs were used at the concentration of 200 mM for each dNTP in the 25 µl reaction mixture.

#### *Tag* DNA polymerase

The amplification reactions are carried out with 2.5 U *Tag* polymerase. *Tag* DNA polymerase ( Promega ) was provided at the concentration of 5 units / µl. The enzyme was diluted to concentration of 2.5 units / µl with steriled deionized water before use.

#### Target DNA

The 0.5 µg of the genomic DNA were used in each reaction.

#### Buffer

The 500 µl of 10 X buffer consist of ; 1 M Tris – HCl, pH 8.8, 3,350 µl ; 1 M ammonium sulfate, 830 µl ; beta – mercaptoethanol, 35 µl ; bovine serum albumin ( 4% ), 125 µl ; and $H_2O$, 560 µl . The buffer can be kept at 4 ° C for 2-4 months or aliquot 200 µl and kept in 20 ° C for 1 year.

Each amplification reaction was carried out in a 200 µl microfuge tube, each tube contained the reaction mixture for the PCR components as follow.

| | | |
|---|---|---|
| $H_2O$ | 13.0 | µl |
| 10 X buffer | 2.8 | µl |
| DMSO | 2.2 | µl |
| 3 mM dNTP | 2.5 | µl |
| primer A ( 50 pmole) | 2.5 | µl |
| primer B or C ( 50 pmole) | 2.5 | µl |
| DNA ( 0.5 µg ) | 2 | µl |
| *Tag* ( 2.5 U ) | 0.5 | µl |
| Total volume | 28 | µl |

The reaction mixture was taken to perform in a DNA . Thermal Cycle ( Perkin – Elmer Cetus model 2000 ). The conditions for denaturation, annealing and polymerization were as follows.

| Cycle | Denaturation | Annealing | Polymerization |
|---|---|---|---|
| first cycle | 95 $^0$ C, 6 min | 68$^0$ C, 1 min | 72$^0$ C, 2.5 min |
| 2$^{nd}$, 25$^{th}$ cycle | 95 $^0$ C, 1 min | 68$^0$ C, 1 min | 72$^0$ C, 2.5 min |
| last cycle | 95 $^0$ C, 1 min | 68$^0$ C, 1 min | 72$^0$ C, 5 min |

The reaction mixture was soaked at 4 ° C until it was kept, The PCR products were then analyzed by 1.5 % agarose gel electrophoresis stained with ethidium bromide. The 1 kb ladder was used as molecular - size standard.

## 2.2.4 Agarose gel electrophoresis

The PCR products were separated in 1.5 % agarose gel. The agarose gel was preprared by dissolving sufficient amount of powder agarose in 0.5 x TBE buffer to the designed concentration and volume. After melting the mixure by microwave and mixed well , the gel was cooled to 50-60 'C then add ethidium bromide, in a ratio of 0.5 mg ethidium bromide per 100 ml of agarose gel before molding. A comb with 5 mm wide and 2 mm thick was put in the melted gel to preprare the slots. After the gel was completly set (45 minute at room temperature), carefully remove the comb and sealing edge of the mold. Then 0.5 x TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the gel to a depth of about 1 mm.The samples were mixed with gel loading buffer and then were slowly loaded into the slots using the automatic micropipettor. The voltage of 4 V/cm (the distance between the electrodes) was usually used and the gel was run for 45 minutes.

## Photography

Photograph of gel were made by using gel doc thermal printer under UV light.

## 2.2.5 Hemoglobin electrophoresis ( hemoglobin typing )

### Preparation of hemolysate

This hemolysate is used for qualitative hemoglobin electrophoresis and for quantification of Hb $A_2$, Hb F, and serum haptoglobin.

### Procedures

1. Obtain 8 ml of EDTA blood, centrifuge blood 2,000 rpm to remove the plasma.
2. Wash cells three times with 0.9 % normal saline. Centrifuge 3,000 for 10 minutes on each washing.
3. Add equal volume of distilled water to washed paced packed red cell . Mix on vortex mixer for 15 seconds and kept it in the room temperature at least 10 minutes.
4. Add carbontetrachloride ( $CCl_4$ ) at a half of total volume. Mix vigorously by vortex for 5 minutes then centrifuge for 15 minutes at 3,000 rpm.
5. Collect hemolysate from the top layer to the new clean tube and ready to use .

### Hemoglobin electrophoresis on cellulose acetate (Schmidt and Brostus, 1974)

### Principle

Detection of abnormal hemoglobins with electrophoretic technics is base on differences of their migration velocities are the result of electric charges of each hemoglobin variant brought about by various amino acid substitutions in the polypeptide chains of the molecule. At this alkaline pH, the relative migration is shown in Figure 27. In this procedure , Tris - EDTA - borate buffer (TEB), pH 8.9 and isotonic strength 0.13, is used. Following electrophoresis 10 minutes at 160 volts, the membrane is stained with Ponceau S. Identify the migration velocities of the unknowns with known hemoglobin controls. The control are Hb A, $A_2$ and F .

## Procedures

1. Fill the two electrode compartments of the cell and the prebuffer tray with refrigerated TEB buffer. Allow the buffer to come to room temperature.
2. Wet the cellulose acetate membrane ( Titan III ), blot with Whatman paper to remove excess surface buffer, and then place on the bridge.
3. Apply hemolysate by using sample applicator at 0.5 cm interval along a transverse mid - line. Allow applicator tip to remain contact with the membrane for 15 seconds before retracting the top by depressing the red button.( The greater protein content of the hemolysate requires a longer time for absorption the samples. )
4. Electrophorese for 10 minutes at 160 volts. Constant current should be about 0.4-0.5 mA / cm.
5. Upon completion of electrophoresis, immerse the membrane immediately in the Ponceau ' s solution for 10 minutes and rinse with 5 % acetic acid until back ground is clear
6. Clearing the strip by immersing in the Isobutanal - Dixan solution for 10 minutes, then placed on the glass plate by avoiding air bubbles.
7. The translucent strip on the glass plate was dried at 100 ° C for 15 minutes which will turn the strip to be transparent and place the transparent membrane in the plastic envelope for a permanent record.

## 2.2.6 Hematological examination

### 2.2.6.1 Hematocrit by microhematocrit method

When anticoagulated whole blood is centrifuged, the space occupied by the packed red blood cells is termed the hematocrit reading and is expressed as the % of red blood cells in a volume of whole blood. It is also, less commonly, known as the packed red cell volume (PCV). The values for the hematocrit closely parallel the values for the hemoglobin and red blood cell count. When whole blood is centrifuged, the heavier red blood cells to the bottom of the tube and the lighter particles (white blood cells and platelets) precipitate out on top of them. When reading the hematocrit, it is important to take the reading only at the top of the red blood cell layer. This is most significant in cases in which there is an extremely elevated white blood cell or platelet count. The white blood cell and platelet layers comprise the buffy coat. As with the hemoglobin and the red blood cell count, the normal values for the hematocrit vary with the age and sex of the individual. Altitude also play a role in that the normal hematocrit for the residents at high altitudes is higher than that of individuals living at sea level, due to decreased oxygen pressure. At birth, the normal range for the hematocrit is 50 to 62 %. This range decreases to 37 to 48 % after 1 year of age . The normal adult hematocrit value gradually increases to the adult levels of 36 to 48 % for women and 39 to 55 % for men . There is a slight decrease in the hematocrit level after 50 years of age.

### Principle

Whole blood is centrifuged for maximum red blood cell packing. The space occupied by the red blood cells is measured and expressed as a percentage of the whole blood volume.

## Specimen

Whole blood sample using ethylenediaminetetra-acetic acid (EDTA) as the anticoagulant.

## Procedures ( Dacie and Lewis, 1968 )

1. Allow well mixed anticoagulated whole blood to enter two microhematocrit tubes 7 cm in length and 1 mm bore until there are approximately two- third' s filled with blood without air bubbles.
2. Seal one end of the microhematocrit tube with the clay material by placing the dry end of the tube into the clay in a vertical position. The plug should be 4 to 6 mm long. Mark certain blood is not forced out the top of the microhematocrit tube during this process.
3. Place the two microhematocrit tubes in the radial grooves of the centrifuge head exactly opposite each other, with the sealed end away from the center of the centrifuge.
4. Centrifuge 11,500 - 15,000 rpm for 5 minutes.
5. Remove the hematocrit tubes as soon as the centrifuge has stopped spinning. Obtain the results for both microhematocrits. Results should agree within $\pm$ 2 % of the hematocrit result. If they do not, repeat the preceding procedure.
6. The percent hematocrit calculated by the follow equation.

$$\textbf{\% Hematocrit} = \frac{\textbf{packed volume in the length}}{\textbf{total volume in the length}} \times 100$$

### 2.2.6.2 Red blood cell count

The red blood cell count ( RBC ) is the number of red blood cells in 1 liter ( L ) of whole blood. The normal red blood count is 3.6 to 5.6 x $10^{12}$ / L for females and 4.2 to 5.8 x $10^{12}$ / L for males. the new born shows an RBC of 5.0 to 6.5 x $10^{12}$ / L at birth, which gradually decreases to 4.3 $\pm$ 0.8 x $10^{12}$ / L at 1 year of age. During childhood and adolescence, the normal values for the RBC are slightly below the normal adult values. There is also a lightly decrease in the RBC after 50 years of age. In addition to the effects of age on the red cell count, strenuous physical activity tends to increase the red cell count. There may also be daily fluctuations with the red count being highest in the morning and at its lowest in the evening. An increased red count is found in polycythemia vera and secondary polycythemia due to the other causes, such as dehydration. The red cell count is below normal in anemia and secondarily in numerous other disorders.

### Principle

Blood is diluted with a isotonic fluid. The diluted specimen is introduced into the hemocytometer chamber, and the erythrocytes are counted. Diluting fluids used for erythrocyte counts do not destroy the leukocytes. There are normally so few that they do not interfere with the enumerations of the erythrocytes. When there are many, they are easily identified and are not counted.

### Procedures ( Babara , 1988 )

1. Dilute 2 µl blood with 400 µl of diluting fluid ( Grower's solution ).
2. Fill both sides of the clean hemocytometer chamber.
3. Count the red blood cells as described in the following steps
    a. Carefully place the filled counting chamber on the microscope stage.
    b. Using low power (10 x objective), place the large center square in the middle of the field of vision. Carefully examine the entire large square for even

distribution of red blood cells.

c. Carefully change to the high dry 40 x objective .

d. Move the counting chamber so that the small upper left corner square is completely in the field of vision. This square is further subdivided into sixteen even small squares. This facilitates cell counting.

e. Count the red cell in the small square, remembering to count the cells on two of the outer margins but excluding those lying on the other two outside edges.

f. Some of the red blood cells may be lying on their sides and, therefore, do not appear as round as the majority of cells in the area. These cells are to be included in the count.

g. If there are any white blood cells in the area being counted, do not include these cells in your count.( the white blood cell is usually much larger than the red blood cell and does not have as smooth an appearance.)

5. Calculate the red blood count for each of the red counts performed and average the two results for the final report. The equation of calculate the red blood cell count show as follow.

$$\text{RBC / L} = \text{RBC in five squares} \times \text{correction for volume} \times \text{correction for dilution} \times 10^{6}$$

6. Calculated the mean corpuscular volume ( MCV) in order to indicate the average volume of the red blood cells in femtoliters ( fL) by the following equation.

$$\text{MCV} = \frac{\text{Hct} \times 10^{3}\ \text{fL}}{\text{RBC/L}} \quad ; \quad \text{Hct} = \text{hematocrit (\%)}$$

Note : The MCV indicates whether the red blood cells appear normocytic, microcytic, or macrocytic. If the MCV is greater than 100 fL, the red blood cells are considered marcrocytic. If the MCV is within the normal range, the red blood cells are normocytic. Normal range for the MCV = 80 - 100 fL.

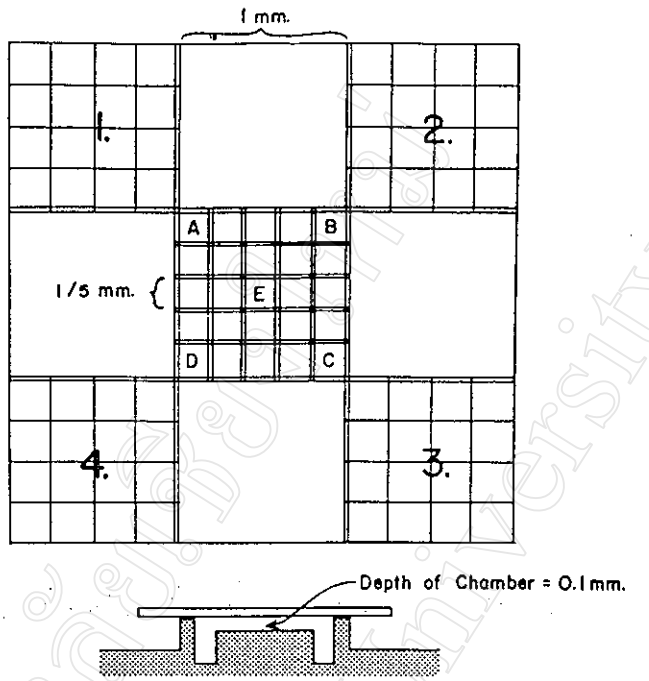


Figure 11. Hemocytometer. Squares 1, 2, 3, and 4 are used for counting leukocytes ; squares A, B, C, D and E are used for counting erytrocytes.

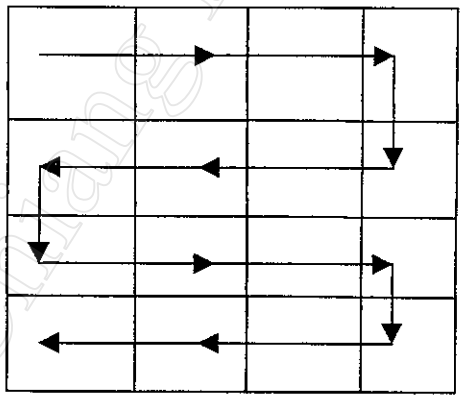

Figure12. Manner of counting erythrocytes in one of small squares.

## 2.2.6.3 Hemoglobinometry by cyanmethemoglobin method

### Principle

Whole blood is add to cyanmethemoglobin (HiCN) reagent (containing potassium cyanide and potassium ferricyanide). The ferricyanide converts the hemoglobin iron from the ferrous state ($Fe^{++}$) to the ferric state ($Fe^{+++}$) to form methemoglobin (Hi) which then combines with potassium cyanide to form stable pigment, cyanmethemoglobin ( HiCN ; hemiglobin cyanide ). The color intensity of this mixture is measured in a spectrophotometer at a wave length of 540 nm. The optical density of the solution is proportional to the concentration of hemoglobin. All forms of hemoglobin are measured with this method except sulfhemoglobin.

### Procedures ( Babara, 1988 )

1. For each sample to be test, pipette exactly 5.0 ml of HiCN reagent an appropriately labeled test tube. Place 5.0 ml of the reagent into a test tube to be used as the blank.
2. Add 0.02 ml of well mixed whole blood to the appropriately labeled tube. Rinse the pipette 3 to 5 times with the HiCN reagent until all blood is removed from the pipette .
3. Mix the preceding solutions well and allow to stand at room temperature for at least 10 minutes in order to allow adequate time for the formation of HiCN.
4. Transfer the mixture to the cuvette and read the optical density in a spectrophotometer at a wavelength of 540 nm using the HiCN reagent in the blank tube to set the optical density (O.D.) at 0.0. Record the readings for the samples from the O.D. scale and refer to the standard curve for actual value of the hemoglobin in g/dl.

**Preparation of Standard Hemoglobin Curve**

Using stock solution of HiCN standard (equivalent to 18 g hemoglobin per 100 ml blood), set up the dilutions to 3.6, 7.2, 10.8, 14.4, and 18 g (Hb)/dl . Plot hemoglobin in g / dl on the horizontal axis against O.D. on the vertical axis this is a straight line curve. A chart should then be made to facilitate reading the test results.

| Tube No. | Standard Hb (ml) | Drabkin's (ml) | g(Hb)/dl | O.D. |
|---|---|---|---|---|
| 1 | 5 | 0 | 18.0 | 0.47 |
| 2 | 4 | 1 | 14.4 | 0.38 |
| 3 | 3 | 2 | 10.8 | 0.29 |
| 4 | 2 | 3 | 7.2 | 0.20 |
| 5 | 1 | 4 | 3.6 | 0.11 |
| 6 | 0 | 5 | 0.0 | 0.00 |

**Table 2.Dilutions and O.D. for Hemoglobin standard curve**

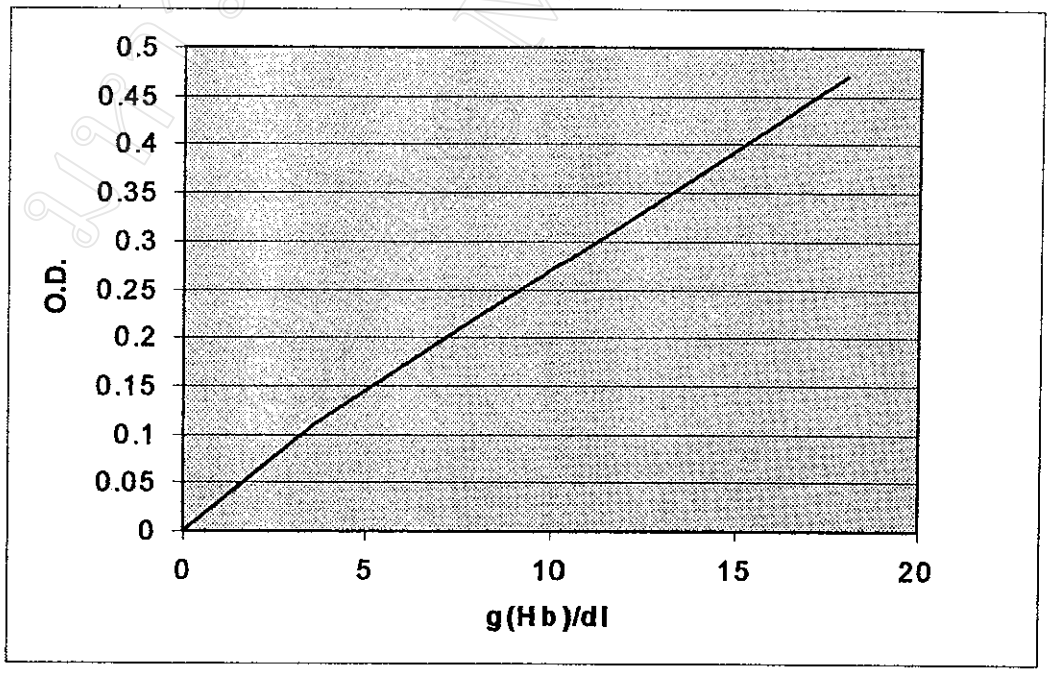


**Figure13. Hemoglobin standard calibration curve**

## 2.2.6.4 Osmotic fragility test ( Babara, 1988 )

The osmotic fragility test using fresh red blood cells is a measure of the ability of the red cells to take up fluid without lysing. This test is employed to help diagnose different types of anemias in which the physical properties of the red blood cell are altered. The main factor affecting the osmotic fragility test is the shape of the red blood cell, which, in turn, is dependent on the volume, surface area, and functional state of the red blood cell membrane. An increased osmotic fragility is found in hemolytic anemias, hereditary spherocytosis, and whenever spherocytes are found. Decrease osmotic fragility occurs following splenectomy, in liver disease, sickle cell anemia, iron-deficiency anemia, thalassemia, polycythemia vera, and condition in which target cells are present. Reticulocytes also shown a decreased osmotic fragility.

### Principle

When red blood cells are placed in an isotonic solution, 0.85 % sodium chloride, fluid will neither enter nor leave the red blood cell. If the red blood cell place in an hypotonic solution0.25 % sodium chloride, however, fluid enters the red blood cell, the cell swells up, and eventually hemolyzes or raptures. A spherocyte, which is almost round, swells up in a hypotonic solution and raptures much more quickly than a normal red blood cell or more quickly than cells that have a large surface area per volume, such as target cells or sickle cells. The fragility of the red blood cell is said to be increased when the rate of hemolysis is increased. When the rate of hemolysis is decreased, the fragility of the red blood cells is considered to be decreased.

Procedures

1. Centrifuged anticoagulated fresh whole blood at 3,000 rpm for 5 minutes.
2. Separated plasma and kept packed red cell .
3. Pipetted10 µl of packed red cell in 10 ml of 0.45 % saline solution mixed well.
4. Measured the O.D. by spectronic 20 at wavelength of 620 nm at 15, and 120 second.
5. Calculated percent of hemolysis by the following equation.

$$\text{\% osmotic fragility ( OF )} = \frac{\text{O.D. 1 - O.D. 2}}{\text{O.D. 1}} \times 100$$

Note O.D.1 = O.D. 620 at 15 second , O.D. 2 = O.D. 2 at 120 second

## 2.2.6.5 Acid elution test for hemeglobin F ( Babara, 1988 )

The acid elution test is employ to determine the distribution of hemoglobin F in the red blood cell: to determine whether hemoglobin F is present in the varying amounts in only some of the red blood cells. It is useful in differentiating hereditary persistence of fetal hemoglobin and in determining the present of fetal red cells in the maternal circulation during pregnancy. Approximately 80% of the total hemoglobin at birth is hemoglobin F. By the age of 4 months, there is approximately 10 % hemoglobin F present, and at the end of infancy only trace amounts remain. Normal adult blood will generally contain less than 2 % hemoglobin F. Increased amounts of hemoglobin F in the red cell are found in hereditary persistence of fetal hemoglobin, sickle cell anemia, thalassemia, various other hemoglobinopathies, aplastic anemia, some leukemias, polycythemia vera, Down's sydrome, in a number of other hematological disorders, and in some complications of pregnancy.

### Principle

Hemoglobin is precipitated inside the red cell by drying and fixing with alcohol. Hemoglobin A and its adult variants were eluted in acid solution while hemoglobin F which was resisted in acid solution remain precipitated inside the cell and densely blue stain with Amidoblack B dye.

### Specimen

Obtain 4 blood smears from venous blood collected in EDTA anticoagulant. Obtain a similar blood specimen for normal and abnormal control at the same time the patient blood collected.

### Procedures

1. Preparation of blood smears

    a. Patient- make four thin (a monolayer of cells) blood smears.

    b. Normal control- Make two thin blood smears from a normal adult.

c. Positive control- Mix two drops of cord blood with two drops of normal, ABO compatible, whole blood. Mark two thin blood smears.

2. Allow the blood smears to air- dry for at least 10 minutes.
3. Fixed the blood smear with 80 % ethyl alcohol in a coplin jar for 2 minutes and fix blood smears in alcohol for 2 minutes
4. Rinse the smears carefully in distilled water and allow to air dry.
5. Place the dry smears to the 0.1 % Amidoblack B dye for 2 minutes.
6. Wash with distilled water and air dry.
7. Examine the slides microscopically, using the oil emersion objective (100x), for the presence of red blood cell containing hemoglobin F.
8. Counting Hb F that was densely blue strain, normal red blood cells will appear as scarcely visible ghost cells.
9. In order to determine the percent of the red blood cells containing hemoglobin F perform the following procedure:
   a. Count the number of red blood cells in 3 to 5 microscopic fields and determine the average of red cells / field
   b. Examine 20 to 25 microscopic fields, counting the number of red cells containing hemoglobin F. Divide to the total number of red cells with hemoglobin F, by the number of field counted. This is the number of red cells containing hemoglobin F /field.
   c. Divide the number of red cells containing hemoglobin F by the average number of red blood cells/field. Multiply this result by 100 to obtain the percent of red blood cells containing hemoglobin F.
10. The degree of hemoglobin F was grading as follows

| | | |
|---|---|---|
| 0-5 cells / field | = | normal |
| 2- 9 % | = | trace |
| 10-20 % | = | 1+ |
| 21-50 % | = | 2+ |
| 51-80 % | = | 3+ |
| > 80 % | = | 4+ |

## 2.2.6.6 Inclusion bodies for hemoglobin H ( Babara, 1988 )

Patients suspected of having thalassemia trait, who have an MCV below 80 fl, are not iron deficient, have a normal hemoglobin electrophoresis, and have a normal levels of hemoglobin F and A2, may have alpha thalassemia. In this disorder, there is and excess of beta chains (due to a deficiency in the production of alpha chains). The excess of beta chains will form beta 4 tetramers (hemoglobin H). Due to the small amount of hemoglobin H formed, it will generally not be seen on cellulose acetate hemoglobin electrophoresis. However, in the presence of oxidant, such as Brilliant cresyl blue, this abnormal hemoglobin will precipitate within the red cell and form many small inclusion. Unstable hemoglobins may also detected and stain with this method . In addition inclusion bodies may be found in the red cells of some patient with enzyme deficiencies e.g. G-6-PD deficiency and after exposure to oxidant drugs, Normal hemoglobin will not denature or precipitate in this procedure and will there fore be negative for inclusion bodies ( Jones, 1981 and Reven, 1973).

## Principle

It is thought that red cells containing hemoglobin H are removed more quickly from the circulation by the recticuloendothelial system. Therefore, the red cell containing hemoglobin H, which are present in blood, are relatively young cells, and, when centrifuged, will lie near the top of the red cell layer. In this procedure, the blood is centrifuged in several microhematocrit tubes. The top layers of the red blood cells are remove and incubated with Brilliant cresyl blue stain. During incubation, hemoglobin H present in the red cell will denature and precipitate within the cell. The hemoglobin H bodies and any other denature hemoglobin will be stain by Brilliant cresyl blue.

## Specimen

Whole blood, using EDTA as the anticoagulant. Excess amounts of anticoagulant may interfere with the staining process. Fresh blood samples, less than 8 hours old are preferable.

## Procedures

1. Add 2 drops of fresh anticoagulated blood to 2 drops of filtered stain (1 % Brilliant cresyl blue ) in microcentrifuge tube.
2. Incubated at 37 ° C in water bath for 1 hour.
3. Prepare the wet smear and examined under light microscope

Note : Hb H inclusions appear as multiple greenish blue inclusions. Ten percent to 100 of the red cells of a patient with Hb H disease may contain the inclusion bodies. Recticulocytes can be seen, but are distinguished by their blue filamentous material. Preformed hemoglobin inclusions seen in a majority of spleenectomized patients appears as large, spherical, single inclusions.