ACCURACY OF LYMPHOCYTE COUNTS FROM UNICEL DXH 800 IN β-THALASSEMIA/HBE PATIENTS HAVING VARIOUS NUMBERS OF NUCLEATED RED BLOOD CELLS

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To Whom It May Concern

Mr. Sunil Timilsena, who enrolled in Master of Science in Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal, conducted successfully his M.Sc. thesis work entitled "Accuracy of lymphocyte counts from UniCelDxH 800 in β-thalassemia/HbE patients having various numbers of nucleated red blood cells" in my laboratory- Center of Excellence of Flow Cytometry, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, for the partial fulfillment of his academic programme. The work conducted by Mr. Timilsena was mutually supervised by me and his co-supervisor Dr.Egarit Noulsri.

As an external supervisor, I wish him for successful submission of his thesis.

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Declaration

I hereby declare that the thesis entitled "**ACCURACY OF LYMPHOCYTE COUNTS FROM UNICEL DXH 800 IN β-THALASSEMIA/HbE PATIENTS HAVING VARIOUS NUMBERS OF NUCLEATED RED BLOOD CELLS**" is based on the work carried out by me. This work has not been submitted in candidature for any other degree. The research work has been carried out at Centre of Excellence for Flow Cytometry, Siriraj Medical Research, School of Medicine, Mahidol University, Bangkok under, the supervision of Prof. Dr. Kovit Pattanapanyasat, and Prof. Dr. Krishna Das Manandhar, and co- supervision of Dr. Egarit Noulsri. I will have no objection for availability of the thesis for photocopy and inter-library loan for the purpose of scholarly research.

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Glossary acronyms

%	: Percentage
µ	: Micro
µg	: Micro-gram
µl	: Micro-Liter
^{0} C	: Degree Celsius
Ab	: Antibody
APC	: Allophycocyanin
BM	: Bone Marrow
bp	: Base pair
BT	: Beta thalassemia
BTI	: Beta Thalassemia Intermedia
BTMi	: Beta Thalassemia Minor
CBC	: Complete Blood Count
CD	: Cluster of differentiation
cpm	: Counts per minute
DN	: CD8- CD4- / Double Negative
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
dNTPs	: Nucleotide TriPhosphates
DP	: Double Positive
EDTA	: Ethylene Diamine Tetra Acetic acid
FACS	: Flow Assisted Cell Sorting
FITC	: Fluorescein Isothiocyanate
FL1, FL2, etc.	: Fluorescence parameter 1, 2, etc., on the flow cytometer
FMO	: Fluorescence minus One
FRET	: Fluorescence resonance energy transfer
FSC	: Forward Scatter
gm	: Gram
Hb	: Hemoglobin
HIV	: Human immunodeficiency virus
hrs	: Hours
Kb	: Kilobase
Min	: Minutes

ml	: Milliliters
Neg	: Negative
NR	:Non-Reactive
NRBC	: Nucleated Red Blood Cells
NS	: Not seen
PBS	: Phosphate buffered saline
PCR	: Polymerase Chain Reaction
PE	: Phycoerythrin
PerCP	: Peridin-chlorophyll-protein Complex
R.B.C.	: Red blood cells
Rpm	: Revolutions per minute
S	: Seconds
SCT	: Sickle cell thalassemia
SP	: Single Positive
SSC	: Side Scatter
TM	: Beta-Thalassemia Major
UV	: Ultraviolet
WBC	: White blood cells
α	: Alpha
β	: Beta
γ	: Gamma
δ	: Delta
ε	: Epsilon

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ABSTRACT

Background: The UniCel DxH-800 is an automated cell counter widely used around the world in hospitals. However, there is no clear account on the influence of the different levels of NRBCs on the lymphocyte count using DxH-800; particularly on β-thalassemia/ HbE patients.

Objective: The objective of this study was to compare the lymphocytes counts in β thalassemia/ HbE patients,using Unicel DxH- 800 and a standard flow cytometer, having various ranges of NRBCs.

Methods: The study analyzed the 25 healthy samples and 69 β-thalassemia/ HbE samples.Multiplex-ARMS PCR was used for the confirmation of β-thalassemia/ HbE patients. After confirmation the number of lymphocytes were determined using UniCel DxH -800 and a standard flow cytometer using counting beads.

Results: The regression analysis of the lymphocyte counts using two approaches, in healthy volunteers, showed an r^2 of 0.85 and a $P<0.0001$ and a Bland-Altman plot showed mean bias of +264 cells/µL. In case of β-thalassemia/ HbE patients, the counts of lymphocyte obtained using an Unicel DxH-800 and flow cytometer showed an r^2 of 0.06, and a $P= 0.028$, and a Bland- Altman plot showed the mean bias of +1,509 cells/µL. Further, a high degree of incongruity in the lymphocyte count was seen in β thalassemia/ HbE patients with the NRBCs number greater than 100,001 cells/µL

Conclusions: The UniCel DxH-800 enumerated the lymphocytes well in β-thalassemia/ HbE samples with various number of NRBCs but when the NRBCs number exceeded 100,001 cell/ µL, interference was observed in the accuracy of lymphocyte enumeration.

Key words: Automated cell counter;β- thalassemia; flow cytometry; lymphocyte;

nucleated red blood cell

CHAPTER 1
INTRODUCTION

1.1 Background

The lymphocytes are the key components of the immune system. They can be further categorized into B cells, T cells and Natural killer cells. The T cells mediate cellular immunity while the B cells are necessary for humoral immunity (Ben-Yosef et al., 2016). After these cells are activated, they differentiate into distinct effector subtypes and provide immune responses against pathogen. Natural killer cells are the part of the innate immunity and show a cytolytic function against those cells infected with tumor and virus(Paul & Lal, 2017).Lymphocyte count is a part of a complete blood count and is expressed as the percentage of lymphocytes to the total number of white blood cells. It has been widely recognized that rise in lymphocyte concentration has an association with inflammation whereas low absolute count is associated higher rate of infection after trauma and surgery(Cronkite & Strutt, 2018; Koyasu & Moro, 2012).

Keeping in mind this association, the accurate enumeration of the lymphocyte count is of utmost importance for the clinical management of these patients.

Nucleated red blood cells (NRBCs) are the red blood cells with which failed to exclude nucleus before exiting from the bone marrow or liver (Cronkite & Strutt, 2018; Danise et al., 2011). NRBCs are commonly found in peripheral blood circulation of patients with severe anemia, extramedullary erythropoiesis in which blood elements are produced outside the bone with the expansion into normal tissues, splenectomy,leukemias, myelodysplastic syndromes and some kinds of lymphoma.They are also found in other conditions of hematopoetic stress such as sepsis or massive haemorrhages (Sohawon, Lau, Lau & Bowden, 2012; Zhu et al., 2012)

NRBC have the size and nucleus similar to that of lymphocytes. Due to this similar characterstic feature of NRBC and lymphocytes, many hematology analyzers misclassify NRBC and produce a wrong total white blood cell and lymphocyte count. In the positive scenario, when the sample is flagged, the NRBC in the blood film is counted manually and the total white blood cell and lymphocyte count are corrected mathematically. If the sample is not flagged, NRBCs may remain undetected and the total white blood cell and lymphocyte count may be falsely read (Sysmex-Europe, 2019, September 22).In severe β-thalassemia patients cell counter analysis has demonstrated that the number of NRBCs can go up to 500 cells/100 white blood (Tantanate & Klinbua, 2015).

Hemoglobin E (HbE)is an extremely common variant of hemoglobin and is found have high frequency throughout many countries of Asia. The coinheritance of HbE with β-thalassemia, a condition called hemoglobin E. β-thalassemia, covers approximately 50% of the clinically severe β-thalassemia disorders in Asia and the world (S. Fucharoen & Weatherall, 2012). β-thalassemia is prevalent in Mediterranean countries, the middle east, Central Asia, India, Southern China and the Far East as well as countries along the north coast of Africa and in south America (Fernández et al., 2008)

As increased number of lymphocytes is associated with the inflammation in patients (Cronkite & Strutt, 2018; Koyasu & Moro, 2012). In such associations, accurate enumeration of lymphocyte is essential for the therapeutic treatment of patients. Several approaches are there for performing lymphocytes enumeration, but the most widely used methods are flow cytometry and automated cell counters (Gulati et al.,

2011; Nicholson et al., 1997). Flow cytometry uses monoclonal antibodies to identify the the total population of WBCs and then granulocytes, monocytes and lymphocytes are categorized according to their size and internal complexity. Further reference microbeads, whose concentrations are known, are used to calculate the number of cells. Studies have shown this approach to be accurate for the enumeration of WBCs in the blood containing high number of NRBCs (Simson & Groner, 1995).

Many modern instruments are available in market for performing automated cell counting. The Unicel DxH- 800 is one of the most widely used automated cell counters in medical laboratories around the world (Barnes et al., 2010; Brown et al., 2014; Hedley et al., 2011; Tan et al., 2011). This product from Beckman coulter incorporates new electronic and mechanical designs and employs the advanced algorithm technology to analyze complete blood counts (CBCs), WBCs differentials, NRBCs and reticulocytes. DxH-800 was designed, taking care of the major performance and has enhanced hardware system compared to previous LH series analyzer. The system captures 29 individual measurements per cell analyzed. The improved sensitivity and specificity of the system helps to reliably assess the abnormal cell population, particularly was found better at detecting samples consisting of the blast cells (Barnes et al., 2010).

The DxH-800 for us is among the modern and advanced hematology analyzers incorporating new technology. And as it is one of the widely used analyzer in medical laboratories around the world. The assessment of the performance of the system in different hematological cases is of prime importance. Several studies have evaluated the instrument's performance (Bruegel et al., 2015; Da Rin et al., 2017; Meintker et al., 2013). However, effect of NRBCs on lymphocyte counts using Unicel DxH-800 has not been fully elucidated, particularly in the cases with higher NRBCs.

In this study, we first confirmed the β thalassemic/ HbE samples using the amplification refractory mutation system (ARMS) PCR. Then lymphocytes count obtained using flow cytometrywas compared to Beckman Coulter Unicel DxH-800 to check the accuracy of this instrument while enumerating lymphocytes in β- thalassemia/HbE patients with various numbers of NRBCs

1.2 Current studies

Modern hematology analyzers are able to executethousands of complete blood count in a single day in a fully automated manner providing clinical data in a short turnaround time. However, the samples with high number of nucleated RBCs or the immature granulocytes often require manual intervention such as the review of the smear and manual differential cell count (Tan et al., 2011)

Nucleated red blood cells have been the subject of research in hematological and biomedical departments in hospitals, clinics and medical laboratories around the world. As NRBCs are derived from the hematopoietic stem cells and are the precursors of reticulocytes and Red Blood Cells(RBCs), concerns have been rising about these subsets of bone marrow populations. Studies have been carried out on the quantification of NRBCs and clinical relevance in umbilical cord transplantation. Stevens and coworkers showed that the NRBCs content is correlated with CD34+ and colony forming cell numbers. NRBCs were also found to be predictive of myeloid engraftment speed (Stevens et al., 2002).

Marolleau and his team pointed out the importance of NRBCs enumeration in quality control (QC) cord blood units (Larghero et al., 2006). It is known that NRBCs can compromise the count of white blood cells (WBC) and can also interfere with the quantification of CD34+ cells as the fractionof CD34+ cells is related to the count of nucleated cell. Thus NRBC quantification is presumed to improve the quality control of marrow graft.

Marolleau's team then targeted to quantify NRBCs in allogenic marrow graft using simple and rapid flow cytometric method to analyze the probable influence of marrow manipulation on recovery of NRBC in both ABO matched and major ABO mismatched cases (Marolleau et al., 2007).

Stachon and his team from Ruhr University, Germany did the research, first of its kind, screening NRBCs in the blood of the hospitalized patients for mechanized blood analysis. The highest number of NRBCs were found in ICU patients from general accident and surgery and showed that detection of NRBC in hospitalized patients was related to significantly increased mortality (21.1%) (Stachon, Sondermann, Imohl, & Krieg, 2002)

Montiero Junior JG and his co- workers in2015 showed that NRBCs are the markers of all- cause in- hospital mortality of patients admitted to a cardiac ICU (Monteiro Junior et al., 2015)

Menk et. al in 2018, demonstrated that NRBCs may predict Acute Respiratory distress syndrome (ARDS) related mortality with higher predictive power and suggested that their presence in the blood might be the marker of disease severity (Menk et al., 2018)

Thus accurate, reproducible and timely reporting of NRBCs has been the crucial function of the clinical hematology laboratory (Kratz et al., 2006).

Taking care of these key functions of clinical hematology laboratories, modern hematology analyzers are being developed to solve the challenge of exact detection andaccurate enumeration of NRBCs. And studies are being carried out to check the reliability of these machines adhering to CLSI rules and validating results.

Buoro and coworkers in 2015, evaluated the NRBCs count by Sysmex XE- 2100 comparing it with optical microscopy. And showed the excellent performance of the machine for NRBCs counting particularly in the patients with haemoglobinopathies and neonates (Buoro et al., 2015).

The comparative study performed at Lucile Packard Children's Hospital, tertiary care hospital that serves children at Stanford University Medical Center, Stanford, CA showed correlation coefficients for neutrophils (r= 0.9996), lymphocytes (r= 0.984), monocytes (r= 0.815), eosinophils (r= 0.840) and basophils (r=0.049), between DxH 800 and Abbott Cell - Dyn Sapphire.

The Cell-Dyn Sapphire is a fully automated hematology analyzer which holds the capacity to analyze upto 106 samples per hour. The system employs multi-angle polarized scatter separation and 3 color fluorescence technologies to provide analysis of the WBC count and differential.

In the detection of NRBCs, DxH -800 was favored due to its higher sensitivity and efficiency in NRBC detection as the detectable NRBCs were enumerated with the manual count.And in the lab where the NRBCs values were used interchangeably between

manual count and of instrument, Sapphire was preferred due to its numerical accuracy. (Tan et al., 2011)

Study carried by Karakukcu and his coworkers in 2015 showed that the hematology analyzers Sysmex XE- 2100 and Advia 2120i can be used tomonitor NRBC% for transfusion therapy for the patients with beta thalassemia major (Karakukcu et al., 2015).

Several disorders are linked with NRBCs and the investigations are carried out depending upon their associated influence. Keeping in mind the similar size of NRBCs and lymphocytes our study aimed to find the influence of NRBCs in lymphocytes enumeration. We took the beta thalassemia/HbE samples as the, the NRBCs in these samples were found to reach 500 NRBCs per 100 WBCs) (Tantanate & Klinbua, 2015). And the influence of NRBCs was evaluated using UnicelDxH- 800, as it is one of most widely used hematology analyzers around the world (Barnes et al., 2010; Brown et al., 2014; Hedley et al., 2011; Tan et al., 2011).

1.3 Hypothesis

1.3.1Null Hypothesis:

There is no influence of nucleated red blood cells in lymphocytes enumeration while enumerating lymphocytes using Unicel DxH- 800in beta thalassemic/HbE patients.

1.3.2 Alternative Hypothesis:

There is the influence of nucleated red blood cells in lymphocyte enumeration while enumerating lymphocytes using Unicel DxH-80 in beta thalassemic/ HbE patients

1.4 Objectives

1.4.1 General Objective

The objective of this research was to investigate if there is the influence of nucleated red blood cells in lymphocyte enumeration, while enumerating lymphocytes using Unicel DxH- 800 in beta thalassemic / HbE patients.

1.4.2 Specific Objectives

- Detect the beta thalassemia/ HbE samples by MARMS PCR.
- To compare the NRBCs count obtained from Manual count and Unicel DxH-800**.**
- To compare the values oflymphocyte, count in healthy donors using Unicel DxH-800 and a standard flow cytometer.
- To compare the lymphocytes counts in β thalassemia/ HbE patients, using Unicel DxH- 800 and a standard flow cytometer, having various ranges of NRBCs.
- To observe the effect of NRBCs on identification of lymphocytes.

1.5 Rationale and scope of the study

Nucleated Red Blood cells (NRBCs) are the subset of bone marrow cell populations which failed to exclude their nuclei before coming out from the nucleus. NRBCs are the progenitor cells which later give rise to red blood cells and the reticulocytes. NRBCs are associated with many maladies and disorders. They have been spotted in the peripheral blood circulation of patients with extramedullary erythropoiesis (a case in which the blood elements are produced outside the bone with expansion into normal tissues), splenectomy, leukemia, myelodysplastic syndromes, severe anemia and in some kind of lymphomas. They have also been traced in case of hematopoietic stress such as massive hemorrhages and sepsis.

Along with the aforementioned cases, quantification of NRBCs have been done and analyzed in the cases of - transfusion dependent beta thalassemia, severe anemia, patients admitted in cardiac ICU, pregnant woman, cases of mortality and respiratory distress syndrome

Thus, quantification of NRBCs has been of increased importance, particularly in hematological laboratories in clinical settings. Extensive researches are being carried out regarding the exact identification and accurate quantification of NRBCs. As the modern labs employ new and advanced hematological analyzers for enumeration of cells; their performance, while enumerating NRBCs, have been the subject of investigation. Further as NRBCs have the size and nucleus similar to that of lymphocytes. Due to this similar characteristic feature of NRBC and lymphocytes, many hematology analyzers misclassify NRBC and produce a wrong total white blood cell and lymphocyte count. Studies have been done to check the precision in enumerating NRBCs and their presence and significance in several diseases and disorders. But there are no studies carried out to investigate their influence in lymphocyte enumeration. Our study focuses on the cases the hematology analyzers can misclassify the lymphocyte and NRBCs and read them as one.

Severe β-thalassemia patients' cell counter analysis has demonstrated that the number of NRBCs can go up to 500 cells/100 white blood. Thus for our study we chose the beta thalassemia samples with an aim to deduce clear picture of the influence of NRBCs in enumerating lymphocyte population. The hematology analyzer used was Unicel DxH-800 from Beckman coulter, which is among the most widely used hematology analyzers around the world.

This kind of study must be carried out as the part of quality control program in hematology and medical laboratories. Our study is indicative of the case that, only calibration and routine check of the hematology analyzers is not sufficient for reproducible and accurate WBCs and lymphocyte count. The properties of these cells must also be taken care of.

Such studies can be of great assistance in troubleshooting the confusions in proper diagnosis. For instance, 258/incase of HIV infected thalassemia patients, where count of lymphocytes is very crucial and when sample has higher number of NRBCs, analyzer might show the increased lymphocyte count which then can lead to misdiagnosis and improper clinical management of the patient.

To sum up, it will be better than not to make it mandatory and beneficial to carry out the performance study of the analyzers for enumeration and influence of NRBCs on the

lymphocytes count in the medical laboratories, clinics and hospitals, which in turn will greatly assist clinicians and medical personnel for proper diagnosis of the maladies.

CHAPTER 2
LITERATURE REVIEW

2.1 Thalassemia

Thalassemia was first described 90 years ago by Cooley and lee, many account have been described since then and an ample amount has been learnt (Olivieri, 1999; Rund & Rachmilewitz, 2005). Significant work has been done in thalassemia in the past 50 years of time though the cellular and molecular basis was initially unknown. Thalassemia, sickle- cell disease, and other disorders of hemoglobin are the most prevalent monogenic disease worldwide. High frequency of inherited hemoglobin variants in some of the regions have shown their heterozygote resistance to *Plasmodium falciparum*malaria, and studies have shown that this is the case for αthalassemia,β thalassemia and hemoglobin E. 1-5 % of global population are estimated to be the carrier of the β thalassemia mutation (Modell & Darlison, 2008; Vichinsky, 2007; Weatherall, 2010).

Hemoglobin synthesis, at the molecular level, is controlled by two multigene clusters on chromosome 16, which codes for the globins and the chromosome 11 which codes for β globins. The genes in the chromosomes are arranged in such a way that they are expressed to produce different hemoglobin tetramers during embryo, fetal and adult life. Unequal-meiotic cross over results from the high homology of the- globin genes, which is the reason for deletions that can cause α thalassemia. In the cluster of β globin gene ε gene is expressed in early embryos, towards the downstream from ε gene are two ϒ. These genes are found to express in fetal hemoglobin (Hb F, α_2 γ_2), the form of hemoglobin that pre-dominates through the most of gestation period. The δ- gene product comprises the only a minor component of hemoglobin, Hb A2 ($\alpha_2\delta_2$) which is useful in diagnosing thalassemia. HBA ($\alpha_2\beta_2$) is the major hemoglobincomponent in thered bloodcells of the adult. Embryonic hemoglobin$\beta_2\varepsilon_2$, $\alpha_2\varepsilon_2$, $\beta_2\Upsilon_2$are formed during the first month of gestation in erythroid cells which are primarily found in the yolk sac. In rest of the fetal life,erythropoiesis shifts from the liver and spleen to the bone marrow. RBCs in this condition contain HbF ($\alpha_2\Upsilon_2$). β- globin expression begins before the birth and completes when baby becomes of 6 months. After this, the 95% of the hemoglobin in the RBCs is adult HbA ($\alpha_2\beta_2$), and rest containing two minor components, Hb A2 and HbF (Taher et al., 2018). Hb F is mostly limited to a small number of RBCs called F cells. The reason behind defective haemoglobin and α thalassemia or β thalassemia is the underlying molecular defects in the α globin or the β globin gene clusters. Besides these, there are also independent intrinsic and extrinsic factors that contribute to the severity of the clinical forms. The thalassemias are also manifested from co–inheritance with variants structure of hemoglobin S, C and E (S. Fucharoen & Weatherall, 2012).

2.2 Beta Thalassemia

Beta- thalassemia one of the most common autosomal recessive disorders worldwide prevalent in Mediterranean, Middle-East, Transcaucasus, Central Asia, Indian subcontinent and Far East. It is also found to be commonplace among people of African ancestry. The highest frequency of the disorder is reported in Cyprus (14%), Sardinia (12%), and South East Asia.Beta thalassemia is characterized by the reduced (+) or the

absence (0) of the beta globin chain in the hemoglobin tetramer protein, which is made up of two alpha globin and two beta globin chains ($\alpha_2\beta_2$)(D. J. Weatherall & J B Clegg, 2001).

The reduction of the beta chain leads to the microcytic hypochromic anemia, manifestation of nucleated red blood cells in the peripheral blood smear and decrease in the level of hemoglobin A (HB A)(Origa & Comitini, 2019). Clinically, Beta Thalassemia is categorized into three groups based on varying level of severity i.e carrier, intermedia and major (Cao & Galanello, 2010).

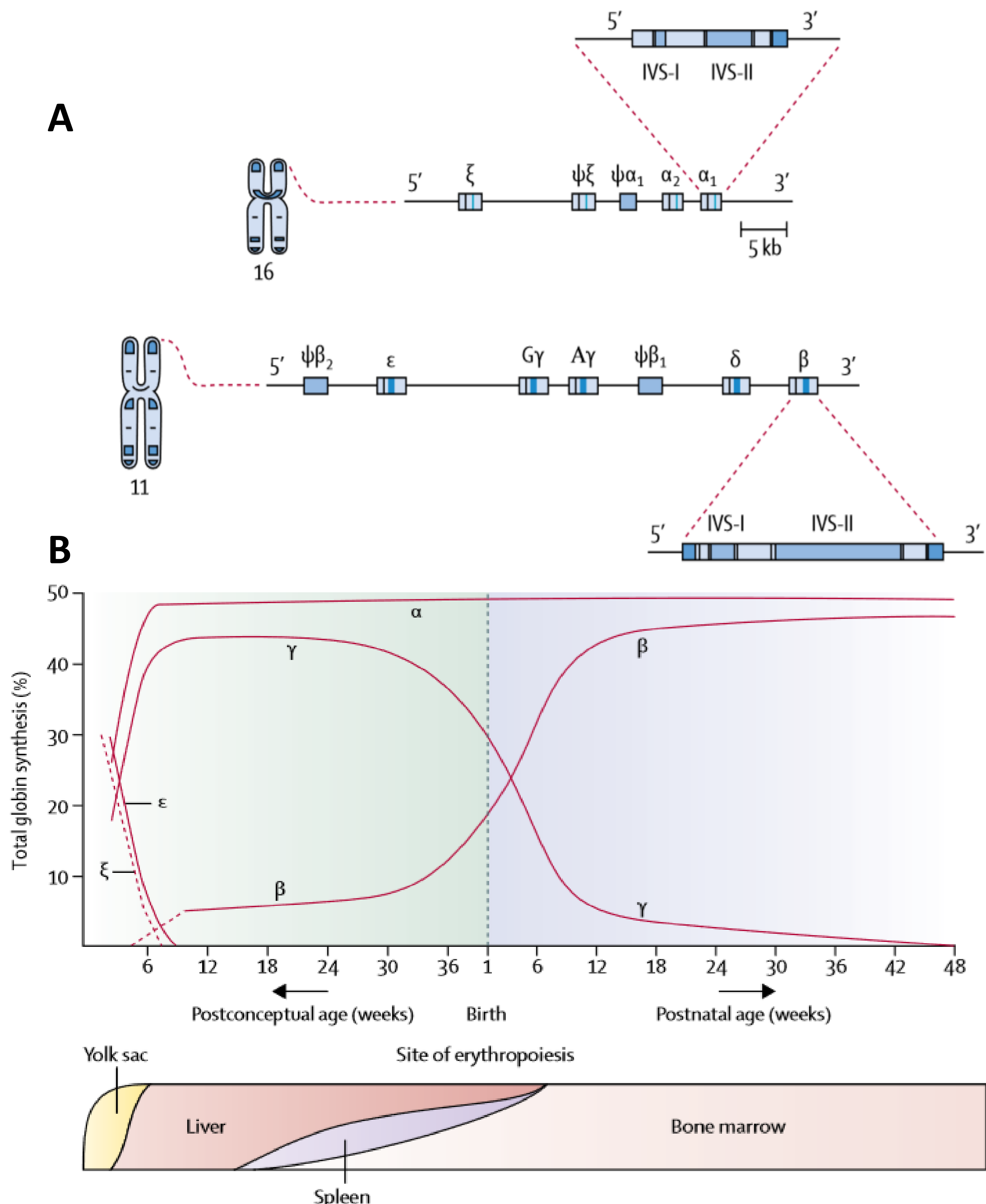


Figure 1 Synthesis of globin at the molecular level. (A) Organization of the α globin and β globin family in chromosome 16 and 11 respectively. (B) Site of erythropoiesis and pattern of globin synthesis during development source : (Taher, Weatherall, & Cappellini, 2018)

Heterozygosity for beta thalassemia is the reason for carrier state which is asymptomatic and has unique hematological features. Thalassemia intermedia is characterized by varying range of severity from asymptomatic to the serious transfusion conditions. Thalassemia major is the severe condition and is also known as transfusion dependent anemia.

The degree of imbalance between alpha and non-alpha chains determine the severity of beta thalassemia. When the beta globin chains are in reduced level in the red blood cells precursors the unassembled alpha chains cause oxidative damage to cell membrane and thus resulting in apoptosis.

The beta globin gene is located on the short arm of chromosome number 11, in the region that also comprises delta globin gene, fetal gamma genes (A gamma and G gamma genes), embryonic epsilon gene and a pseudogene, psiB1. More than 200 mutations leading to the disease describe its molecular heterogeneity. Single nucleotide substitutions, insertion of oligonucleotides leading to frameshift or deletions arefrequently observed mutations.

But the gross gene deletion leading to beta thalassemia is a rare phenomenon. The genetic factors located outside the beta globin cluster could affect the phenotype of beta thalassemia and the factors that are best described are the ones disturbing iron, bilirubin and bone metabolisms.

Life- long red blood cells transfusions and iron chelation therapy to shun the increasing level of iron are the clinical remedies for beta thalassemia (Cao & Galanello, 2010).

2.2.1 Classification

2.2.1.1 Beta- thalassemia major

Thalassemia major or thalassemia intermedia are the results of thehomozygotes case for beta thalassemia. Individuals with beta-thalassemia major are the ones who require regular blood transfusion to survive and come to medical attention within first 2 years. Those who do not need medical attention within fist 2 years do not require blood transfusion and are diagnosed -thalassemia intermedia. Distinction between thalassemia major and thalassemia intermedia at the presentation is a serious issue that must be carefully pursued, which otherwise might lead to unnecessary blood transfusion in thalassemia intermedia and start early transfusion in thalassemia major. Differentiation can be done with analysis of the genotype of the alpha and beta loci and checking for the bettering genetic factors.

Infants who are affected with thalassemia major cannot thrive and go on becoming increasingly pale. They can have feeding problems, irritability, recurrent bouts of fever and splenomegaly may cause enlargement of the abdomen.If the Hb concentration can be maintained 95-100 g/L through transfusion, then growth and development will be normal until 10-11 years of age. After 10-11 years, the affected individuals can be prone to severe complicationsdue to iron overload which depends on their compliance with chelation therapy.

Iron overload can retard the growth and inhibits the sexual maturation, invite those complications observed in adults with HFE- associated hereditary hemochromatosis (HH): involvement of the heart (pericarditis and dilated myocardiopathy), liver (fibrosis,

chronic hepatitis and fibrosis), and endocrine glands (resulting in diabetes mellitus and inefficiency of the pituitary, parathyroid, thyroid and, rarely, adrenal glands). The introduction of vaccination for hepatitis B and development of the screening methods for blood donor based on viral nucleic acid enzymatic amplificationhave significantly reduced the probable infectious complications due to hepatitis B and C virus and HIV, which are relatively common in old patients. Other complications include lung hypertension (secondary to chronic hemolysis), hypersplenism(usually related to late and irregular transfusions), venous thrombosis (occurring specially after splenectomy),osteoporosis (which is associated with many diseases such as hypogonadism, diabetes mellitus, bone marrow expansion (strong association), hypothyroidism, hypoparathyroidism, low insulin like growth factor 1, cardiac dysfunction)(Origa et al., 2005; Voskaridou et al., 2006). Liver viral infection, iron overload and longer survival also increase the risk for hepatocellular carcinoma(Borgna-Pignatti et al., 2004). Individuals who got satisfactory transfusion treatment and treated with proper chelation therapy live beyond the age of 30 years. The most severe complication that arises due to iron overload is myocardial disease caused by trans fusional siderosis, which can be life threatening complication in beta thalassemia. In fact, 71% of the deaths in individuals with beta-thalassemia major are reported to be from cardiac complications (Borgna-Pignatti et al., 2004).

Thalassemia major is currently only seen in some developing countries, where long term long term tansfusion programs are not available. Individuals die before the third decade if they are not transfused regularly. Those individuals who are untreated or poorly transfused show pallor, hepatosplenomegaly, jaundice, growth retardation, brown pigmentation of the skin, poor musculature, genu valgum, development of masses from extramedullary hematopoiesis, leg ulcers and the skeletal changes that result from expansion of the bone marrow. The skeletal changes comprise craniofacial changes (bridge depression of the nose, mongoloid slant of the eye, hypertrophy of the maxillae that can expose the upper teeth) and deformities of the long bones of the legs(D. J. Weatherall & J B Clegg, 2001).

2.2.1.2 Beta thalassemia intermedia state

Patients having thalassemia intermedia manifest heterogeneous clinical symptoms. Principle symptoms are cholelithiasis, enlargement of liver and spleen, moderate to severe skeletal changes, leg ulcers, pallor, extramedullary masses of hyperplastic erythroid marrow, jaundice, a tendency to develop osteoporosis and osteopenia, thrombotic complications resulting from a hypercoagulable state because of the composition of lipid membrane of the abnormal red blood cells (particularly in case of splenectomized patients)(Eldor & Rachmilewitz, 2002; D. J. Weatherall & J B Clegg, 2001).

In thalassemia intermedia transfusion are not required or only occasionally required. Mainly, iron overload occurs from increased intestinal absorption of iron due to ineffective erythropoiesis.

The mechanism of iron overload in beta-thalassemia has been partly known. The mechanism is controlled by hepcidin, a peptide secreted from hepatocytes, which restricts the uptake of iron in the intestine and the release from the reticuloendothelial system. Hepcidin binds to an iron transporter called ferroprotein which is present on the

surface of absorptive enterocytes, macrophages, and hepatocytes. The complex of hepcidin and ferroprotein complex is internalized and rapidly degraded, which explains defect in absorption of iron in intestines (Ganz, 2003). Bone morphogenetic proteins (BMP), control the transcription of hepcidin. BMP activate SMAD protein complex which is translocated to the nucleus and stimulates the transcription of hepcidin. Iron overload enhances the expression of Hepcidin whereas hypoxia and anemia inhibits its expression (R. H. Wang et al., 2005).

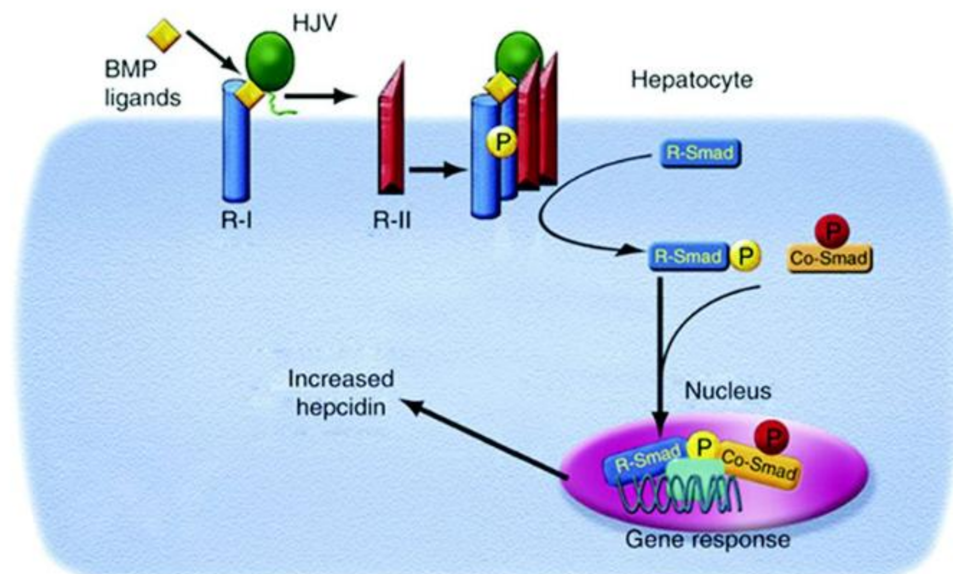


Figure 2 Mechanism showing the control of hepcidin synthesis(Cao & Galanello, 2010).

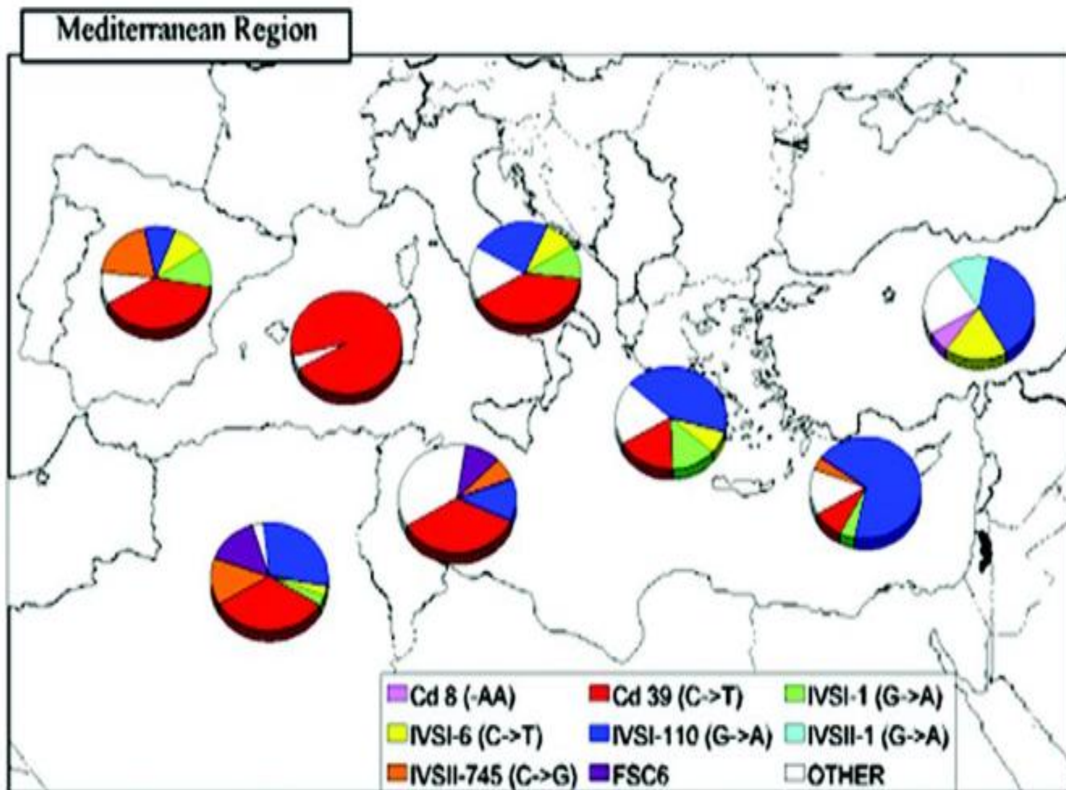
2.2.1.3 Beta thalassemia carrier state

The carrier state of beta- thalassemia is clinically asymptomatic. The hematological features of carrier state are hypochromia (reduced Hb content in the blood cell), increased HbA_2 level (minor component of the adult Hb, made up of two alpha and two delta chains) microcytosis (reduction in the volume of red blood cell) and slightly imbalanced alpha/ beta+ gamma globin chain synthesis (Cao & Galanello, 2010)

The HBB gene mutations are detected by the number of polymerase chain reaction (PCR)-based procedures.

Common methods are the reverse dot blot analysis or the amplification with using a set of primers complementary to the most common mutations in the populations from which the affected individuals originated(Old J et al., 2012).Methods based on microarray and PCR can be of potential substitute due to their rapidity, reproducibility and easy handling (Old J et al., 2012; Ye, Zhang, & Lei, 2007)

If targeted mutation analysis fails to detect the mutation, scanning or sequence analysis can be used. Sensitivity of both mutation scanning and sequence analysis is 99%.



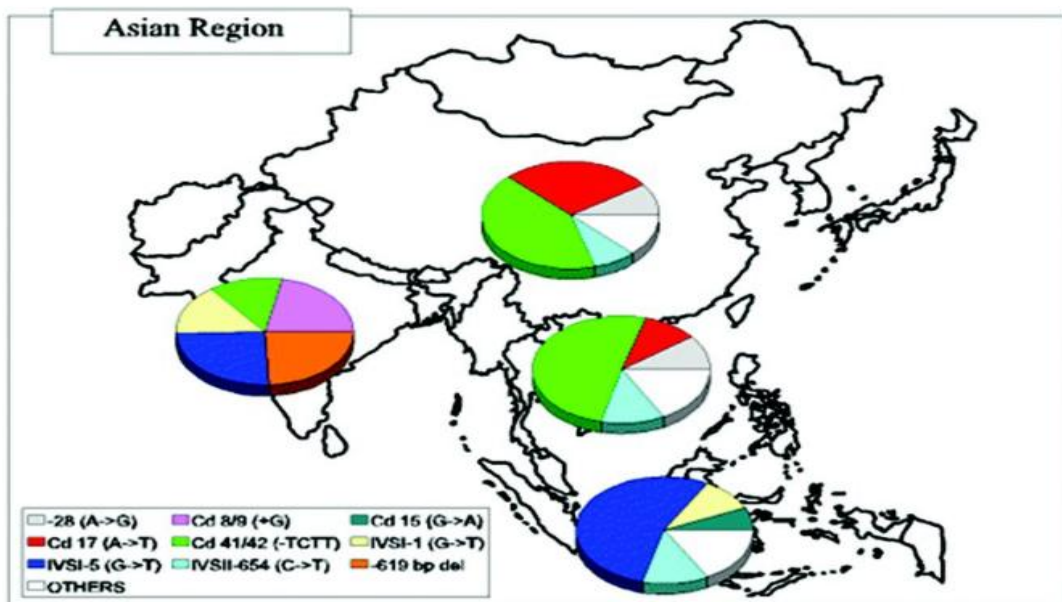


Figure 3 Most common beta-thalassemia mutations in different countries. Source : (Cao & Galanello, 2010)

2.3 Multiplex Amplification- refractory mutation system (ARMS) analysis of point mutation:

The amplification- refractory mutation system (ARMS), which is also known by the other name- allele-specific polymerase chain reaction (ASPCR) is a rapid, reliable and simple method for detecting any mutation that involves single base changes or small deletions. ARMS uses the sequence specific primers that amplifies the test DNA only if the target allele is contained within the sample and does not amplify the non-target allele. The PCR product of ARMS PCR is then observed for the presence or absence of the target allele. Optimized ARMS test are straight forward and reliable to use.

The success of ARMS test depends in the selection of appropriate primer sequences and the reaction conditions. The ARMS technique is based on the observation that the oligonucleotides which are complementary to the given DNA sequence except for a mismatched 3’ terminus will not function as PCR primers under the appropriate conditions. There are guidelines used to produce primers for ARMS that will detect point mutations when used along in combination with the basic protocol. According to the guidelines the oligonucleotides used as a primer should be of around 30 or more base pairs. Primers less than 28 bases long are not recommended while long primers can extend up to 60 mers. In case of mutant specific primers, 3’ terminal base of the ARMS primer should be complementary to the mutation; for the normal specific primer 3’ terminal base should be complementary to the corresponding normal sequence. Normally, additional deliberate mismatches should be introduced at the penultimate base of the ARMS primer to increase the specificity of the ARMS reaction. Because different mismatches have been found to have different destabilizing effects, it is necessary to consider both terminal and penultimate mismatches together. If the mutation-induced terminal mismatch is strong, a weak additional mismatch should be selected. The remainder of the ARMS primer should be complementary to the target sequence.

In case of common primers, the length must be of 30 bases long, should have – 50% G+C content, should have no 3’ complementarity with the ARMS primer or the internal control primers, and no repeated or unusual sequences. (e.g., runs of a single base or palindromes) (Ferrie et al., 1992; Little, 2001).

2.4 Normal physiology of red blood cells development

Red blood cells are important due to their oxygen carrying role in all vertebrates. The hematopoietic progenitors undergo massive division towards erythroid lineage giving rise to approximately 2×10^{11} new erythrocytes on the daily basis. Embryonic and adult are two types of red blood cells shown by the studies related to development (Dzierzak & Philipsen, 2013). Yolk sac is the initial site of development of cells of erythroid lineage. These erythroid cells are with nucleus and are short lived. The epiblast cells which enter through the primitive streak give rise to the mesoderm cells(Lawson, Meneses, & Pedersen, 1991)

The mesoderm cells intrude into to the yolk sac and come in immediacy with endoderm cells. This interaction of two cell layers is pivotal for the inception of erythropoiesis (Baron et al., 2012). Primitive erythrocytes can be spotted in the yolk sac island of

mouse conceptus at 7.5 embryonic day (E7.5) and in conceptus of human at 16-20 days of gestation (Kinder et al., 2001). At the end of gastrulation, the progenitors of primitive erythroid cells develop in the region of blood islands of mammalian yolk sac. As the heartbeat becomes strong enough, the nucleated erythrocytes circulate and mature in steps, nearly in a parallel manner. Up until recently, nucleated erythroblasts were thought of to resemble nucleated erythroid cells of lower vertebrates and thus called primitive. But now it is known that primitive erythroid cells in mammals become devoid of nucleus after several days of entry into the blood stream.

Unlike the primitive erythroid cells, adult (definitive) erythroid cells originate from comparatively complex differentiation order. Hematopoietic stem cells (HSCs) are the source of origin for this hierarchy. A group uncommon HSCs found in bone marrow regularly refills the blood in adult life. HSCs go through long and series of differentiation and proliferation steps to give rise to adult red blood cells. Other functionally unique line of blood cells like lymphocytes, macrophages, granulocytes and megakaryocytes require similar extensive process of differentiation and proliferation. HSCs arise three days later than yolk sac blood island in mouse embryos (Dzierzak & Medvinsky, 2008).

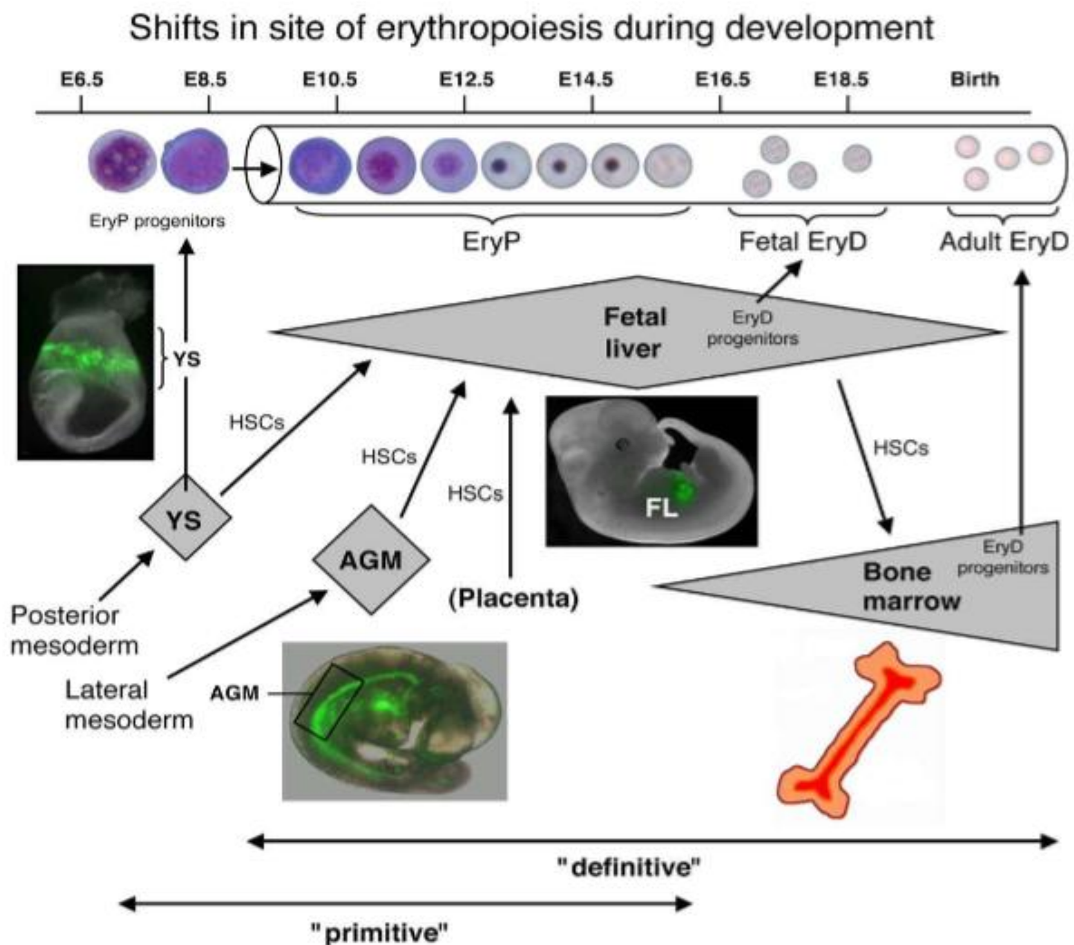


Figure 4: Change in the locations of hematopoiesis during development. (Baron et al., 2012)

The figure 4 illustrates the development of hematopoiesis in the mouse along with the shift in developmental sites – yolk sac, placenta, Aorta- gonad-mesonephros region, placenta, fetal lever and bone marrow. These sites are also closely comparable to

human hematopoiesis. Development of progenitors of ERYp in yolk sac marks the inception of hematopoiesis. The HSCs that develop from the AGM region, large vessels of the embryo and the placenta after the hematopoietic progenitors develop. The green color in the above shown transgenic embryos is due to the GFP Protein which is used as a reporter in the line of ERYp development (Baron, 2013).

2.5 Nucleated red blood cells and pathophysiology

Nucleated Red blood cells (NRBC) are found to circulate in Peripheral Blood in several hematological and non-hematological conditions. Their presence in the blood shows the abnormal immunological conditions indicating blood disorders (Danise et al., 2011). NRBC indicate stress in erythropoiesis and carry significant important in optimizing blood transfusion therapy (Karakukcu et al., 2015).

During ontogenesis NRBCs are found in bone marrow. They are spotted in peripheral blood in several conditions like the metastasized solid cancer, syndromes of thalassemia, extramedullary hematopoiesis, in the case of hematopoietic stress such as in septicemia or hemorrhages followed by acute hypoxic conditions (Christensen et al., 2014; Danise et al., 2011)

NRBCs in the patient with thalassemia are a marker of defective erythropoiesis. They can be used to screen the disease and are useful for optimization of the transfusion therapy Babies born before the 37th week of gestation with the sustained and the higher number of NRBCs are found to have increased mortality rate and prone to disorders (Danise et al., 2009).

Once the NRBCs come in contact with the lysis agent, the outer membrane degrades and the free nuclei get released into the blood, the main reason for the maladies. These free nuclei are of the size <40-50 fl and gets superimposed with clumps of platelets. Results from the hematology Analyzers show that the NRBCs count together with WBC or not as WBC or Counted separately (Kim et al., 1998; F. S. Wang et al., 2003).

Enumerating NRBCs with optical microscopy from the peripheral blood smear takes time, demands specific resources and can be error prone due to observer's biological abnormalities (Briggs, 2009; G. Gulati et al., 2007; Pipitone et al., 2012; Zandecki et al., 2007). The modern instruments used in counting the NRBCs are the hematology analyzers. Different hematology analyzers employ different techniques for enumerating NRBCs. Though hematology analyzers are widely used for counting NRBCs they are not hundred percent sensitive. They may miscalculate the NRBC number when present in low amount and in cases when the lysing method employed by every analyzer cannot lyse the RBCs with same sensitivity (G. L. Gulati et al., 2011; Pipitone et al., 2012)

2.6 Flow cytometry

The use of new instruments in the field of quantitative cytology has greatly improved our understanding in cellular biology. Amongst them, the development of flow cytometry has countless applications in immunology along with fundamental and applied research. From the beginning it employed monoclonal antibodies for the identification of immune competent cells, to study the change in the expression of the surface markers and to distinguish the cells before testing their functions. Further

advancement has been achieved either using single or dual- laser systems, multicolor fluorescence and the facilities of computer for the analysis of multiple parameters. Using this tool, it became possible to analyze and correlate the phases of cell cycle and expression of membrane antigens. Applications have been designed to study the analysis of novel drugs invitro, assessing the immunomodulating treatment and investigations of clinical cases (Cordier, 1986).

Due to the need to culture the microorganisms, classical microbiology techniques are comparatively slow. In addition, the conventional microbiology techniques are difficult with unculturable microorganisms. With the help of flow cytometry microbes can be identified on the basis of their cytometric parameters or by the means of certain fluorochromes that can be used in the independent fashion or bound to specific antibodies. FCM has allowed the development of quantitative procedures for assessing antimicrobial susceptibility and the cytotoxicity of the drug in a rapid, accurate, and highly reproducible way. Moreover, the antimicrobial activity and antimicrobial treatments ex vivo can be monitored with the help of this technique. The most exceptional contribution of FCM is the detection of heterogenous populations with different responses to antimicrobial treatment.

In spite of the advantages, the uses of FCM in clinical microbiology is not widespread due to the lack of access to flow cytometers or due to the lack of insufficient knowledge about the potential of this technique (Alvarez-Barrientos et al., 2000).

Further, FCM has a wide range of applications in cytopathology. Serous effusions are ideal for studies involving the technique as they consist of viable cells in suspension. FCM can be used in the quantitative analysis of molecules related to the responses of chemotherapy, which, together with apoptosis, signifies an important tool for gauging treatment response and prediction of advanced or recurrent cancer (Davidson et al., 2012).

Flow cytometry is a technology that measures the size, the complexity or the granularity and the relative fluorescence intensity of a particle. The optical to electric coupling system determines these parameters based on the scattering of incident laser light from the individual particles. The individual particles come across the laser beam through a stream of fluid. The particles emit fluorescence which is recorded by the detector. A flow cytometer consists of three systems: fluidics, optics and electronics. The fluidics system takes the particles in stream to the laser for exposure. The portion of the fluid which carries the particles is called the sample core. Particles of the size 0.2-150 micrometer are appropriate for detection. Cells must be segregated from tissues before doing the analysis. The optics comprises the lasers to strike the particles, which in turn scatters the light and emits fluorescence, which are directed by the beam splitters and optical filters to the appropriate detectors. The detectors generate the electronic signal proportionate to the optical signals (BDBiosciences, 2002).

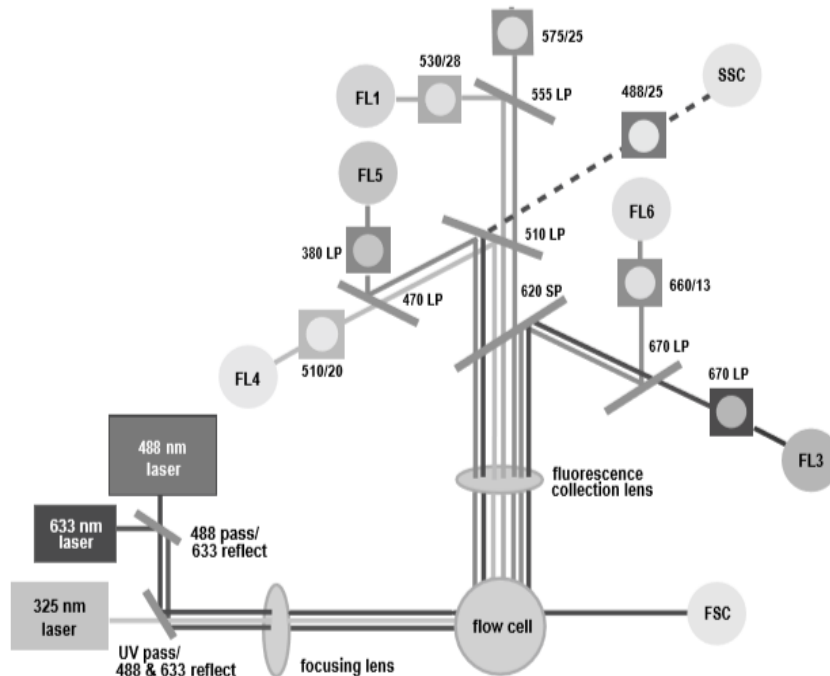


Figure 5: Optical bench diagram of the BD LSR benchtop flow cytometer. Source: (BDBiosciences, 2002)

2.6.1 Fluidics

The fluidics system takes the particles to the laser beam for interception. For optimal interception, the stream carrying particles should pass through the center of the laserbeaming such a way that a single particle passes through the beam at the given time (BDBiosciences, 2002). It can also be the downside of this method that the particles must pass separate and in suspension while passing through the beam. Because if the cells are concentrated in a suspension, single cell suspensions might carry clumps of the cell that tend to aggregate or there may be cells in pseudo-clumps. Even in suspensions of low cell concentration, there is always some probability that the cell might coincide with each other. In a cytometer the fluidics is designed in such a way that, there is decreased probability that the cells will coincide in the point of analysis. In addition, the fluidics should also facilitate the illumination of each cell without obstructing the flow tubing and while cells are flowing in and out of the point of analysis as promptly as possible. One of the ways to confine the cells to a narrow path while passing through the uniformly bright section of the laser beam would be to use a chamber that is optically clear and has a very narrow diameter. But the problem of pushing the cells through the narrow orifice is that , if the cells are large and clumped they tend to block the pathway (Teresa S. Hawley & Hawley, 2004)

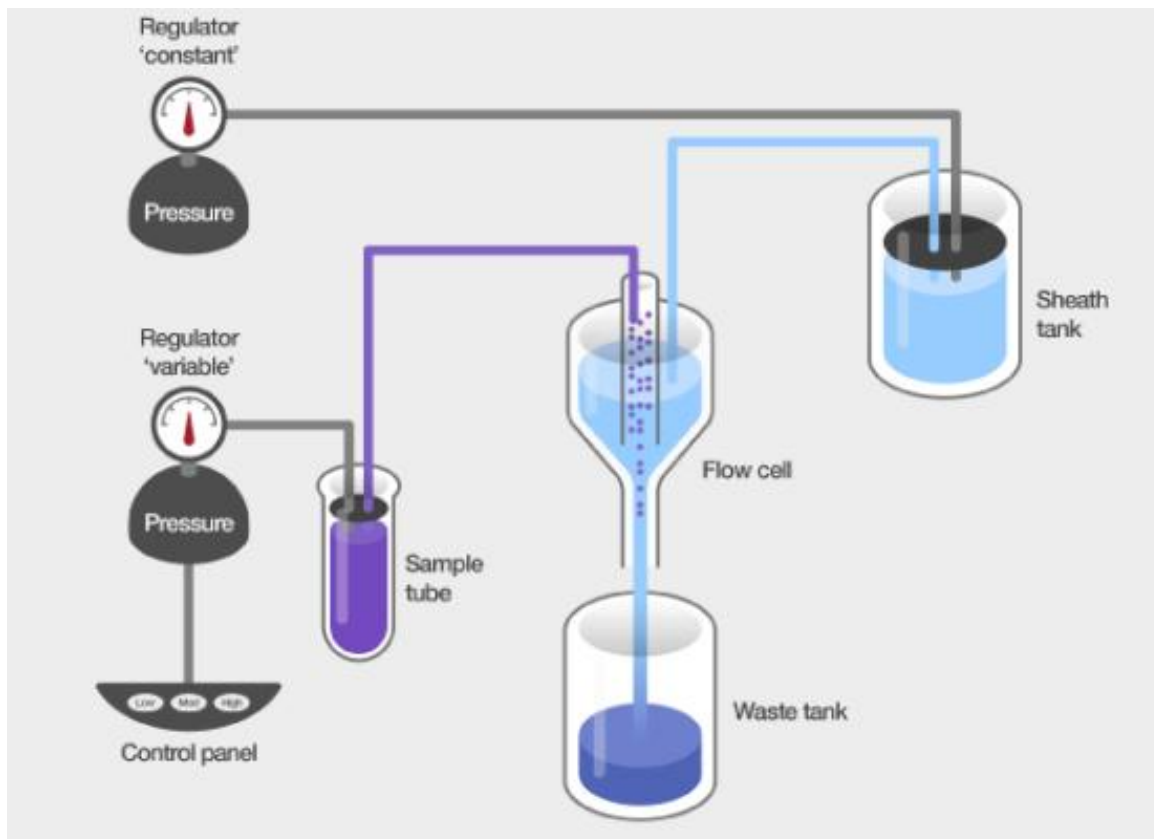


Figure 6 Differential pressure based fluidics system with sheath pressure and sample pressure. Source: https://www.thermofisher.com/ flow cytometry fundamentals

In hydrodynamic focusing the sample is drawn to the center of sheath fluid in flow chamber through the nozzle tip. The flow chamber in flow cytometer is called flow cell. The flow cell is designed in such a way that the sample core gets in the center of sheath fluid in co-axial fashion and yet remains separate.

Even after the use hydrodynamic focusing for the alignment of the cells within a wide stream there is still the requirement for rapid analysis, for better confining of the flow of cells in the center of the laser beam. Also to avoid the co incidence of the multiple cells in the analysis point. These features can be achieved through the design of the flow cell. Some cytometers use optically clear region of the flow cell as in the case of cuvette while other use flow cells where light intersects the stream of the fluid when it comes out of the flow cell through an orifice. In all cases, what flow cell does is – it increases the velocity of the stream, with the diameter of the exit orifice that is narrower than the entrance. The differences in the diameter are usually between 10- and 40- fold, which increases the velocity 100 to 1600 fold. When the stream reaches towards the exit of the flow cell, it narrows in diameter and increases the velocity. Narrowing of the diameter and increment of the velocity tightly confines the path of the cells to the center of the laser beam in such a way that the cells are illuminated similarly and movement of the cells through the laser becomes rapid. In addition, cells in such narrow stream are spread out at distance and are less likely to coincide in the analysis point

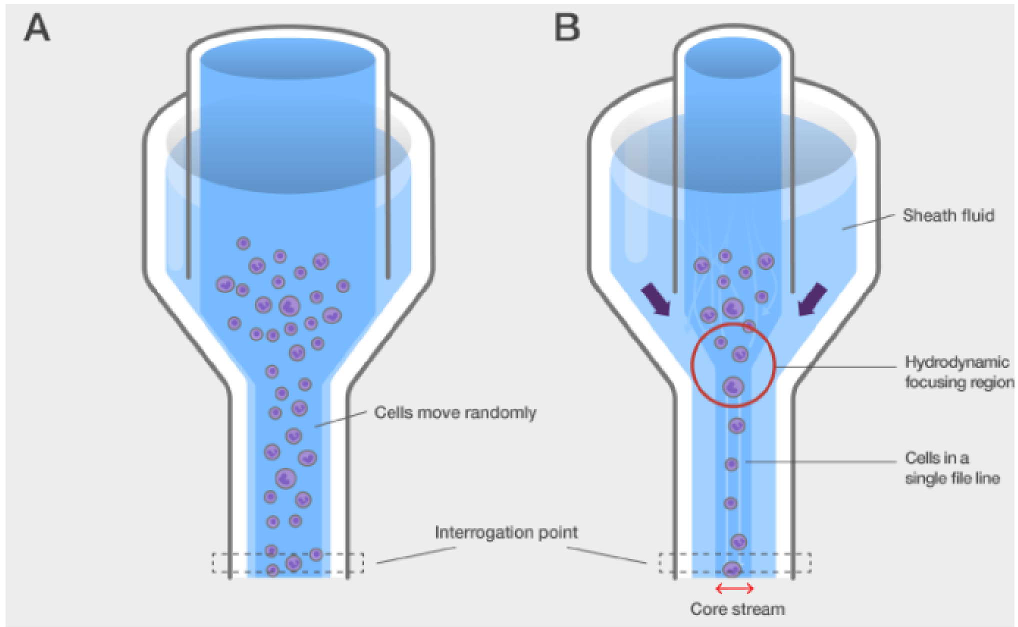


Figure 7: A. Before Hydrodynamic focusing B. After hydrodynamic focusing. Source : https://www.thermofisher.com/ flow cytometry fundamental

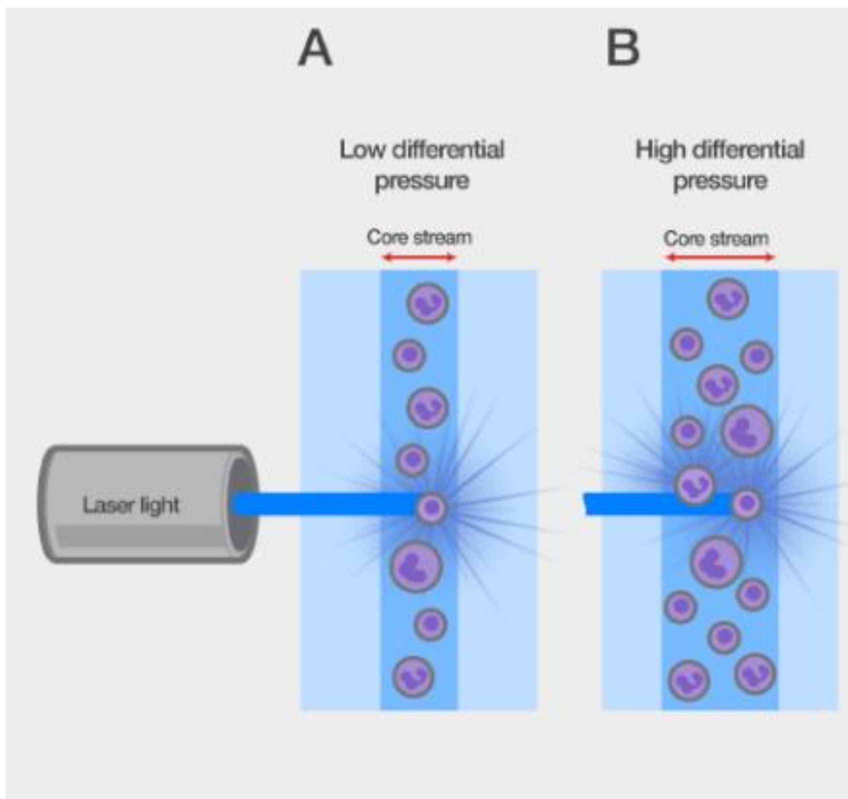


Figure 8: (A) At low pressure, the cells travel through the interrogation point one at a time. (B) Increasing the pressure increases the width of the core stream. Source: https://www.thermofisher.com/ flow cytometry fundamental.

The sample core pressure is always higher than sheath fluid pressure. Flow rate of the sample is controlled by sample pressure regulator by changing the sample pressure relative to the pressure of sheath fluid.

In Benchtop cytometers from the BD company, the sample pressure is applied so that sample moves upward through the optically vivid region of flow cell or cuvette, and the particles pass through the laser beam while they are within the flow cell. Benchtop cytometers are mostly featuredwith the fixed sample pressure settings – LO, MED and HI. BD LSR cytometer has the fine adjustment knob for intermediate pressure.

In case of stream in air cytometer, the sample passes through a small nozzle before being intersected by the laser in open air. The increase in sample pressure increases the width of sample core increasing the flowrate. With the increase in width of the sample core the particles can pass off center of the laser and may strike the laser at less optimal angle. But it can be appropriate depending upon the application.

Generally, a high flow rate is used in qualitative process like immunophenotyping. The samples are acquired more quickly but the data is less resolved as cells could fall off the center of laser. While the small flow rate causes the particles to pass through center and is used experiments where resolution matters much like in case of DNA analysis.

One should always make sure that the fluidic components are operating properly as their operation it is very critical for the particles to strike the laser. The bubbles and debris must be avoided as much as possible

2.6.2 Optics

2.6.2.1 Light Scatter

A particle that comes in the way of light deflects it in several directions. There are several factors that determine the extent of scattering of light. Few of them are physical properties like size and internal complexity of the particle. The cell shape, cell membrane, granule materials inside the cell and nucleus can scatter the light from cell surface and within the cell.

2.6.2.2 Forward scatter(FSC)

The light scattered in the forward direction (forward scatter) is proportional to the size or the area of the particle. The diffracted light from the particle in the forward direction is captured by the photodiode.

2.6.2.3 Side scatter(SSC)

Side scatter is the measure for the granularity or the complexity of the particle. The light that is refracted or reflected from the particle where there is change in the refractive index is the side scatter. The collection of the side scatter light is done at around 90 degrees to the laser light

The measurements of forward scatter and side scatter from the particles allow to segregate the similar populations from the diverse group (BDBiosciences, 2002).

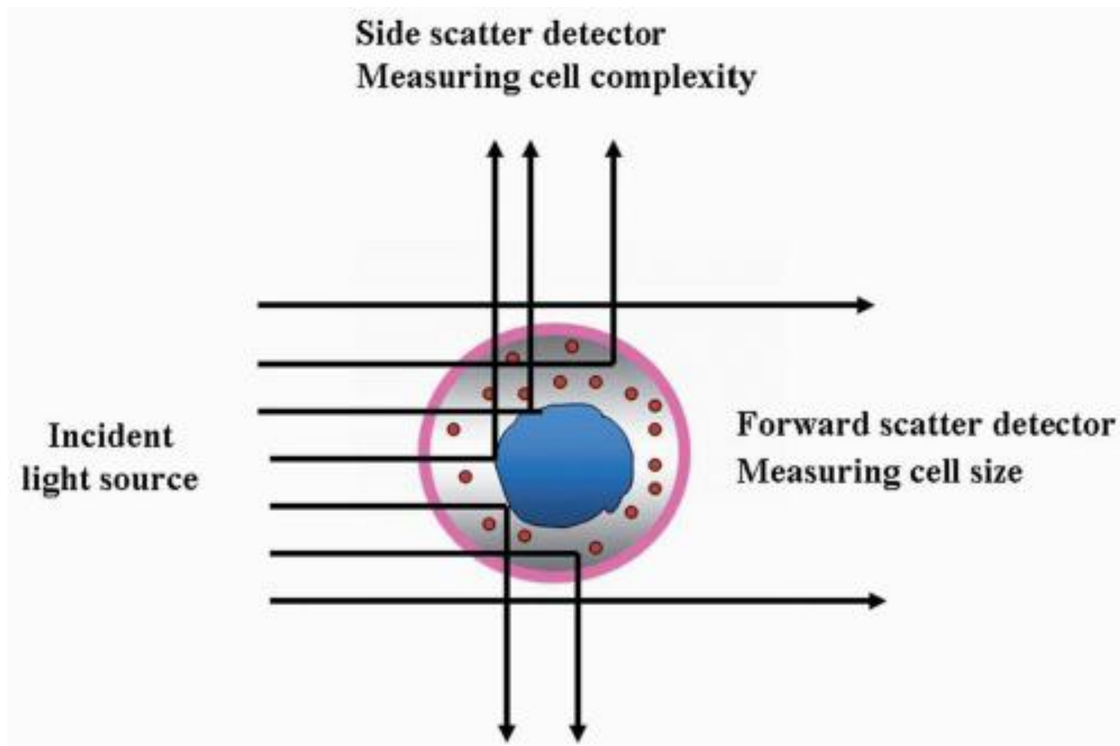


Figure 9: scattering of light in the forward and side directions. Source: https://oncohemakey.com/wp-content/uploads/2016/08/C2-FF4-1.gif

2.6.2.4 Fluorophoresand light

A fluorochrome combined with a given macromolecule is called a fluorophore. Different fluorophores absorb the light at their specific wavelength and emit the light of longer wavelength. Absorption of the light causes excitation which is quickly followed by emission, in nanoseconds, which is called the fluorescence. Several fluorophores are used in flow cytometry, but before considering them for the application, one should understand the absorption and emission principles.

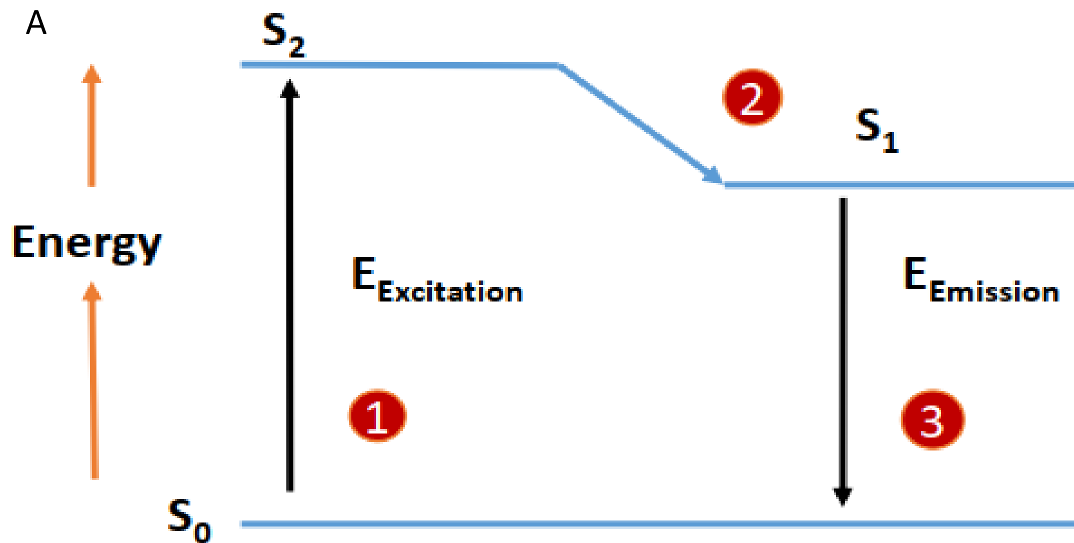
Light is an electromagnetic wave with both frequency and length. The length imparts the color of the light. Visible light has the narrow wavelength band of 380-700 nm between ultraviolet (UV) and infrared (IR) radiation. The visible light can further be dissected in terms of colors – Red, Orange, Yellow, Green, Blue and Violet. Red has the lower energy (longer wavelength) whereas violet has the higher energy.

2.6.2.5 Fluorescence

The electron gets excited to the higher energy level called excited electronic state (S_2, from a resting state (S_0) after the fluorophore absorbs the light, shown in figure 10. Different fluorophores have different energy for this transition. The time for an electron to remain in the excited state depends upon the type of fluorophore. Typically, an electron remains in the excited state for one to two nanoseconds. As conformational change takes place in the fluorophore, the electron decays to lower energy state which is more stable and is called electronic singlet state (S_1). Some of the energy is released in the form of heat during this process. The electron then falls back to the resting state (S_0)/ ground state emitting the energy ($E_{emission}$). The emitted energy is called the

fluorescence. This process continues thousands of time for a single fluorophore which allows the reuse of the fluorophore and hence the intensification of the signal (BIORAD, 2019)

A



B

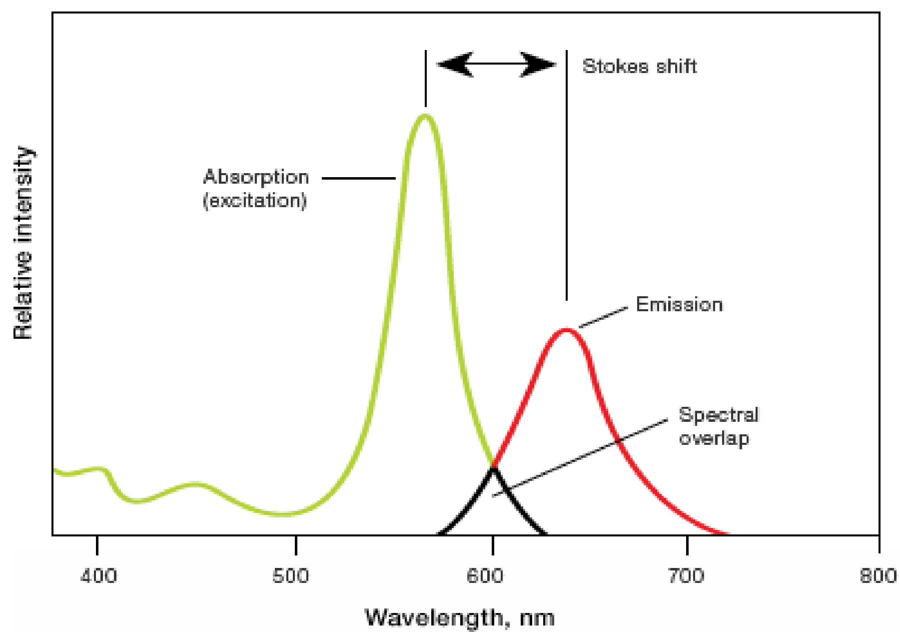


Figure 10. Shows Transition and decay of electron gets excited it moves from ground state (s_0) to the excited electronic state (s_2). Some energy is released as heat, when the electron decays back to the ground state, the energy is emitted in the form of

fluorescence. Stoke shift is defined as the difference between the excitation maxima and emission maxima of a fluorophore.

Every fluorescent compound has its range of wavelength over which it can be excited called absorption spectrum. The energy expended is higher in absorption transition than emitted in the transition causing fluorescence. Thus, for any fluorophore the emission wavelength is longer length excitation wavelength and thus are of different color (BDBiosciences, 2002)

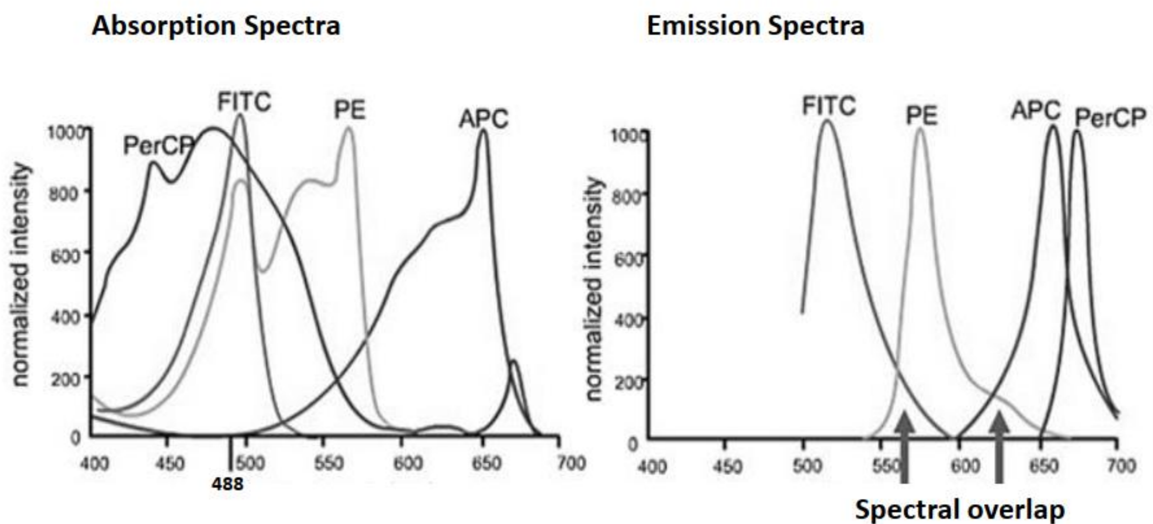


Figure 11: Absorption and Emission spectra of four commonly used fluorophores. Source: (BDBiosciences, 2002)

The laser that is common in use with flow cytometry is the Argon Ion laser. For the reason, the light emitted by the laser is of 488-nm which excites multiple fluorochromes. One of the fluorochromes excited by the laser is fluorescein isothiocyanate(FITC). FITC absorbs light in the wavelength of range 400 to 530nm, but absorbs the highest at the wavelength of 490nm, which is its excitation maximum. Fluorophores are preferably excited at their maximum because the more photons are absorbed the more will be the emission. Maximal absorbance and maximal emission wavelengths are the terms for highest absorption and emission respectively.

Two or more fluorophores can be used simultaneously if each of them can be excited at 488nm and peaks of emission are not in close proximity to each other. One example of such combination is of FITC and PE. The absorption spectra of these two fluorophores are shown in figure 11. Albeit the absorption maximum of PE differs with FITC; at 488nm, the excitation of PE suffices enough to get required fluorescence emission for detection. Moreover, the peak emission of PE is 575nm and of FITC is 520nm, the difference enough apart to detect each signal by different detectors. The quantity of fluorochrome particles and fluorescent signals are proportional to each other

Diverse population of cells in a mixture can be segregated using different fluorochromes. Fluorescent dyes tagged with monoclonal antibodies, specific to antigenic markers on cells, are used to separate different cell populations. The relative percentages of cells can be calculated once they are stained and analysis of FSC and SSC data.

2.6.2.6 Use of fluorescent marker

Fluorescent markers like fluorescent conjugated antibodies are used to target the specific epitope and help in studying the biological properties. Fluorescent markers have wide range of applications such as in, identification and quantification of distinct cell populations, intracellular organelles, cell surface receptors; immunophenotyping; calcium influx experiments; quantifying enzyme activity; measuring nucleic acid content and for apoptosis studies. Single laser can excite the several fluorophores and with the use of filters to capture particular wavelength at proper detector, several parameters can be analyzed in a single time.

2.6.2.7 Single and tandem dyes

Single dyes like FITC, PE, PERCP and APC are being used for several year and currently are facing competition from other more stable and brighter fluorescence dyes.

In tandem dyes a small fluorophore is covalently attached with a larger fluorophore such that when the smaller fluorophore gets excited and reaches its highest excited electronic singlet state, the energy is transferred to the larger fluorophore, activating and causing it to emit the fluorescence. This process is known as Fluorescence Resonance Energy Transfer (FRET). With it, the higher Stokes shift can be achieved increasing the number of colors that can be analyzed from the given laser wavelength.

Table 1 : Fluorophores used in flow cytometry.

Fluorophores	Fluorescence color	Maximal absorbance, nm	Maximal emission, nm	Relative brightness
DyLight 405		400	420	3
Alexa Fluor 405		401	421	3
Pacific Blue		410	455	1
DyLight 488		493	518	4
Alexa Fluor 488		495	519	3
FITC		490	525	3
DyLight 550		562	576	4
PE*		496, 546	578	5
Texas Red		596	615	2
APC		650	661	4
Alexa Fluor 647		650	665	4
Cy5		649	670	3
DyLight 650		654	673	4
PerCP		490	675	2
DyLight 680		692	712	4
Alexa Fluor 700	Infrared	702	723	2
DyLight 755	Infrared	752	778	4
DyLight 800	Infrared	777	794	4

* PE is the same as R-phycoerythrin.

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Source: (BIORAD, 2019)

Table 2: Tandem dyes used in flow cytometry

Fluorophores	Fluorescence color	Maximal absorbance, nm	Maximal emission, nm	Relative brightness
PE–Alexa Fluor 647		496, 546	667	4
PE–Cy5		496, 546	667	5
PE–Cy5.5		496, 546	695	4
PE–Alexa Fluor 700	Infrared	496, 546	723	2
PE–Alexa Fluor 750	Infrared	496, 546	779	4
APC–Alexa Fluor 750	Infrared	650	779	4
PE–Cy7	Infrared	496, 546	785	2
APC–Cy7	Infrared	650	785	2

* PE is the same as R-phycoerythrin.

APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Source: (BIORAD, 2019)

2.6.2.8 Fluorescence Compensation

The amount of signal that a fluorochrome releases in a secondary detector, specific to the other fluorochrome is called the Fluorescence Spillover. Spillover signal is measured in median fluorescence intensity (MdFI). As shown in figure 12.A, the fluorochrome PerCP-Cy5.5 is spilled into the detector of PE-Cy7. This spillover is equivalent to the background in PE-Cy7.

Fluorescence spillover (which can be tackled with compensation) is one of the major problems for scientists which cause the deterioration of the data. Thus, it is of utmost importance in flow cytometry to correctly compensate for the spillover to precisely locate the population, mostly in the case of multicolor experiments. The erroneous compensation of a fluorochrome can transmit to other detectors giving rise to false positive percentage of population through incorrect gating.

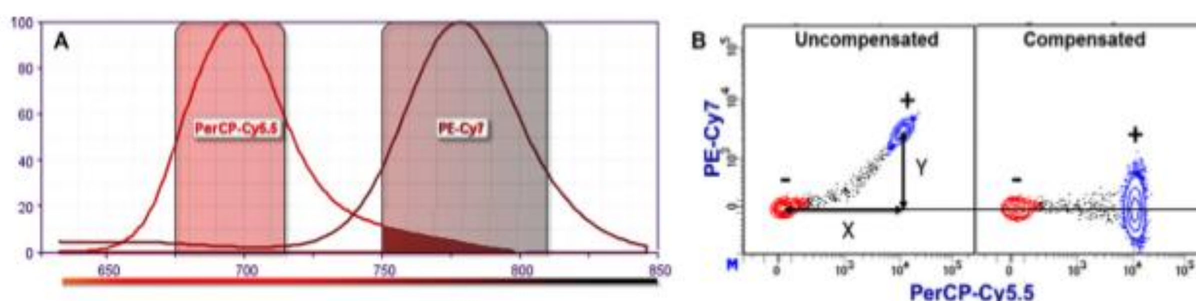


Figure 12: A. The spillover of Cy5.5 into PE- Cy7. B: Compensation. Source: (Cossarizza et al., 2017).

The spillover value (SOV) of PerCP-Cy5.5 into PE- Cy7 can be calculated as Y/X *100%. Compensation as shown figure 12 B, is the process of constructing the compensation matrix using the SOVs, which helps to correct the background due to spillover in every detector.

This process relies on the accuracy of the SOVs determined from the single color controls for compensation. There might be few exceptions but the mathematical

calculation of SOVs is same for all cytometers and software package they employ (Cossarizza et al., 2017).

Acquiring Correct SOVs

Correct SOVs can be acquired by sticking to four simple principles for single-color compensation controls listed below:

The fluorochrome used as a control must be identical to the one used in experiment, i.e with identical fluorescence spectrum. For instance, though Alexa flour® 488 and FITC similar spectrum, Alexa flour compensation control cannot be used for FITC or vice versa. Other instances are Allophycocyanin (APC)/ Alexa flour®647 and APC CY-7/APC-H7. While using tandem reagents, where one can encounter significant spectral differences in lot to lot, it can cause the varied values in the SOV. Thus, to deal with such cases it is suggested to use single color lot specific compensation controls.

It is required that the auto fluorescence of the negative and positive population be alike. i.e. Lymphocytes and cell lines must not be the used for the same control. If a given stained cell lines are used a positive control, then the unstained cells of the same line must be used as a negative control. The positive control is expected to be as bright as possible (Cossarizza et al., 2017).

2.6.3 Electronics

2.6.3.1 Signal and Pulse processing

The fluorescent light and scattered signals generated by the particle are manifested in a stream of electrons (current) from the anode of PMT. The scale of the current is proportional the number of photons that strike the photocathode and therefore is also proportional to the intensity of scatter or fluorescence produced from the particle. Once the particle reaches the laser, the current output of the detector (photomultiplier tube (PMT) starts increasing and escalates further to the maximum when the particle is at the center of the laser.

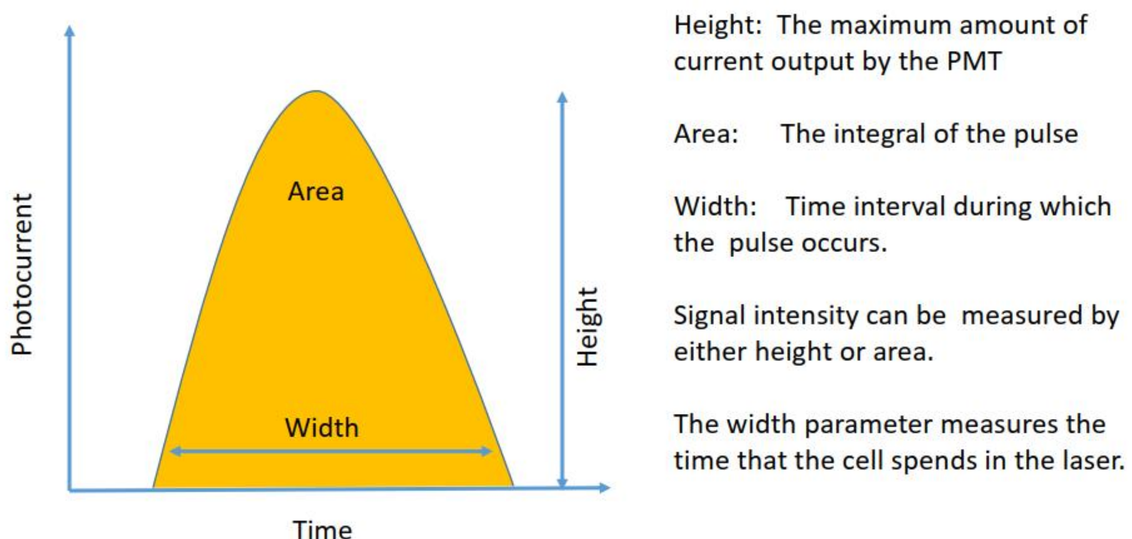


Figure 13: Quantification of pulse from its Height, Area and Width. Source: (BIORAD, 2019)

All pulses produced by particles cannot be accepted as events. The PMTs are very sensitive and can detect the signals from other sources. These sources can be debris, very small particles, dust and stray light. Usually, the number of pulses generated by these particles is higher than the number of pulses produced from particles in investigation. So inclusion of these pulses could mar the relevant data. Thus it is necessary to avoid these meddlesome data. This is achieved by assigning a level in that parameter as the threshold. The pulses that fail to exceed the level of threshold are overlooked. While those pulses that exceed the threshold level are processed by all detectors.

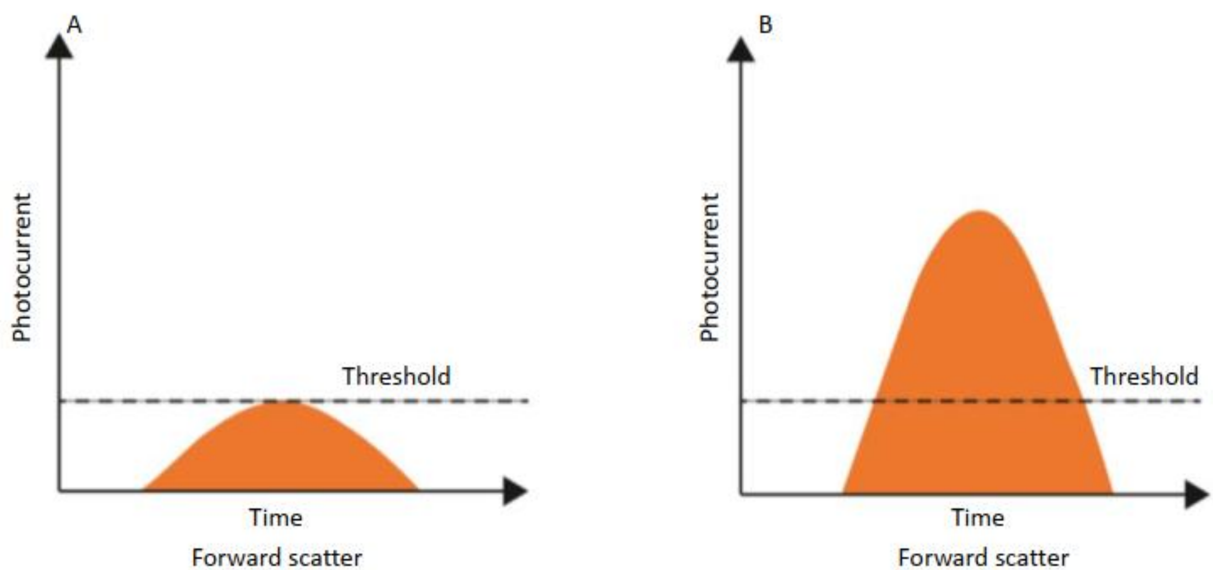


Figure 14: Excluded pulse (A). Processed Pulse (B). Source: (BIORAD, 2019).

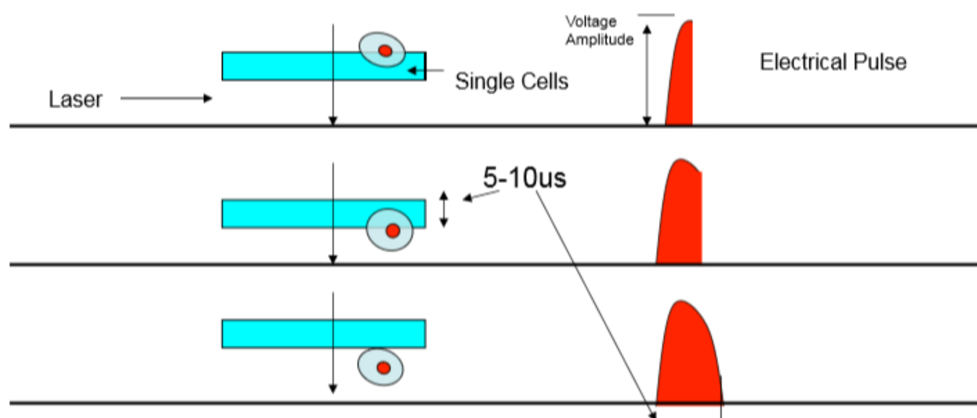


Figure 15.The passage of cell from laser and the generation of the pulse. Source: Basic principles in flow cytometry, Hector Nolla.

After the generation of the pulses they must be quantified for fluorescent signals to be displayed on plots, analyzed and finally interpreted. This task is carried out by the signal processing electronics. Most of the flow cytometers and cell sorters now depend upon the digital system. The process of converting the analog current from PMT to digital version is called Sampling. This is done by analog to digital converter (ADC). At the given instant, a sample of pulse catches the signal, turns it into a digital value and stores it. All the samples together represent the entire pulse and the signal from the particle.

The quantification of the entire pulse is done by the electronics. Height, width and area of the pulse are calculated. Height and area or the maximum and integral, respectively are taken for measuring the signal intensity as their values are proportional to the number of photons that interact with the PMT. The width is proportional to the time that the particle spends in the laser. It can be used for doublet (the particles that are held together so closely that the system recognizes it as a single pulse and event from singlets).

Fluorescence amplification uses the log amplification as it compresses the strong signals and expand the weak signals and gives out the distribution which can be displayed easily on a histogram. In case like DNA analysis, where the minuscule differences must be assessed, linear scale is used.

Measurement from every detector is called parameter. The parameter can be shown as width, area and height values on the plots – histogram and dot plots in flow cytometry. These are used for comparing populations, measuring the fluorescent intensity and segregate the sorting populations (BIORAD, 2019).

2.6.4 Application of flow cytometry in immunophenotyping

2.6.4.1 CD4 immunophenotyping in HIV

Flow cytometry is regarded as the gold standard for enumerating both the absolute number and the percentage of $CD4^{+}$ T cells using three or four color immunophenotyping. It is deemed reliable due to its accuracy and reproducibility and is also the choice when the large processing of samples is required.

$CD4^{+}$ counting using flow cytometers requires routine maintenance, additional equipment and costly reagents, which makes it an expensive technique, but the development of simple and affordable flow cytometers has helped to install the technique in resource qualified settings (Pattanapanyasat, 2012).

There has been significant development in the enumeration techniques of $CD4^{+}$ cells, over the last 25 years. In all these years, the flow cytometry has developed from a complex, expensive, requiring specifically trained personnel, to simple, affordable bench top instruments that can be operated by the individuals with minimal training.

By this time, it is well recognized that the AIDS progresses, decreasing the $CD4^{+}$ count, which plays the crucial role in regulating the immune system and its response. The binding of HIV to the CD4 antigen and chemokine receptor 4 (CXCR4) or the chemokine receptor 5 (CCR5) present on $CD4^{+}$ T cells for replication leads to the dilapidation of the immune system. Thus, observing the $CD4^{+}$ T cells during the HIV infection is important in studying the disease progression and response to the Antiretroviral therapy (Pattanapanyasat, 2012).Two methods are generally used for generating $CD4^{+}$ T Cells count using flow cytometry.

2.6.4.2 Dual Platform Approach:

The dual platform for counting $CD4^+$ T cells employs a flow cytometer and a hematology analyzer. Flow cytometer gives the percentage of $CD4^+$ T cells and hemataology analyzer gives the absolute lymphocyte count. Thus, absolute $CD4^+$ T cells count is calculated by multiplying the $CD4^+$ T % with absolute lymphocyte count. The $CD4^+$T % value is more important than the absolute value, as percentage value does not vary as significantly as absolute value (Malone et al., 1990; Moodley et al., 1997). Based on this, World Health Organization (WHO) recommended the use of %$CD4^+$T- lymphocytes in casting decisions towardsthe inception of Antiretroviral Therapy (ART) in infants below five years of age (WHO, 2006).

Yet, dual platform can be error prone as it relies on hematology analyzer for enumerating absolute $CD4^+$ T cells (Brando et al., 2000; Gelman & Wilkening, 2000; Malone et al., 1990). Single platform relies only on flow cytometer from which both absolute and $CD4^+$ percentage can be obtained using a single tube (Bergeron et al., 2002; Nicholson et al., 1997). As $CD4^+$% is calculated from the lymphocyte population, it is required that the lymphocyte population is pure. A trustworthy method, developed by Loken et al is used to assess lymphocytes based on the differential expression of CD45 (Loken et al., 1990), also called CD45 gating. CD45 is a pan-leukocyte marker expressed in all leukocyte population at different intensities but densely expressed in lymphocyte population.

Three color immunophenotyping uses three monoclonal antibodies tagged with fluorescent dyes, used to label. A cocktail of three mAbs is available commercially as TriTESTTM (from Beckton Dickinson (Nillakupt et al.) Biosciences; sanjose, CA, USA). The mAbs in the TriTEST are anti-CD3 conjugated with fluorecein isothiocyanate (FITC), anti-CD4 tagged with phycoerythrin (PE), and anti-CD45tagged with peridinin chlorophyll protein (PerCP).

Cocktail of these antibodies are also available from the Beckman coulter flow cytometer with the name Opticlone TM (Beckman Coulter (Igout et al.), Miami, FL, USA) and consists anti-CD3-FITC, anti-CD4-PE, and anti-CD45-phycoerythrin-cyanin 5.1 (PC5). Cocktail of four mAbs, from BD as BD MultiTEST™ for CD3-FITC, CD8-PE, CD45-PerCP, and CD4 - Allophycocyanin (APC) or BC Cyto-Stat tetraCHROME™mAbs to CD45-FITC, CD RD1 (phycoerythrin), CD8 phycoerythrin-Texas Red-x (ECD), and CD3-PC5 can be selected while labelling blood sample.

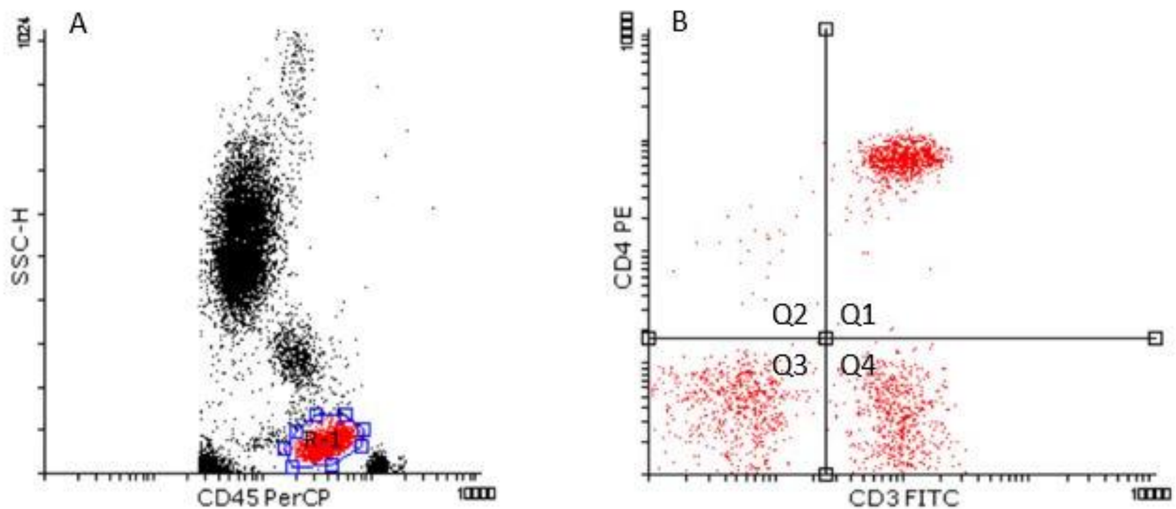


Figure 16: Representation of the two parameter cytometry dot plot of a normal sample

Figure 16. shows the representation of the two parameter cytometry dot plot of a normal sample stained with BD Tri-test of mAbS TO CD3-FITC/CD4-PE/CD45-PerCP threecolor reagent. The SSC-H/CD45-PerCP dot plot In figure 16A shows all the leukocyte population, with lymphocyte population in the region R-1. Determination of % $CD3^+/CD4^+$ population in upper right quadrant Q1, $CD4^+$ population in quadrant Q2, $CD3^-$ $/CD4^-$ population in Q3 and $CD3^+$ population in Q4 obtained from R1 gated population.

Once the staining is completed with the lyse-no-wash method the sample is introduced into the BD flow cytometer. Then the threshold value is set for the fluorochrome (PerCP) conjugated to the CD45, in the logarithmic scale, covering almost all leukocyte population in the linear side scatter (SSC-H) against log CD45 plot. Then, the gate is created around the lymphocyte population as in figure 16 A defining them as high CD45 and low SSC-H (R1). Cells outside this gate are considered- monocytes, with intermediate CD45 and medium SSC-H value and, granulocytes with low CD45 and high SSC-H value. After establishing the lymphocyte gate, the percentage of $CD3^+/CD4^+$ T (double positive) can be automatically generated in the upper right corner in two parameter dot plot (Figure 16 B). If the target is to find out the percentage of $CD3^+/CD8^+$ double positive population then another mAb reagent panel, CD3- FITC/CD8-PE/CD45-PerCP for flow cytometers from BD or CD45-FITC/CD3-PC5/CD8-ECD for flow cytometers from BC, is required.

In immunophenotyping technique with four- color, lymphocyte gate that corresponds to CD45 bright and SSC-H low ($CD45^{bright}/SSC\text{-}H^{low}$) is created to contain all lymphocytes and some contaminating non-lymphocytes. Following, a two parameter dot plot of SSC-H against FSC-H light scatter, gated on region R1 is created with region R2 to comprise all CD45 bright lymphocytes (R2) (figure 17 B).The logical gating based on the events from R1*R2 is used to generate percentage of the double positive population of both $CD3^+/CD4^+$ and $CD3^+/CD8^+$. For calculating the absolute $CD4^+$ lymphocyte count, the $\%CD3^+/CD4^+$ is multiplied by the absolute lymphocyte count generated from the hematological analyzer. Use of CD45 gating technique is worth noticing and has several advantages. This technique is found to be reliable in distinguishing lymphocyte in the

region $CD45^{bright}$/$SSC\text{-}H^{low}$ even if sample comprises debris along with non-lymphocytes population (Pattanapanyasat et al.,1994) and this permits the use of lyse no wash method for staining. Further, the percentage lymphocyte can be calculated from the leukocyte population. And, as this method does not require the use of isotype control, the cost is also reduced.

The dual platform relies on three test results: the total white blood cells and lymphocyte percentage from the hematological analyzer and the percentage of $CD4^+$ T lymphocyte from flow cytometer. It has been well known that generating leukocyte count and differential count using hematological analyzer could hinder the actual count of $CD4^+$ T lymphocytes subpopulation, as hematological analyzer is found to include variable factors, interfering and deviating the actual count of $CD4^+$ T lymphocytes. Such are the issues encountered during inter-laboratory studies, employing different hematology analyzers (Whitby et al., 2002). Studying the individual patient follow up, dual platform approach resulted in higher variability compared to values of %$CD4^+$ T lymphocyte (Pearson' coefficient variation (CV) in the range 20-33% versus CV of 10-16%).

Moreover, the lymphocyte population demarcated by hematological analyzer and the flow cytometer might differ from each other. Yet, hematological analyzers are still recommended in guidelines of several institutions (Gelman & Wilkening, 2000) and are in use as they are cheaper compared to single-platform approach.

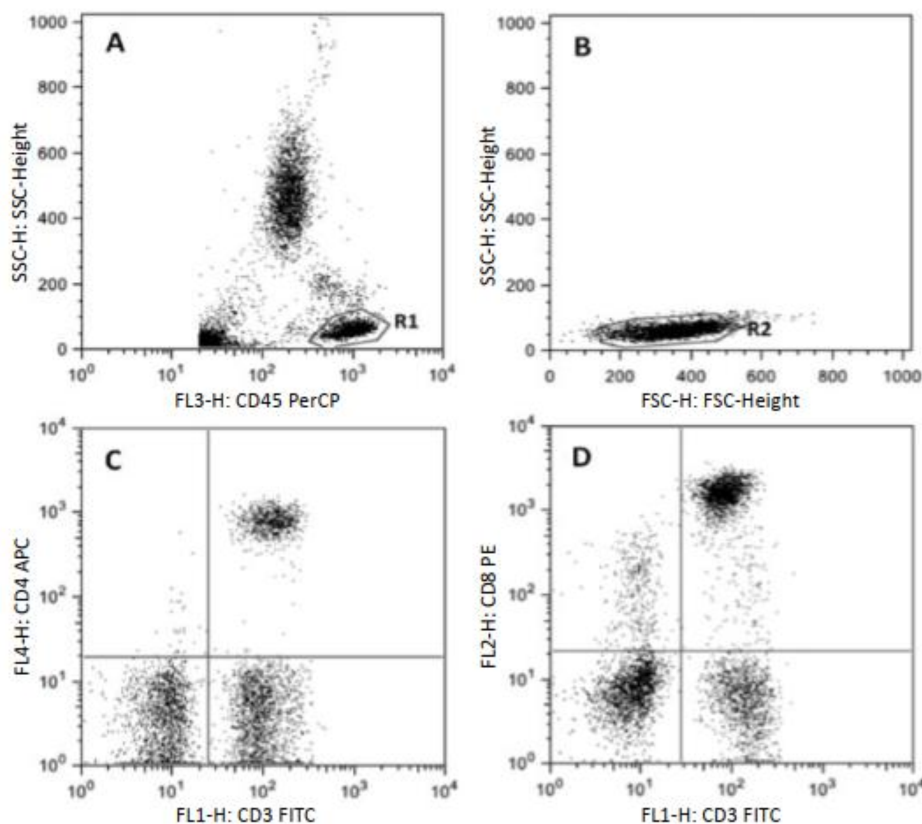


Figure 17 : Two parameter flow cytometric dot plot of whole blood. Source: (Pattanapanyasat et. al)

Figure 17 shows the two- parameter flow cytometric dot plots represent whole blood stained with BD multiTEST of mAbs CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC. (A) The dot plot CD45 PerCP/SSC-H manifests the leukocyte population and region R1 showing the lymphocyte population. (B) The dot plot FSC-H vs SSC-H shows the pure $CD45^+$ lymphocyte population in the region (R2) obtained from R1 gated population of cells. The logical gating R1*R2 is used to identify both $CD3^+/CD4^+$ (C) and $CD3^+/CD8^+$ T-lymphocytes .

2.6.4.3 Single platform approach:

Single platform approach generates the $CD4^+$ T lymphocytes count without the use of hematological analyzer. This is done by the adding the known number microbeads or micro fluorospheres to the CD4 stained samples or by taking a precisely determined volume of the blood sample for $CD4^+$ count. This approach demands high level of pipetting accuracy and requires strict control over the final dilution factor. The reverse pipetting technique or the preprogrammed electronic pipette is considered most reliable.

Currently, an increasing number of laboratories use single platform approach (Mandy et al., 2002)due to higher reliability and the reproducibility compared to dual platform approach.

Alternatives for single-platform approach

Glutaraldehyde-fixed chicken red blood cells as bio beads

The cost of using single platform bead based approach is more expensive (US $ 25-30) than using dual platform approach that employs hematological analyzer (US $ 15-20). An extra cost of US $ 6-8 (local price) in using the reference microbeads (in lyophilized form or in liquid suspension) averts the wide use of single platform bead based approach in countries with qualified resources. The other single platform approach, volumetric FCM system, is simple, reliable and cost effective but the unavailability of the new machine in developing countries and an additional cost involved with its installation makes it less likely to be an affordable option.

Thus, FCMs that uses dual platform approach are the first choice in many developing countries like India and Thailand though these systems are reported to have several drawbacks including inaccuracy, inconvenience and variability in results due to use of different hematological analyzers with different protocols (Whitby et al., 2002).

Red blood cells (RBCs), from chicken and rainbow trout become fluorescent after they are treated with glutaraldehyde. The fluorescence is from the compounds formed after the aldehyde binds to the amino group of protein in the RBCs. These fluorescent RBCs are often used in calibration of FCM and as standard for determining DNA content (Noguchi & Browne, 1978; Pattanapanyasat et al., 2010). And as they fluoresce with brightness tantamount to immunofluorescently stained cells, they are also used in stimulation of the cells labelled with fluorescent antibodies. Further, chicken red blood cells are easy to fix with glutaraldehyde and are cheap (Pattanapanyasat et al., 2010)

Preparation of Glutaraldehyde-fixed CRBCs

Fourteen week old Gallus gallus domesticus chicken was venipuctured in wing to draw twenty milliliters of fresh whole blood using a 50 ml syringe that has 5ml of heparin solution (1000 U/ml). The guide for Research/Teaching Proposals Involving Animal subjects [Siriraj Animal Care and Use Committee (SI ACUC)] was followed for keeping, maintaining and treating chicken. The blood was transferred to a 50-ml centrifuge tube after heparinized and was centrifuged for 5 minutes at room temperature (24°to 26°C). A Pasteur pipette was used to remove supernatant and buffy coat consisting all white blood cells. The pellet of CRBC was washed with phosphate buffer saline (PBS) with pH 7.4 for 2times. The pellet was then resuspended in 25ml of PBS.25 ml of 0.2% freshly prepared glutaraldehyde solution (prepared with addition of 0.8ml of 25% glutaraldehyde in 100ml PBS} was added to the suspension of CRBC. The fixed CRBCs were left for overnight incubation with gentle shaking, washed with PBS and resuspended in 50 ml 0.1 M glycine solution (which is prepared by dissolving sodium glycinate, 9.8 g in 900 ml distilled water) for 30 min at 4°C. After centrifugation at 250 × g the fixed CRBC pellet was washed twice with PBS containing 1% sodium azide (sigma), and was suspended in 20ml volume of PBS- azide solution. Lastly, Guava Personal analyzer (Guava Technologies, CA, USA) employing Viacount software (Guava)was used to determine the density of glutaraldehyde fixed CRBC solution. The final density of the cell CRBC solution was adjusted to 12×10^{6}CRBCs/mL. Aliquots (1mL each) of glutaraldehyde fixed chicken red blood cells solution were prepared and stored between the temperature of 4°C to 8°C to be used as stocks. Cells in each aliquot were counted for 10 times using the Guava and the same lot of CRBCs with the density of 11715/µL or 58575/5µL was used for this study. An internal control set of check beads from Guava was used *to* measure the precision and accuracy of the Guava system before subjecting glutaraldehyde –fixed CRBCs to the count. In the settings, where Guava facility is not available, density of glutaraldehyde-fixed CRBCs can be calculated by using the reference microbeads [eg, TruCOUNT from Becton Dickinson Biosciences (BDB), San Jose mixed with the known volume of fixed of CRBCs and prepared for count on FCM.

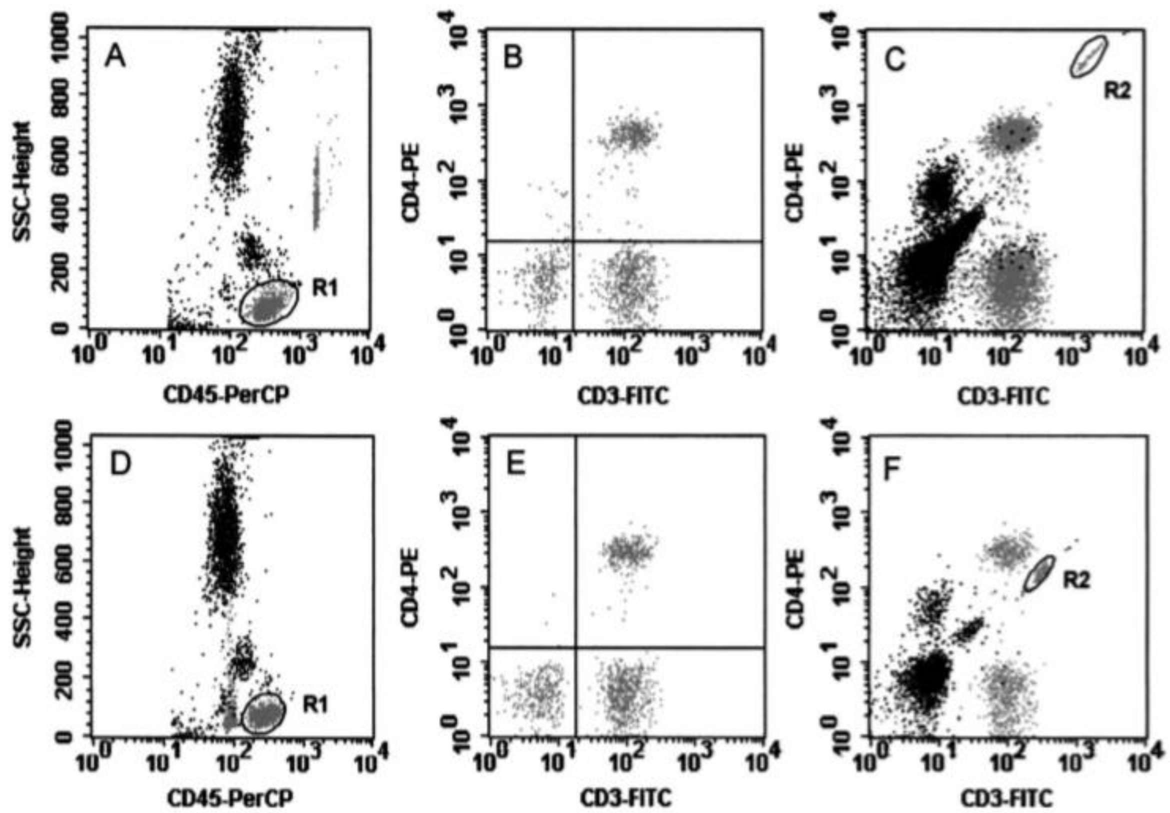


Figure 18: The two parameter dot plots with microbeads and glutaraldehyde-fixed CRBCs. Source:(Pattanapanyasat et al., 2010)

The two parameter dot plots figure 18- (A-C) show MultiSET software algorithm of the 3-color Tritest/ Trucount method, plots(D-F) show algorithm of CellQUEST software of three-color Tritest/CRBC method. The Region R2, plots (C, F) showTrucount microbeads and glutaraldehyde-fixed CRBCs and the region R1, plots (A, D) depicts the lymphocytes. (Pattanapanyasat et al., 2010).

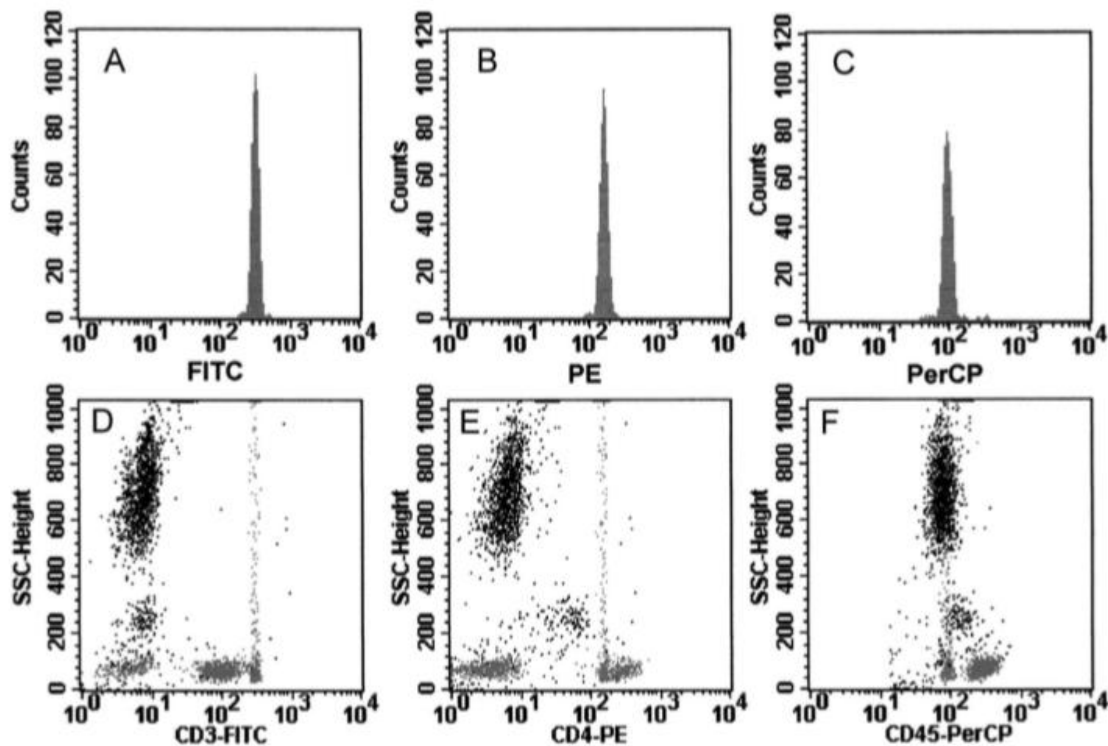


Figure 19: Fluorescence from glutaraldehyde fixed CRBCs. Source: (Pattanapanyasat et al., 2010)

Histograms in figure 19 showing spontaneous fluorescence from glutaraldehyde-fixed CRBCs. Histogram plots A, B and C show auto fluorescence of CRBCs into FITC, PE and PerCP channels respectively. This auto fluorescence of CRBCs is compared with white blood cells stained with corresponding FITC (D), PE(E), and PerCP (F) conjugated monoclonal antibody. This autofluorescence of CRBCs is found to be higher than FITC, but lower than CD4-PE and PerCP (Pattanapanyasat et al., 2010).

2.7 Hematology analyzers

"*. . . for the blood is the life*", Deuteronomy 12:23

Even in ancient times, blood was deemed a remarkable body fluid that was the essence of life, with enigmatic properties that provided the sustenance for life. Understanding those enigmatic properties only became possible when components of blood were characterized based on their appearance and number. All these critical steps led to the development of microscopy facilitating the visualization of blood cells which was followed by subsequent advancement of the techniques to measure physical properties of the elements formed. And commensurate advancement in electronics was achieved for capturing the information. Visualization of individual human and plant cells including bacteria became possible with the development of optical microscope. Even the microscope made 300 years ago, by Van Leeuwenhoek, allowed the observation that the blood comprises of small red globules. And eventually their size was determined. The next two centuries witnessed further developments in optical microscopy like the addition of an eye piece to form a compound microscope, improvised optics, with

objective lenses consisting of several lenses to correct for possible distortions (Green & Wachsmann-Hogiu, 2015).

The first quantitative method for blood testing was the counting of blood cells and has been widely used in the clinical settings. Initial methods involved the wet sample preparation on the slide chamber, visualization and using optical microscope for manual counting. Later the counting employed the flow method together with scattering/fluorescence technique or the impedance technique that could enumerate the cells one by one.

The credit for the first blood count is honored to Karl Vierordt at the University of Tubingen, who published research articles on the topic in 1852. In his method, he used the capillary tube to draw the blood and spread the known volume into a slide, and did the microscopic analysis. Later on, method was improvised, including sample preparation and refinements of the counting chamber. These improvements made the count more accurate but slower (Green & Wachsmann-Hogiu, 2015). Gray reviewed and published the subsequent modification. The dyes invented by Paul Ehrlich in the late 1870s for the first time allowed the differentiation between different white cell types. It was clear by the time that the number of blood cells varies in many diseases and thus is important to do the blood count more accurately. Manual blood counts, comparatively laborious, involved keeping the known volume of the blood in the chamber and counting an individual cell. The total count is then calculated from the geometry of the chamber and the known volume. Though manual count is still in practice today because of its simplicity, it is labor intensive, time consuming and prone to errors. Hematologists and engineers started to work together, as the automated counting methods became a necessity, to find the solution to the problem. Over the subsequent decades, impedance measurements, flow-based cytometers using light or both were developed. These machines could enumerate large number of parameters including erythrocytes, leukocytes and thrombocytes and could differentiate the various subtypes of leukocytes. Also, the qualitative differences found in the red blood cells in size, shape and degree of hemoglobinization indicated certain types of anemia and the capacity to concurrently and independently quantify the concentration of hemoglobin and hematocrit (or packed cell volume), together with the count of red cells. This provided Maxwell Wintrobe with indices of red cells that encompassed mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. With this, anemia could be classified into subtypes – microcytic, normocytic, or macrocytic; and hypochromic, normochromic, or even hyperchromic as per the level of hemoglobinization. Later quantification of the size distribution of red cell populations as red blood cell distribution width was also possible which led to further categorization into homogeneous and heterogeneous morphologic subtype (Bessman, 1981).

2.8 Why do the blood count?

A complete blood count (CBC) gives a rapid and cost- effective assessment of various parameters of a patient's health as well as important hints on the presence of diseases. CBC gives the number of three basic cell types along with the shape, size and level of hemoglobinization of RBC and WBCs with different morphologies; also called WBC differential. CBC provides clinicians with the data which, for the most of the cases, is the basis of diagnosis of a patient disease and responses to treatment. At its very basic level

the CBC provides information on various abnormalities like anemia- low RBC levels, erythrocytosis - high RBC levels, leukopenia - low WBC levels, Leukocytosis- high WBC levels, thrombocytopenia- low platelet levels and thrombocytosis- high platelet levels. Further the data on high and low absolute numbers of different kind of leukocyte types provide plentiful information on type of disease, whether neoplastic, inflammatory, infectious or other. Thus blood cell count is a key indicator of maladies in many illnesses and therefore is vital for forming diagnosis, screening for the aberrations in patients' health and for monitoring the progress or treatment. For instance, in aberrations regarding the leukocytes, the reported leukocyte number and the differential count of the leukocytes helps in the assessment of infections as well as in gauging the hematologic malignancies like leukemias (Green & Wachsmann-Hogiu, 2015).

2.9 Automation of CBC

Complete blood count was deemed to enter the automation considering the development in technical abilities to measure, record, report and higher throughput mechanisms were available. Further improvements in these steps of automation has led to further advancement in both quality and quantity of the information obtained from a CBC.

Modern blood counting instruments can simulate the qualitative information obtained through conventional microscopy with the quantitative measurements of the numbers, properties of the cellular components of the blood providing the multiparameter assessment of the circulating blood (Green & Wachsmann-Hogiu, 2015).

Table 3 : A brief history of leading breakthroughs leading to automation in blood count

Discoverer, Year	Methodology
Vierordt, 1852	Microscope
Oliver, 1896	Light scattering and absorption measured by eye
Marcandier et al, 1928	Light scattering and absorption measured with a photodetector
Coulter, 1953	Impedance measurement
Fulwyler, 1965	Impedance measurement and electrostatic cell sorting
Dittrich & Goehde, 1968	Fluorescene-based flow cytometry

Julius et al, 1972	Fluorescence- activated cell sorting
George & Groner, 1973	Light scattering in flow cytometry

Source: (Green & Wachsmann-Hogiu, 2015)

2.9.1 Automated counting methods

Historically, the cells in the body fluids have been counted using the hemocytometer. This method like other microscopic manual count is the subject of inters observer variability and poor reproducibility.

The prototypes of the first automated counters were developed towards the end of nineteenth century and most of the advancement were made throughout the twentieth century. These machines measured the cells using either the light scattered and absorbed by the blood cells or the changes in the electrical currents due to the flow of blood cells flowing through the electrically charged small opening (Green & Wachsmann-Hogiu, 2015)

2.9.1.1 Methods based on optical measurements

George oliver proposed a new method for blood counting based on the measurement of the loss of light due to scattering and absorption in a test tube filled with diluted blood (Oliver, 1896).The test served as the groundwork for the automation of the automated blood count, it also avoided the need of manual individual counting of cells for RBC enumeration. The use of test was limited by its inability to accurately quantify the loss of light as well as the problems related to variations in the shape, size or the haemoglobin contents. Nonetheless, the development of novel detectors in the 1920s, rejuvenated the interest in light scattering and absorption to be used for the counting of blood cells. In 1928, Marcandier and colleagues showed that if the photometer is calibrated properly, blood count can be derived by measuring the transmitted light through a solution of diluted blood (Automated Hematology Analyzers: State of the Art, 2015 March). However, the problem was as described by Oliver, the count will be inaccurate if the cells varied in the size, shape or the haemoglobin content(Oliver, 1896). In 1965, Fulwyler developed a device that could isolate the cells in droplets, based on their size(Fulwyler, 1965). Ditrich and Gonde took it further by coupling a laser beam to the flow device and successfully demonstrated fluorescence-based cytometry. High speed of the flow could pass thousands of cells through the laser beam per second and this made high throughput cytometry a reality. This advancement could be seen as watershed in the transition from manual to automated blood counting. Julius and colleagues in early 1970s showed the sorting based on the fluorescence, which he named fluorescence activated cell sorting. Fluorescence labels allowed identification along with the separation of many types of cells and thus added a new dimension in the blood count. The technology could use 11 fluorophores simultaneously, and with the addition of deconvolution algorithm that enables separation of overlapping spectra the number could be even large (Julius et al., 1972). George and Groner also implemented the light scattering in flow cytometers for the discrimination of different types WBCs based on

their size/ scattering properties. Further with the use of light scattering measurements at two different angles they obtained hemoglobin content (high angle scatter) and red cell size (low angle scatter) after the cells were isovolumetrically sphered (Green & Wachsmann-Hogiu, 2015).

2.9.1.2 Methods based on electrical measurements

In 1940s, Wallace counter was trying to assess the particulates in paint. Driven from his experience in navy and seeing the effects of atomic bombs in Second World War, he started to look for the ways to count the blood cells. He discovered a simple tool useful for screening the blood of large numbers of people. The discovery was named the coulter effect, given after his name. The coulter principle is based on the principle that cells are the poor conductor of electricity compared to the saline solution and when an individual cell passes through the orifice at the same time as an electric current, produce the change (decrease) in the current due to increase in the electrical impedance. The change in impedance is then proportional to the volume of the particle, which served as the foundation for the size based counting and separation. This simple but smart work laid the groundwork for the subsequent development of automated cell counters. After the coulter patented his work, many other developments took place, including the flow cell principle in which cells were direct through the flow chamber instead of being passed through an orifice (Green & Wachsmann-Hogiu, 2015).

The particles, green in color, pass through the electrical sensing zone, shown in purple.

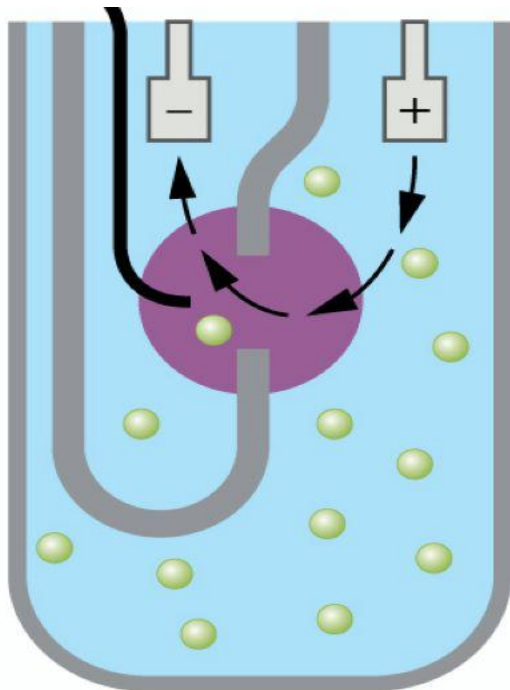


Figure 20 : Schematic overview of coulter principle

The system was modified in 1965 by Fulwylerwho used the coulter counter for measuring the cell volume and then separated the cells into the droplets of the medium (Fulwyler, 1965). This is because the charge of the droplets is related to the cell volume and once the electrostatic force is applied, the droplets can deflect them into a collection vessel, cells can then be sorted and reused later. This method served as a groundwork for development of automated cell sorting techniques (Julius et al., 1972).

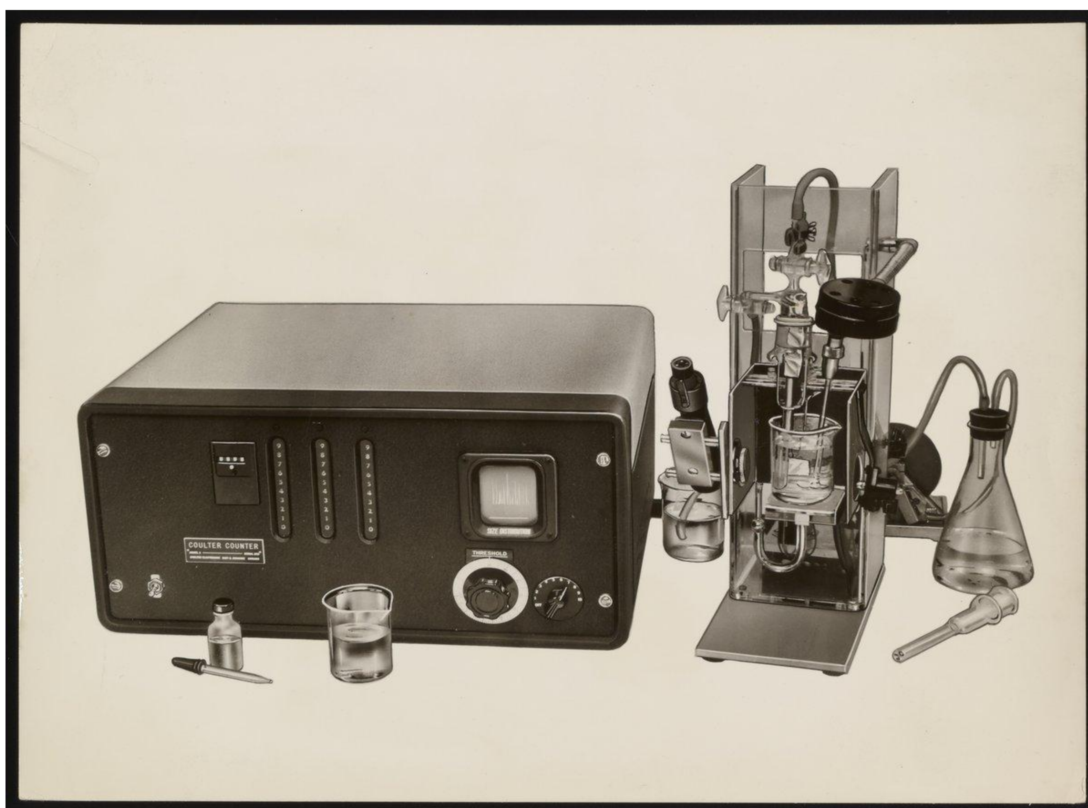


Figure 21: Coulter counter model. Source:https://digital.sciencehistory.org/works/f1881m41k

Figure 21 shows is the Photograph of the historic Coulter Counter Model, A cell Counter from Beckman Historical Collection, Department Of Archives. The instrument draws liquid containing particles through the channel. Each particle releases the electrical charge which is measured and counted (Coulter Counter Model A Cell Counter)

2.10 UniCel® DxH 800 coulter® cellular analysis system.

Unicel® DxH 800 is a hematology analyzer from the Beckman Coulter Inc. that integrates electronic and mechanical design with unconventional algorithm for performing CBC, white blood cell (WBC) differential, nucleated red blood cell (NRBC), and reticulocyte analysis (Hedley et al., 2011).UniCel DxH 800 is engineered for dependable operation. Both calibration and quality control process are simplified by single aspiration pathway which eliminates mode to mode issues. Sample aspiration in UniCel DxH 800 has the unique design which reduces the aspiration volume which can be beneficial for pediatrics sample. With several patents already granted and some in process, the UniCel

DxH 800 platform is designed with new and proprietary technologies that give high accuracy, specificity and sensitivity. The platform gives the four times higher resolution with High Definition Cellular Analysis system. Further, the flow cytometric Digital Morphology (FCDM) enhances the performance as it provides ten times more data than existing technologies, which reduces the review rates. It employs multi-angle scatter technology, has the facility of onboard reagents and has novel algorithms that gives unequaled NRBC and neither requires special nor expensive stains. The platform has the data fusion capability which corrects for most of the common interferences in laboratories ensuring first pass accuracy and efficiency.

Figure 22: Unicel DxH 800. Source: https://media.beckmancoulter.com/Unicel DxH 800

UniCel® DxH 800 Coulter® Cellular Analysis System Specifications

Available Test Menu

CBC	WBC, UWBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, RDW-SD, PLT, MPV
Differential	NE, LY, MO, EO, BA, NRBC, NE#, LY#, MO#, EO#, BA#, NRBC#
Retic	RET, RET#, MRV, IRF
Body Fluids (Spinal, Serous and Synovial)	TNC, RBC

Throughput

Up to 100 samples/hr

Aspiration Volumes

Open/Closed Vial 165 µL

Power

90–264 VAC and 48–62 HZ

DxH 800 System with System Manager and printer draws 1,185 watts

DxH 800 System with System Manager, printer and UPS draws 2,390 watts

Weight and Dimensions	Depth	Width	Height	Weight
Specimen Processing Module	78.7 cm (31.0 in)	76.2 cm (30.0 in)	96.5 cm (38.0 in)	~117.5 kg (260 lb)
Pneumatic Supply	51.0 cm (20.0 in)	21.6 cm (8.5 in)	36.8 cm (14.5 in)	~18.4 kg (40.5 lb)
Optional Floor Stand	78.7 cm (31.0 in)	76.2 cm (30.0 in)	83.8 cm (33.0 in)	~125.6 kg (277 lb)

Whole Blood Performance – Measuring Range

WBC	0.000 – 400.000 X 10^3 µL
RBC	0.000 – 8.500 X 10^6 µL
HGB	0.00 – 25.50 g/dL
MCV	50.00 – 150.00 fL
PLT	0.0 – 3,000.0 X 10^3 µL
NRBC	0.00 – 600.00/100 WBC
Retic	0.000 – 30.000 %

Data Management

Computer	Microsoft® Windows® XP
Database	40,000 Patient results with Graphics
Quality Assurance	QC with Levy-Jennings Graph, XB/XM for Moving Averages, Daily Check, Intelligent Quality Monitoring

Sample Transport Capabilities

Capacity	20 five-tube cassettes
Bar Codes	Digital Bar Code with 2D Bar Code capability
Sample ID	Up to 22 characters

Optional floor stand helps organize consumables and makes changing reagents easy.

Figure 23: Unicel DxH-800 features. Source: DxH 800 Catalogue

The challenging part of complete blood count is the accurate enumeration and identification of white blood cells (WBCs), platelets (PLTs) and nucleated red blood cells(NRBCs). The confirmatory steps for the accurate count of these parameters is time-consuming, costs more and the delayed patient report might have negative impact on the patient management. The new hematology analyzer from Beckman Coulter Unicel® DxH 800 Coulter® Cellular Analysis System has the combination of advanced hardware technology, innovative computer algorithms, impedance technology, conductivity, flow cell volume, conductivity and five light scatter measurements to analyze PLTs, WBCs, and NRBCs. With these technologies combined, it helps to define a signature position of NRBCs, detect discrete populations of WBC and common cellular interferences. The efficiency of the hematology laboratory depends highly upon accurate automated count of WBC, PLT and NRBC, especially in the presence of interference (Bodey, 2009; Segal et al., 2005).

CHAPTER 3
MATERIAL AND METHODS

3.1Sample collection

Beta Thalassemia samples were collected from the Pediatrics department of Siriraj Hospital, tertiary hospital in Thailand. 25 healthy samples and 69 β-thalassemia/ HbE samples were collected.

3.2 Ethical consideration

The study was approved by the Institutional Reviewer Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The patients were from the Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University. Patients' diagnoses were based on hemoglobin typing and molecular characterization. Written informed consent was taken from patients after which, 3 ml of peripheral blood was collected from each into vacutainer tubes containing K_2EDTA as an anticoagulant (Becton Dickinson; Franklin Lake, N, USA)

Time period between the collection and immunophenotyping.

Samples were processed for the first step within five hours of collection, and were stored at 4°C in between.

3.3 Conformation of β Thal samples

3.3.1 Genomic DNA extraction from blood

Genomic DNA was extracted using salting out method. 3-7 ml of the sample blood was brought to the centrifuge tube. 7-10 ml 1X RBC lysis buffer was added to the centrifuge tube. The tube was kept in inverted position for ten minutes at room temperature. The tube was centrifuged at 4000 rpm for ten minutes. Supernatant was discarded and 1X RBC lysis buffer same as aforementioned volume (7-10 ml) was added to the pellet and centrifuged again at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet was kept. 3ml of 1X Nuclei lysis buffer was added followed by 25µl of 20 mg/ml Proteinase K. The solutions were mixed well. 200 µl of 10% SDS was added and incubated at 37°C for overnight (can also be kept at 60°C for 2 hours or in 57°C for 4 hours). 1ml saturated 6N NaCl was added, mixed and kept in 4°C for 20-30 minutes. The tube was centrifuged for 10 minutes at 4000 rpm. Supernatant was then transferred to the new centrifuge tube. 9 to 10 ml of 2X absolute ethanol was added and inverted for 10 minutes. Precipitate DNA was transferred to micro tube. Precipitated DNA was washed by 1ml75% ethanol. The tube was centrifuged at 6000 rpm for 1min in 4°C. The supernatant was discarded. The precipitated DNA was left to dry for 2 hours. The DNA was dissolved in sterile MilliQ water. The DNA concentration was measured by nanodrop.

3.3.2 ARMS PCR for detecting mutations

The PCR was first optimized to obtain all possible amplicons.

3.3.2.1 PCR conditions for ARMS PCR for Group 1:

Table 4: PCR conditions for ARMS PCR for Group 1

Stages	Steps	Temperature	Time	Cycles
1.	Initial Denaturation	95°C	15 min	
2.	Denaturation	94°C	0.45 sec	30 cycles
3.	Annealing	65°C	0.45 sec	
4.	Extension	72°C	1.30 min	
5.	Final Extension	72°C	10.0 min	
6.	Hold	10°C	10.0 min	

3.3.2.2 PCR conditions for ARMS PCR for Group 2:

Table 5: Conditions for ARMS PCR for group 2 mutations

Stages	Steps	Temperature	Time	Cycles
1.	Initial Denaturation	95°C	15 min	
2.	Denaturation	94°C	0.45 sec	30 cycles
3.	Annealing	60°C	0.45 sec	
4.	Extension	72°C	1.30 min	

5.	Final Extension	72°C	10 min	
6.	Hold	10°C	10min	

3.3.2.3 PCR Conditions for ARMS PCR for group 3

Table 6: Conditions for ARMS PCR for group 3 mutations.

Stages	Steps	Temperature	Time	Cycles
1.	Initial Denaturation	94°C	10 mins	
2.	Denaturation	95°C	45 secs	30 cycles
3.	Annealing	62°C	45 secs	
4.	Extension	72°C	1 min	
5.	Final Extension	72°C	10 mins	
6.	Hold	10°C	10 mins	

3.3.2.4 PCR conditions for group 4

Table 7: Conditions for ARMS PCR for group 4 mutations

Stages	Steps	Temperature	Time	Cycles
1.	Initial Denaturation	94°C	10 mins	
2.	Denaturation	95°C	45 secs	30 cycles
3.	Annealing	62°C	45 secs	
4.	Extension	72°C	1 min	
5.	Final Extension	72°C	10 mins	

6.	Hold	10°C	10 mins	

3.3.2.5 Primers for group 1

Table 8: Primers for group 1 mutations

Group 1	Primers
Beta71/72 R	5'- GGTTGTCCAGGTGAGCCAGGCCATCAGTT-3'
Beta41/42 R	5'- GAGTGGACAGATCCCCAAAGGACTCAACCT - 3'
BetaIVSI-5 R	5' - CTCCTTAAACCTGTCTTGTAACCTTGTTAG -3'
BetaIVSI-1 R	5'- TTAAACCTGTCTTGTAACCTTGATACGAAA-3'
Beta17 R	5'- CTCACCACCAACTTCATCCACGTTCAGCTA-3'
Beta8/9 R	5'-CCTTGCCCCACACGGCAGTAACGGCACACC-3'
Beta -28 R	5'-TAA GCA ATA GAT GGC TCT GCC CTG AGTTC-3'
Primer F1	5'- TGAAGTCCAACTCCTAAGCCAGTG-3'

3.3.2.6 Primers for group 2

Table 9: Primers for Group 2 mutations

Group2	Primers
IVS2 654 R	5'-GAA TAA CAG TGA TAA TTT CTG GGT TAA CGT-3'
CD 26 HbE R	5'- TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT-3'
Primer F2	5'- TGA AGT CCA ACT CCT AAG CCA GTG- 3'

3.3.2.7 Primers for group 3

Table 10: Primers for group 3 mutations

Group 3	Primer
Beta 95 R	5'- GGATCCACGTGCAGCTTTG-3'
Beta 43 R	5'- TGGACAGATCCCCAAAGGACTA-3'
Beta 35 R	5' -GAACCTCTGGGTCCAAGGT-3'
Beta 26 R	5'- ACCTGCCCAGGGCCTA-3'
Beta 19 R	5'- CACCAACTTCATCCACGCTC - 3'
Primer F3	5'- TCCAACTCCTAAGCCAGTGC-3'

3.3.2.8 Primers for group 4

Table 11: Primers for group 4 mutations

Group 4	Primer
Beta 27/28 R	5'- TGGTGGTGAGGCCCCT -3'
Beta 41R	5'- ACCCTTGGACCCAGAGGTTT - 3'
Primer F4	5'- CGATCCTGAGACTTCCACACTG - 3'

3.4 Enumeration with Unicel DxH-800

Complete blood counts were evaluated using a UniCel DxH-800 (Beckman Coulter Inc.; Brea, CA, USA) according to the routine procedure used in the Department of Pediatrics, Faculty of Medicine, Siriraj Hospital. Before the samples were analyzed, both inter- and intra-laboratory quality controls were performed on the instrument.

3.4.1 Corrected WBCs

Blood smears were prepared and then stained with Wright-Giemsa (Miles Laboratories; Elkhart, IN, USA). In all, 200 cell WBC deferential counts were randomly performed using different slides and the two medical technologists. To determine their percentages, the NRBCs were counted microscopically using the laboratory's routine methods. Corrected WBCs were calculated using the following formula: corrected WBC/µL = (estimated WBCs/µL × 100)/(100 + number of NRBCs in 100 WBCs).

3.4.2 Statistical analysis

The data were graphed and analyzed using GraphPad Prism version 5.0.1 (GraphPad Software, Inc.; San Diego, CA, USA). The results were expressed as mean ± standard error (SE). Linear regression was used to determine the association between the number of WBCs and the lymphocyte counts obtained using the DxH-800 and those obtained using flow cytometry. A Bland-Altman test was used to address the bias between the two approaches.

3.5 Immunophenotyping

3.5.1Sample staining

20µl of the BD tritest™CD3 fluorescein isothiocyanate (fITC)/CD4 Phycoerythrin (PE)/CD45 Peridinin chlorophyll protein(PerCP) was pipetted into a trucount tube, just above the stainless steel retainer without touching the pellet (Note: One should be careful that BD trucount bead pellet is intact and within the metal retainer at the bottom of the tube, if the case is not so then BD trucount tube must be discarded and replaced with another). Blood sample was vortexed for 10 seconds and 50 µl was pipetted into the trucount tube with reverse pipetting technique. (Note: Accurate pipetting is critical when using a BD Trucount tube, reverse pipetting technique is used to pipette the sample onto the side of the tube just above the retainer. For reverse

pipetting, the button is depressed to the second stop. When the button is released, excess sample is drawn up into the tip. The button is then pressed to the first stop to expel a precise volume of sample; this leaves excess sample in the tip). The trucounttube was vortexed for few seconds and incubated for 15 minutes at room temperature (20°C-25°C) at dark. 450 µl of FACS lysing solution was added. The tube was vortexed and incubated for 15 minutes in dark at room temperature (20°C-25°C). The sample is now ready to be analyzed on the flow cytometer.

3.5.2 Flow cytometry

Facs Calibur™ BDflow cytometer was used for the flow cytometry. The machine was operated in air- conditioned room at the temperature of 22°C. The machine was turned on at first and then the CPU, and left for 15 minutes for proper laser heating. The machine was pressurized. Priming was done three times at high pressure to clean the clogs in the sample injection port (SIP).

Cells were vortexed thoroughly at low speed to reduce aggregation before running them on the flow cytometer.

Compensation was done to check the spillover of one fluorescence compound into the detector of the next fluorescence compound. For compensation, cells were stained with those antibodies which were later on used for the experiment. Three sample tubes (12*75 mm) were taken and labeled. Monoclonal antibodies (5 µl) for CD3, CD4 and CD45 were pipetted separately in their respective labeled tubes. 50 µl of the sample was pipetted into each of the three tubes. The tubes were gently vortexed and incubated in dark at room temperature for 15 minutes. 450

Four dot plots were created i) FSC vs SSC ii) CD45 vs SSC iii) CD3 vs CD4 iv) Time vs Count. FSC vs SSC plot was created to differentiate the cell populations in terms of their size and complexity. CD45 (pan leukocyte marker) vs SSC plot was used to find lymphocytes population. The lymphocyte population appeared as bright cluster with low SSC value and monocytes and granulocytes also appeared as distinct clusters. (Note: one should not proceed if there is little or no separation between the clusters). Further lymphocyte gated CD3 VS CD4 plot was created to find the CD4 lymphocyte subpopulations. Before saving the data a Time vs Count plot was used to check the flowrate. Data was saved only after flow rate was constant. Cells were acquired and list mode data was analyzed using the software, BD Cell Quest. Before acquisition, voltage, threshold and ampgain were adjusted to ensure that the populations of interest were included.

Samples can be stored in the dark at room temperature, if they are not being analyzed directly after preparation.

3.5.3 Calculating Absolute Lymphocytes Count

During the analysis, the absolute count (cells/µl) of the lymphocyte population in the sample was calculated by comparing cellular events to the bead events. Manual data analysis was done using BD cell quest. (Note: BD multiset software can determine the absolute counts automatically). Number of positive lymphocyte events was divided by the number of bead events and was multiplied by the BD Trucount bead concentration.

Absolute count of the cell population was obtained using the following equation.

$A = X/Y * N/V$, Where,

X is the number of lymphocyte events.

Y is the number of bead events

N is the number of beads per test, which is found on the BD Trucount foil pouch and vary from lot to lot.

V is the test volume.

CHAPTER 4
RESULTS

Multiplex Amplification- refractory mutation system analysis of sample. The β-thalassemia samples were checked for the spectrum of mutations using the Multiplexed Amplification Refractory Mutation System – Polymerase Chain Reaction (MARMS- PCR) Assay. The samples are categorized into customized groups according to the primers used and the mutations detected. The gel picture below shows the mutation detected after MARMS PCR for group 1.

4.1 Detection of Group 1 mutations

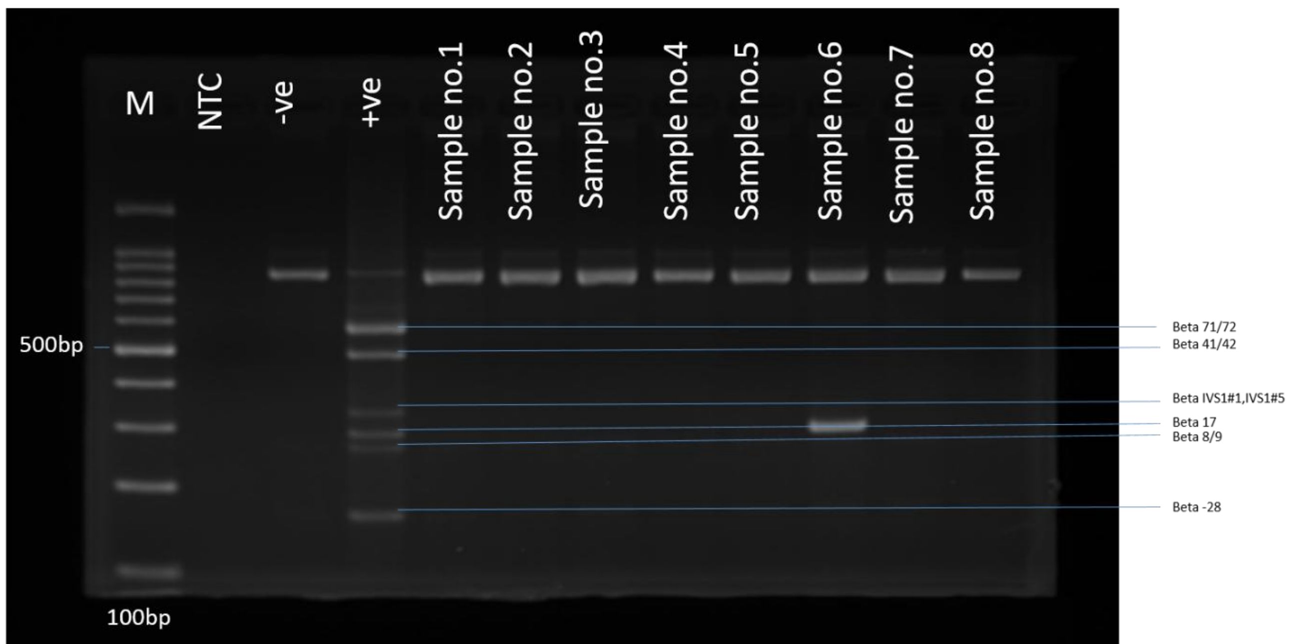


Figure 24: 2% Agarose gel electrophoresis shows amplification of samples for group 1 mutations

In this figure Lane M is the 100bp DNA ladder. NTC is the the lane withnon-template control. Lane -Ve has the PCR product of samples with no mutation. +Ve is positive control for mutations classified as group one. Lanes with sample no.1 to sample no. 8 are the PCR products, with sample no. 6 positive for Beta 17 mutation.

4.2 Detection of Group 2 mutations

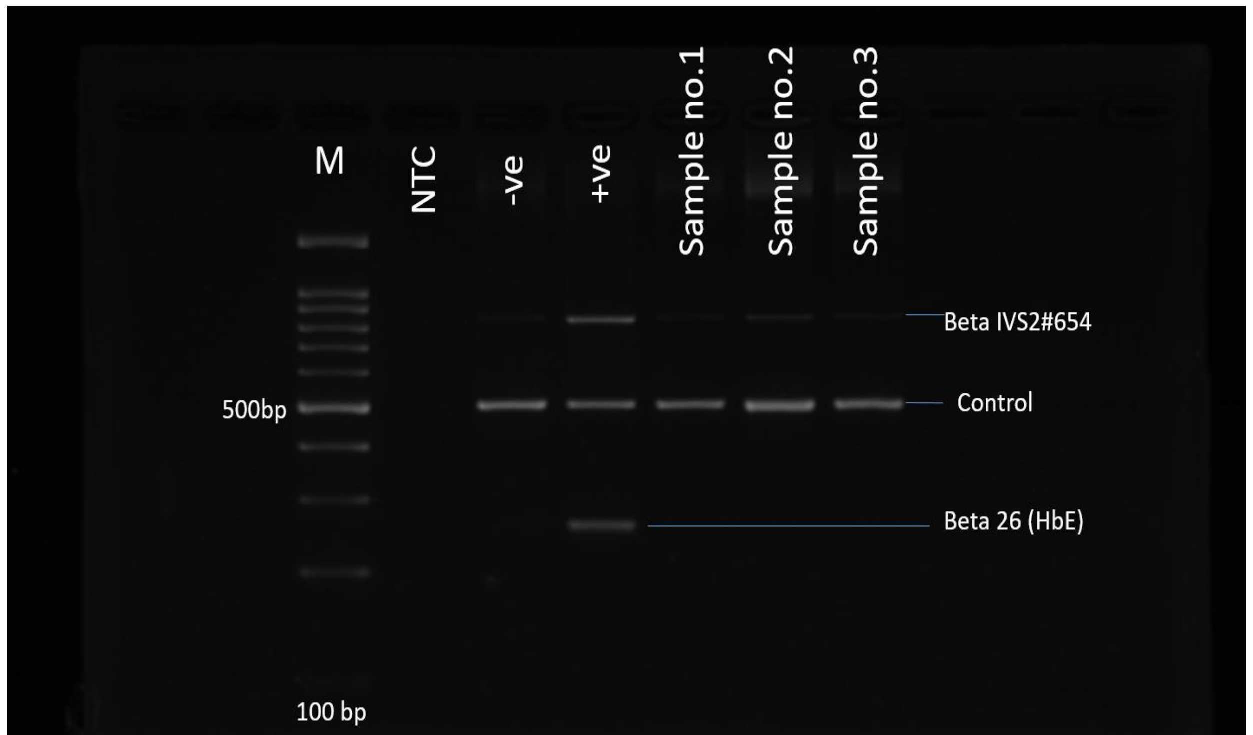


Figure 25 : 2% Agarose gel electrophoresis shows amplification of samples for group 2 mutations

In figure25 lane M has the 100bp DNA ladder. NTC is the nontemplate control. Lane –Ve is the PCR product with no beta 26(HbE) mutation whereas +Ve is the positive control for group 2 mutations . Lanes with sample 1 to sample 3 are found negative for beta 26 (HbE) mutation.

4.3Detection of Group 3+4 mutations

Group 3+4

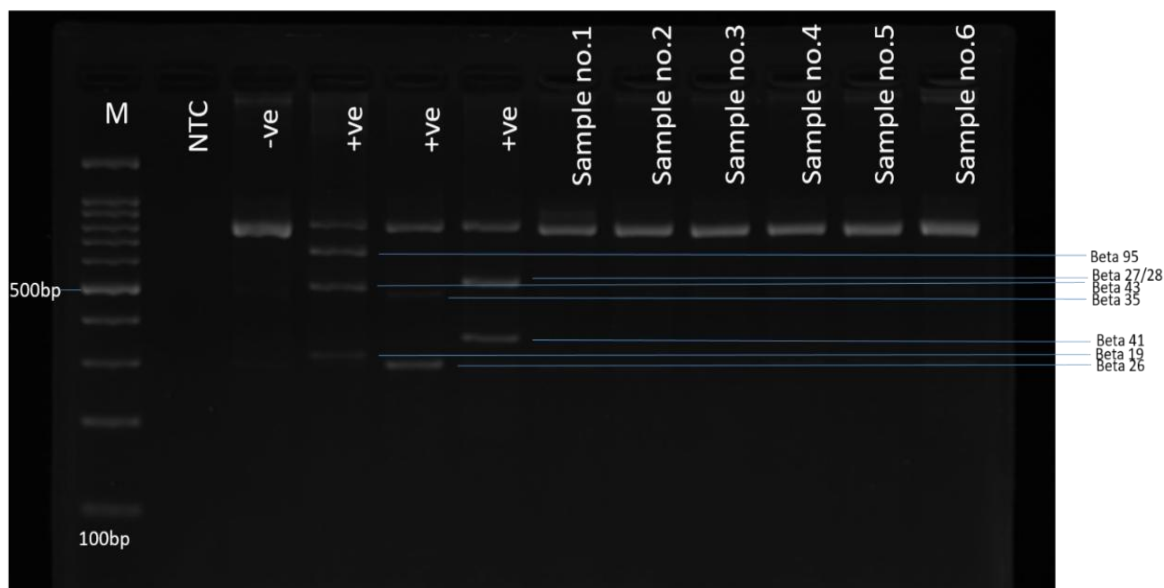


Figure 26: 2% Agarose gel electrophoresis shows amplification of samples for group 3+4 mutations .

In this figure, lane M has the 100bp ladder. NTC is the non-template control. –Ve is the lane with PCR product without mutations. Three positive lanes comprise PCR product positive for group (3+4) mutations. Lanes with specimens 1- 6 are found negative for group 3+4 mutations.

Table 12 : Table showing the codon, mutation base and product size in beta- Thal samples

Codon	Percentage, (n=69)	Mutation base	Product size
		Group 1	
71/72	7.24	+ A	569
41/42	34.78	-TTCT	476
IVSI-5	4.34	G-C	319
IVSI-1	2.89	G-T	315
17	15.94	A-T	275
8/9	1.44	+G	250
-28	10.14	A-G	145
		Group 2	
IVS2-654	2.89	C-T	826
HbE	All	G-C	266
		Group 3	
95	1.44	+A	649
43	2.89	G-T	497

35	4.34	C-A	472
26	4.34	G-T	310
19	2.89	A-G	292
Group 4			
27/28	4.34	+C	516
41	4.34	-C	351

4.4 Hematological parameters of healthy and β-thalassemia/HbE patients

In our study, 69 β-thalassemia/HbE patients and 25 healthy volunteers were taken. Table 1 summarizes the hematological data of all the subjects. We found that the RBC counts and RBC indices in the β-thalassemia/HbE patients were significantly lower than those in the healthy volunteers. In contrast, the RDWs, WBC counts, and platelets in the β-thalassemia/HbE patients were significantly higher than those in the healthy volunteers. In addition, the number of NRBCs/100 leukocytes in β-thalassemia/HbE patients ranged from 3–1766.

Table 13: Data on hematological parameters of healthy volunteers and β-thalassemia/HbE patients

	Healthy (n=25)	β-thalassemia/HbE (n=60)	*p*-value
RBC ($\times 10^6$/µL)	4.62±60287 (4.12-5.39)	3.42±63939 (2.53-5)	< 0.0001
Hemoglobin (g/dL)	13.28±0.25 (10-17)	7.94±0.17 (5-11)	< 0.0001
Hematocrit (%)	39.25±0.68 (32-50)	25.48±0.47 (17-35)	< 0.0001
MCV (fL)	84.84±0.72 (77-96)	74.72±0.83 (59-88)	< 0.0001
MCH (pg)	25.53±0.3 (24-32)	23.09±0.34 (16-29)	< 0.0001
MCHC (g/dL)	33.44±0.19 (29-35)	30.9±0.28 (24-35)	< 0.0001
RDW (%)	13.97±0.2 (12-17)	26.16±0.81 (15-42)	< 0.0001
Estimated WBC (/µL)	7,203±440 (2,900-13,300)	17,945±1,983 (3,300-108,900)	< 0.0001
Corrected WBC (/µL)	-	6,396±414 (1,676-15,825)	-
Lymphocyte (/µL)	2807± 197 (1,400-5,700)	6080±793 (700-41,200)	0.0077
Platelet (/µL)	252,938±13,812 (103,000-572,000)	480,274±27,074 (42,900-988,000)	0.03
NRBCs/ 100 WBCs	0	256±45 (3-1,766)	-

Data represents mean ± standard error (min – max)

4.5 Correlation and agreement between NRBCs count obtained from Manual count and Unicel DxH-800.

We investigated the accuracy of the NRBC counts by comparing the NRBC counts obtained using the DxH-800 with those obtained using the manual approach in β-thalassemia/HbE patients. The results demonstrated an excellent correlation between the two methods, with an r^2 0.96, a *y* =1.15x +26.93, and a *p*< 0.0001 (Figure 27A). A Bland-Altman analysis showed a mean bias of +52 cells/µL and a limit of agreement (LOA) ranging from -372 to +226 cells/µL (Figure 27B).

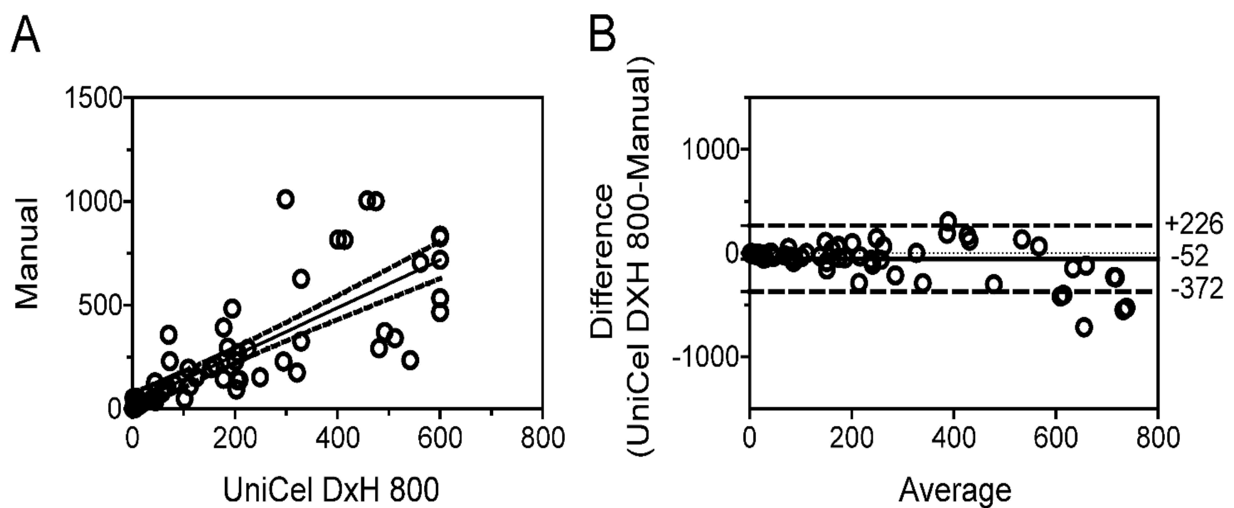


Figure 27: A. Comparison of NRBC counts obtained using DxH-800 and the manual count. B. A Bland Altman plot for two methods.

Excellent correlation was observed with of r^2 0.96, y= 1.15x+26.93 and a p<0.0001.B. A Bland Altman plot showing mean bias of +52cell/ul and the limit of agreement ranging from -372 to +226.

4.6 Correlation and agreement between lymphocyte counts obtained using DxH-800 and flow cytometry in healthy volunteers and β-thalassemia/HbE patients

Analysis of the results from healthy donors showed a good correlation (r^2 = 0.85, y = 0.8x +243.4, *p*< 0.0001) between lymphocyte counts obtained using a UniCel DxH-800 and counts obtained using flow cytometry (Figure 28 A). A Bland-Altman plot demonstrated a mean bias of +264 cells/µL and an LOA that ranged from -536 to +1066 cells/µL (Figure 28 B).

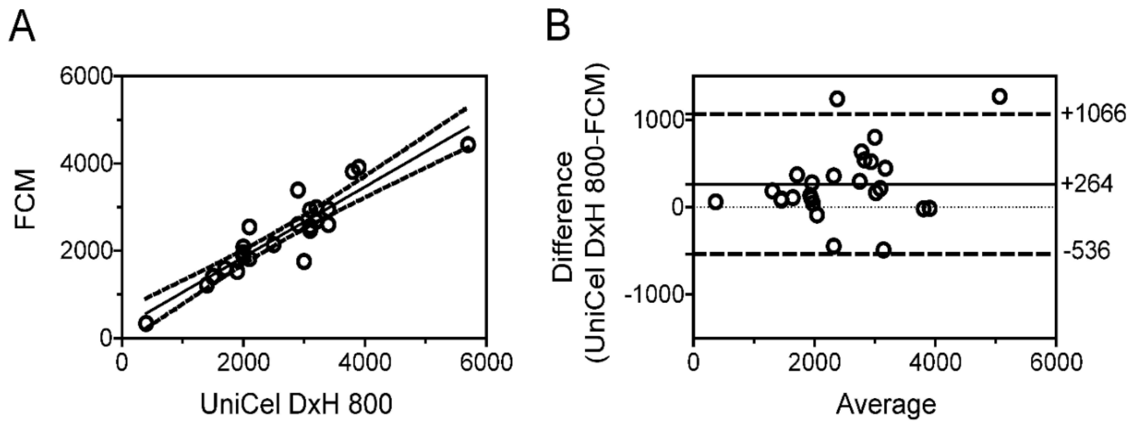


Figure 28: .A. Plot showing the correlation between the lymphocyte count obtained from FCM and UniCel DxH 800 in healthy donors. B. A Bland altman plot for two methods.

Regression analysis showed, $r^2 = 0.85$, y = 0.8x +243.4 and p< 0.0001between two methods in healthy donor. B. A Bland -Altman plot showing mean bias of +264 cells/µL and an LOA that ranging from -536 to +1066 cells/µL

For the β-thalassemia/HbE patients, linear regression analysis showed an $r^2 = 0.06$, a *y* = 0.11x +3642 and a *p* = 0.028 (Figure 3A). The Bland-Altman analysis showed a mean bias and an LOA of +1509 cells/µL and ranging from -11,684 to +14,704 cells/µL (Figure 3B). To further evaluate the effect of the NRBC levels on lymphocyte counts obtained using the DxH-800, the β-thalassemia/HbE patients were categorized into four groups according to the number of their NRBC counts, and the agreement of the counts obtained using flow cytometer and the DxH-800 were compared.

The results showed that the patients with the NRBCs count > 100,001 had the highest disagreement with the mean bias of +10,203 cells/µL and LOA of -9,138 to +25,546 cells/µL (FIGURE 3.) While rest of the three groups (NRBCs < 1,000; 1,001-10,000; and 10,001-100,000 cells/µL) had the mean biases of +516 cells/µL; -3,335 to +4,368 cells/µL, -578 cells/µL; -3962 to +2,805 cells/µL, and -308 cells/µL; -8,694 to +8,078 cells/µL, respectively. In addition, Bland Altman plot the differences values on either side of the zero line in the group with NRBCs < 1,000; 1,001-10,000 and 10,0001-100,000 cells/µL.

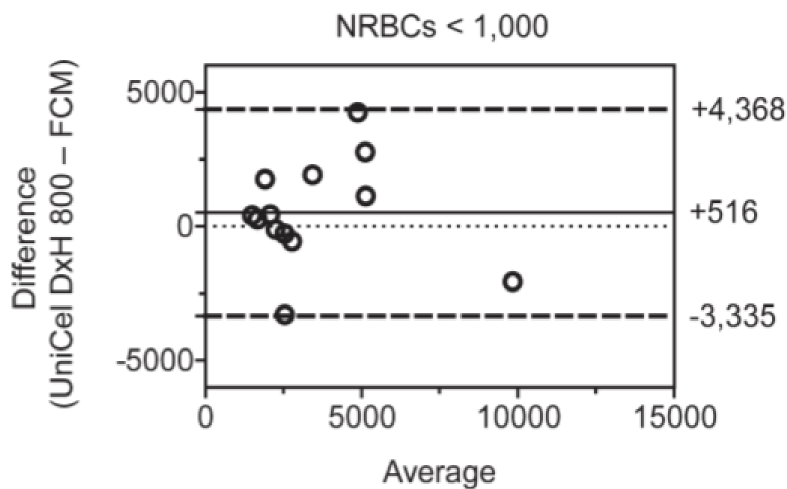


Figure 29: Bland-Altman plots showing the differences against the average of the lymphocyte counts obtained using the UniCel DxH-800 and a standard flow cytometer in β-thalassemia/HbE patients who have NRBCs < 1,000

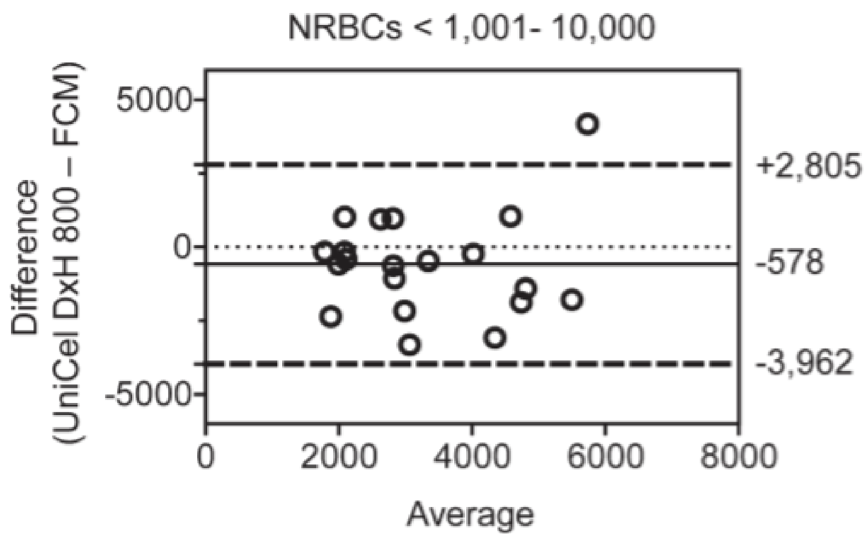


Figure 30: Bland-Altman plots showing the differences against the average of the lymphocyte counts obtained using the UniCel DxH-800 and a standard flow cytometer in β-thalassemia/HbE patients who have NRBCs < 1,001-10,001.

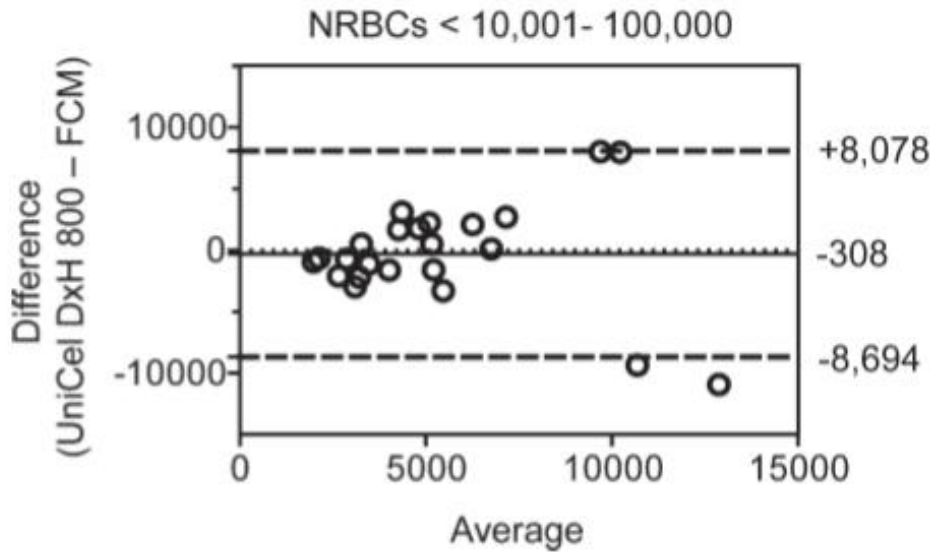


Figure 31: Bland-Altman plots showing the differences against the average of the lymphocyte counts obtained using the UniCel DxH-800 and a standard flow cytometer in β-thalassemia/HbE patients who have NRBCs < 10,001-100,000

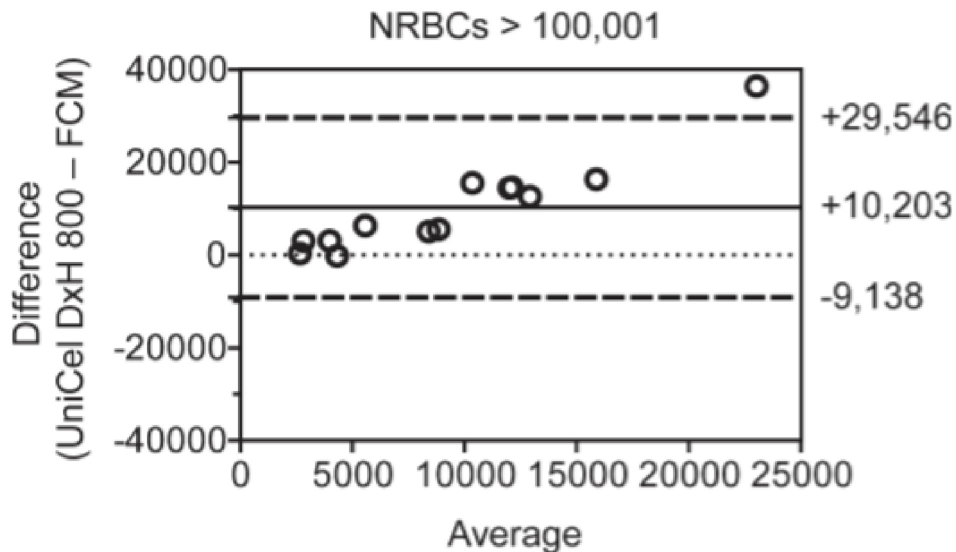


Figure 32: Bland-Altman plots showing the differences against the average of the lymphocyte counts obtained using the UniCel DxH-800 and a standard flow cytometer in β-thalassemia/HbE patients who have NRBCs

4.7 Effect of NRBCs on identification of lymphocytes

Further, to investigate the effect of the number of NRBCs on the identification of lymphocytes, FSC and SSC characteristics of lymphocytes was compared in blood specimens obtained from healthy volunteers and β-thalassemia/HbE patients. In the samples from healthy volunteers, the lymphocyte population was clearly separated from other leukocyte populations according to their CD45 intensities and FSC and SSC characteristics (Figure 4A). In specimens from β-thalassemia/HbE patients, the

lymphocyte population could be classified using CD45 vs. SSC (Figure 4B). However, the backgating analysis demonstrated the similarity of the FSC and SSC characteristics of the lymphocytes and the non-lysed NRBCs, suggesting an influence of the NRBCs on lymphocyte enumeration.

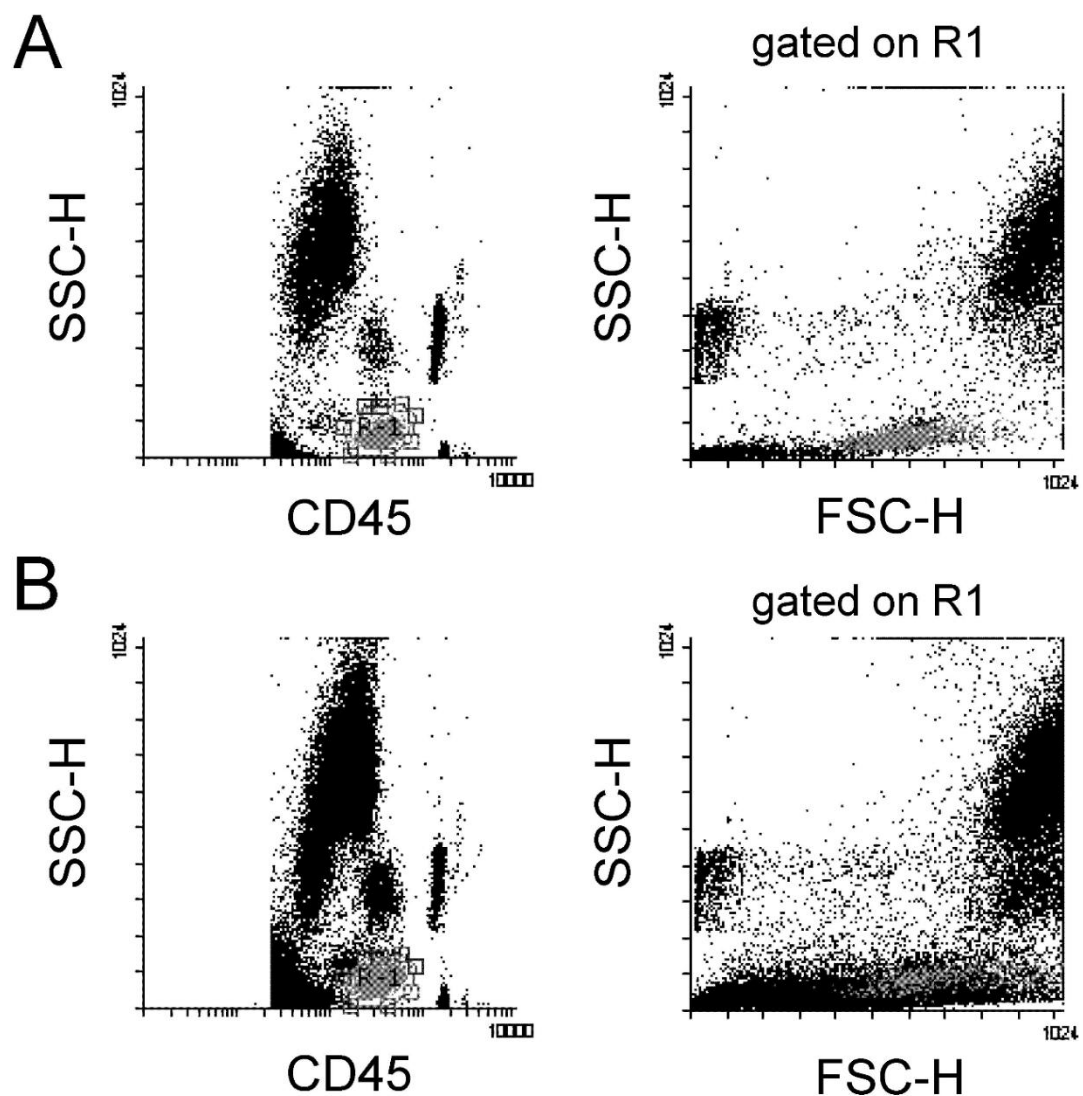


Figure 33: Effects of NRBCs on lymphocyte enumeration. CD45 vs. SSC and FSC vs. SSC dot plots showing the lymphocyte population (R1) in specimens from healthy volunteers (A) and β-thalassemia/HbE patients who had high numbers of NRBCs (B)

CHAPTER 5
DISCUSSION

β-Thalassemia results from the genetic defects in hemoglobin that occurs due to the mutation in β-gene of autosomal chromosome 11(D. J. Weatherall & J. B. Clegg, 2001). Hemoglobinopathies are emerging as a public concern around the world. 320,000 babies are estimated to be born with a clinically significant hemoglobin disorder every year (Modell & Darlison, 2008). Almost 80% of these occur in developing countries. According to Modell and Darlison al least 5.2% of the world population, which accounts over 360 million carry a significant hemoglobin variant (Modell & Darlison, 2008). And there are more than 100 million beta thalassemia carriers with the global frequency of 1.5%. The inherited beta thalassemias along with hemoglobin E and sickle cell anemia disorders are the single gene disorders that are most frequently reported around the world. Each year 50,000 new born patients are detected with severe form of the thalassemia, i.e beta thalassemia major and the HbE beta thalassemia. Further, due to the high rate of migration these disorder is spreading in non-endemic part of the world (Colah R et al., 2010).

Thalassemias in many Asian countries result from the co-inheritance of beta thalassemia and HbE. HbE is the most predominant hemoglobin variant in eastern parts of Indian sub-continent, Bangladesh and other South Asian Countries (Olivieri et al., 2011)

Colah R and his co- workers considered South Asia to be the hotspot of hemoglobinopathies (Colah R et al., 2010) and is home to approximately 1.7 billion world's population. In India, carriers of beta thalassemia are estimated to be 2.78 – 4 %, which accounts to be approximately 30-48 million whereas Pakistani carriers are estimated to be 5-7% which translates to be approximately 5-12 million.

Beta thalassemia is one the most common genetic disorders in Thailand. Many clinical phenotypes have been detected in Thailand which ranged from silent carrier to the manifested conditions which included severe case of beta- thalassemia major and mild beta-thalassemia intermedia (G. Fucharoen et al., 1990)

Our research was conducted in Siriraj hospital in Bangkok, which is tertiary hospital in Thailand. Samples were collected from the department of pediatrics. The samples were screened for 16 beta thalassemia/HbE mutations according to the routine procedures at the department.

Multiplex ARMS PCR was used for screening the sample for the mutations as shown in the mutation table in result section. Prior to this, DNA was extracted from the blood samples using the salting out method. Total Three MARMS PCR reactions were made for the mutations - Group 1, Group 2 and Group 3+4. In MARMS Group 1, we screened for71/72 (+A), 41/42 (-TTCT), IVSI-5 (G>C), IVSI-1 (G>T), 17 (A>T), 8/9 (+G) and -28 (A>G) mutations. In MARMS Group2- we screened for IVS2-654 (C>T) and HbE (G>C). In MARMS Group 3+4, we screened for 95 (+A), 43(G>T), 35(C>A), 26(G>T), 19(A>G), 27/28 (+C) and 41 (-C) mutations. Forward primer F1 was used with Group1 Reverse primers, forward primer F2 with Group 2 reverse primers, forward primer F3 with Group 3 reverse primers and forward primer F4 with Group reverse primers. And a pair of control primers was also used for each MARMS group. Out of 69 beta thalassemic/HbE mutations, 34.78% were 41/42 (-TTCT), 15.94 were 17 (A>T), 10.14% were -28 (A>G) and7.24% were 71/72 (+A). IVS2-654 (C>T), IVSI-1(G>T), 43(G>T) and 19(A>G) mutations

comprised 2.89% whereas 8/9 (+G) and 95 (+A) mutations comprised 1.44%. Our study showed that the most common mutation detected was the 41/42 (-TTCT) which is in agreement with the study conducted by Traivaree et. al on genotype phenotype correlation among beta thalassemia and beta thalassemia HbE disease. Boonyawat and his co- workers also showed that the most common mutation was codon 41/42 (-TTCT) followed by codon 17(A>T) which is also in agreement with our results.

Once β thalassemia/HbE mutations were confirmed we further went to investigate the influence of NRBCs in the lymphocyte enumeration in the samples, using Unicel DxH-800.

Our investigation aimed to investigate the effects of NRBC levels on the accuracy of lymphocyte counts obtained using the Beckman Coulter UniCel DxH-800 in β-thalassemia/HbE patients. The results suggested the accuracy of lymphocyte counts using the instrument in both healthy volunteers and β-thalassemia/HbE patients. However, a high number of NRBCs was associated with a discrepancy in the lymphocyte counts obtained using the UniCel DxH-800 cell counter and standard flow cytometry.

β-thalassemia/HbE is one of the most prevalent diseases in South-Asian and other countries (Laosombat et al., 1992; Nillakupt et al., 2012), and patients show varying degrees of anemia and abnormally high numbers of RBCs (McGann et al., 2017; Noulsri et al., 2018). Higher number of NRBCs in severe β-thalassemia/HbE has been shown to interfere with the lymphocyte counts obtained using several automated cell counters(de Keijzer & van der Meer, 2002; Igout et al., 2004). Thus from the view point of quality control in lymphocyte enumeration, it becomes important to know the particular number or the range of NRBCs that interferes in lymphocytes enumeration. However, there are no studies investigating the effects of the numbers of NRBCs in β-thalassemia/HbE patients on lymphocyte counts obtained using the UniCel DxH-800. Knowing the limitations of an automated cell counter is important in managing laboratory routine. In the present study, in both healthy volunteers and β-thalassemia/HbE patients, lymphocyte counts correlated well whether they were obtained using a UniCel DxH-800 or the standard flow cytometry approach, suggesting that the UniCel DxH-800 performs well. However, despite the high accuracy, discrepancies were observed between the lymphocyte counts obtained using standard flow cytometry and those obtained using the UniCel DxH-800 when NRBCs > 100,001 cells/µL. For this group the plot showed that the difference between the methods tended to increase as the average increased. Collectively, these data suggested an influence of high numbers of NRBCs on the lymphocyte counts obtained using the UniCel DxH-800. This finding was similar to those of previous studies, which suggested that the performance of the DxH-800 in leukocyte counts must be improved (Da Rin et al., 2017; Hotton et al., 2013). The results of the present study suggested that the interpretation of lymphocyte counts made to monitor the immune systems of β-thalassemia/HbE patients who have increased NRBC numbers should carefully consider variations that may occur due to the methodology used. Accurate leukocyte counts are important in β-thalassemia for several reasons. First, infection is a common complication and a major cause of death in β-thalassemia/HbE patients (Wanachiwanawin, 2000), making monitoring of WBC counts necessary to diagnose this complication in these patients. Second, it is extremely important to enumerate the lymphocytes accurately in case of β-thalassemia/HbE HIV patients for the management of the patients. (Tiensiwakul et al.,

2009). In such cases misdiagnosis can also lead to wrong prescription and improper management of the patient, which later on can be fatal.

After demonstrating the influence of high numbers of NRBCs on lymphocyte counts obtained using the UniCel DxH-800, we next went to determine whether high numbers of NRBCs interfered with lymphocyte classification. The results obtained showed that a mixed population of lymphocytes and NRBCs according to their FSC and SSC characteristics, suggesting that physical characteristics cannot be used to discriminate between lymphocytes and NRBCs. This finding is important in explaining the discrepancies in lymphocyte counts between the UniCel DxH-800 and standard flow cytometry in specimens that had high numbers of NRBCs. The principle behind automated cell counters is based on electrical impedance when each cell passes through a small aperture, which causes an electronic pulse (Chabot-Richards & George, 2015; DeNicola, 2011; Green & Wachsmann-Hogiu, 2015). The size and number of pulses, respectively, indicate cell volume and number of cells. To enumerate leukocytes, the RBCs are lysed using the lysis buffer before counting and sizing. The differentiation of the leukocyte population is determined using data on both impedance and optical measurement. Considering the presence of high non-lytic NRBCs in a differential scatter gram could result in increased leukocyte counts. In contrast to the automated counter approach, the flow cytometry uses the molecular marker of the leukocyte, CD45, to classify the leukocyte population and differentiate leukocytes from non-leukocytes. This approach increases the accuracy when enumerating the leukocyte population(Kalva et al., 2012). Trying to address this limitation of automated cell counters, a new model of automated cell counter incorporates advanced technology to improve the accuracy of leukocyte enumeration. Studies have been carried out that show the good efficiency of this technology (Bruegel et al., 2015; Da Rin et al., 2017; Hwang et al., 2016). But still it is important to evaluate the efficiency of this technology in the subjects with increased numbers of NRBCs, particularly in β-thalassemia/HbE patients.

Our study is indicative that the technology incorporated in hematology analyzers must be carefully examined before bringing into the market. Our study on the influence of NRBCs in lymphocyte enumeration of β-thalassemia/HbE patients can draw attention and cast the light in designing new technologies so that most erratic of the cases can be addressed.

The present study had several limitations. We used MARMS in detecting the β-thalassemia/HbE mutations. Every molecular diagnostic tool comes with its own limitation. Molecuar analysis is considered highly accurate in diagnosis but can be time consuming and expensive without proper planning. Allele- specific oligonucleotide probes (ASO) dot blot hybridization is consistent and easy in case of populations predominated by Although modern automated cell counters are available on the market (Ben-Yosef et al., 2016; Rastogi et al., 2017), the present study focused only on evaluating the performance of the DxH-800 instrument against that of flow cytometry. This was because the DxH-800 instrument is currently among one most widely used coulter counter around the world (Barnes et al., 2010; Brown et al., 2014; Hedley et al., 2011). In addition, the present study examined the increased number of NRBCs only in β-thalassemia/HbE disease. Apart from β-thalassemia/HbE disease, other types of patients have shown increased numbers of NRBCs in their peripheral blood circulation, including cancer patients and pediatric patients, but the present study did not examine these patient (Constantino & Cogionis, 2000; Danise et al., 2011). Studies must be

carried out on these samples and confirm the influence of NRBCs on the lymphocyte counts obtained using automated cell counters.

In summary, the results of the present study demonstrated good performance by the UniCel DxH-800. However, these results also detected that the number of NRBCs could interfere with the accuracy of lymphocyte counts when the number of NRBCs reached 100,001 cells/µL. Therefore, in specimens that have high numbers of NRBCs, manual examination is recommended to confirm the lymphocyte counts.

CHAPTER 6
SUMMARY

Due to the similar size of the NRBCs and the lymphocytes, there is a chance that automated hematology analyzers misclassify NRBCs as lymphocytes. We aimed to study the influence patterns of the NRBCs on lymphocyte enumeration. As a representative of modern day hematology analyzer we took Unicel DxH-800 from Beckman coulter which is also one of the most widely used hematology analyzers around the world. And as beta thalassemic blood have high number of NRBCs content, such these were considered appropriate for the study and taken as samples.

Beta thalassemia/ HbE was confirmed using the ARMS PCR. CBC count of the samples was done using Unicel DxH-800.NRBCs count obtained using Unicel DxH-800 were compared with the NRBCs count obtained using manual count. We found an excellent co-relation between the two methods. Bland Altman plot showed the mean bias of +52 cell/ul and the limit of agreement ranging from -372 to +226.

Analysis of the results from healthy donors showed a good correlation (r^2 = 0.85, y = 0.8x +243.4, p< 0.0001) between lymphocyte counts obtained using a UniCel DxH-800 and counts obtained using flow cytometry. A Bland-Altman plot demonstrated a mean bias of +264 cells/µL and an LOA that ranged from -536 to +1066 cells/µl.

In case of β-thalassemia/HbE patients, linear regression analysis showed an r^2 = 0.06, a y = 0.11x +3642 and a p = 0.028 . Analysis of Bland-Altman plot showed a mean bias and an LOA of +1509 cells/µL and ranging from -11,684 to +14,704 cells/µL

To evaluate the effect of NRBCs levels on lymphocyte counts obtained using the DxH-800, the β-thalassemia/HbE samples were divided into four groups according to the number of their NRBC counts. We found that the the patients with the NRBCs count > 100,001 had the highest disagreement with the mean bias of +10,203 cells/µL and LOA of -9,138 to +25,546 cells/µL.

Further,we investigated the influence of the NRBCs on the identification of lymphocytes.We compared the FSC and SSC characteristics of lymphocytes in blood from healthy volunteers and β-thalassemia/HbE patients. In case of the samples from the healthy volunteers the lymphocyte population was clearly separated from rest of the leukocyte populations according to their CD45 intensities and FSC and SSC characteristic. In case of β-thalassemia/HbE patients, the lymphocyte population could be classified using CD45 vs. SSC. But the backgating analysis showed the similarity of the FSC and SSC characteristics of the lymphocytes and the non-lysed NRBCs, which strongly suggests the influence of NRBCs in lymphocyte enumeration.

CHAPTER 7
CONCLUSION

The world has moved towards the automated culture with the development of the advanced technologies. Automation has touched every part of lives and has been the integral part of health, particularly in diagnostics. Companies are incorporating new and refined technology with an aim to receive accurate and reproducible results. But before using the machines, it is important to assess and know the limitation of the technology being used. It is essential to analyze several cases for multiple times and do comparative study to know the limitations.

Our research aimed at assessing the efficiency of the technology used in enumeration of the cells in hematology.Our target was to calculate the efficiency of Unicel DxH- 800 from Beckman coulter in enumerating the lymphocytes in the presence of NRBCs. As the size of NRBCs and lymphocyte are similar. There are chances that the hematology analyzer might misread NRBCs as lymphocyte. The main purpose of taking Unicel DxH - 800 is due to its wide spread use around the world, in medical laboratories. This automated cell counter includes new electronic and mechanical designs and uses advanced algorithm technology to analyze complete blood counts (CBCs), WBCs differentials, NRBCs, and reticulocytes. And samples were β- Thalassemia/ HbE as NRBCs count in β Thal patients were reported to as high as 100,000 cells/µl and more.

From our experiment we concluded thatUniCel DxH-800 performed well in enumerating lymphocytes but had its limitations. Our results showed that the number of NRBCs could interfere with the accuracy of lymphocyte counts when the number of NRBCs reached 100,001 cells/µL. Therefore, in specimens with high numbers of NRBCs, manual examination is recommended to confirm the lymphocyte counts. And manual counts must be carried out multiple times by a trained health professional.

After clearifying the influence of high numbers of NRBCs on lymphocyte counts obtained using the UniCel DxH-800, we then checked whether high numbers of NRBCs interfered with lymphocyte classification. The results showed a mixed population of lymphocytes and NRBCs according to their FSC and SSC characteristics, which suggests that physical characteristics cannot be used to discriminate between lymphocytes and NRBCs. This finding is important in explaining the discrepancies in lymphocyte counts between the UniCel DxH-800 and standard flow cytometry in specimens that had high numbers of NRBCs.

From our investigation, we recommend that all medical laboratories around the world must know the limitation of the automated cell counter being used. At first quality control processes should be run in the machine as assigned by the company protocols and following laboratory guidelines. Then after, case studies must be carried out for lymphocytes enumeration if excessively high NRBCs are reported.

APPENDIX

Common Protocols on flow cytometry

Sample Preparation

Single cells must be suspended at a density of 105–107 cells/ml to keep the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2,000–20,000 cells/second. Higher sort speeds can result in lower yield or recovery.

Phosphate buffered saline (PBS) is a common suspension buffer. The most straightforward samples for flow cytometry include no adherent cells from culture, waterborne microorganisms, bacteria, and yeast. Even whole blood is easy to use — red cells are usually removed by a simple lysis step. It is then possible to quickly identify lymphocytes, granulocytes, and monocytes by their FSC and SSC characteristics.

However, researchers may also wish to analyze cells from solid tissues, for example, liver or tumors. In order to produce single cells, the solid material must be disaggregated. This can be done either mechanically or enzymatically. Mechanical disaggregation is suitable for loosely bound structures such as adherent cells from culture, bone marrow, and lymphoid tissue. It involves passing a suspension of chopped tissue through a fine-gauge needle several times followed by grinding and sonication as necessary.

Enzymes are used to disrupt protein-protein interactions and the extracellular matrix that holds cells together. Their action depends on factors including pH, temperature, and cofactors, so care must be taken when choosing an enzyme. For example, pepsin works optimally between pH 1 .5 and 2 .5, but the acidic conditions would damage cells if left unneutralized for too long, and cell surface antigens of interest might be lost. Chelators like ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis(2-aminoethylether)- N, N, N', N'-tetraacetic acid (EGTA) can remove divalent cations responsible for maintaining cell function and integrity, but their presence may inhibit certain enzymes. For example, collagenase requires Ca2+ for activity. Optimizing the isolation of an epitope under investigation via disaggregation, either enzymatic or mechanical, is often a trial and error process.

To study intracellular components, for example, cytokines, by flow cytometry, the plasma membrane of the cell must be permeabilized to allow dyes or antibody molecules through while retaining the cell's overall integrity. Low concentrations (up to 0 .1%) of nonionic detergents like saponin are suitable. In summary, the method for sample preparation will depend on the starting material and the nature of the epitope. Although it is not possible to describe every method here, some standard protocols are provided in this chapter.

Preparation of Cells for Flow Cytometry

Single cells must be suspended at a density of 105–107 cells/ml to keep the narrow bores of the flow cytometer and its tubing from clogging up. The concentration also influences the rate of flow sorting, which typically progresses at 2,000–20,000 cells/second. Phosphate buffered saline (PBS) is a common suspension buffer.

The most straightforward samples for flow cytometry are no adherent cells from tissue cell culture. Here we describe methods for both tissue culture cell lines grown in suspension and adherent tissue culture cell lines. Analysis may be required of cells derived from other sources. Protocol FC4 provides instructions for the preparation of human peripheral blood mononuclear

cells and protocol FC5 for the preparation of peritoneal macrophages, bone marrow, thymus, and spleen cells.

It is recommended that all containers that have come into contact with human blood or cells should be considered hazardous waste and discarded appropriately.

The following should be considered when designing your flow cytometry experiments:

1. Fc receptors need to be blocked with FcR blocking reagents such as Mouse Herblock FcR Reagent (AbD Serotec product code BUF041) when working with cell types such as spleen cells.

2. Appropriate controls should always be carried out, including:

– Isotype controls to determine specificity of staining

– Unstained cells to monitor autofluorescence

For all multicolor flow cytometry experiments it is advisable to include compensation controls and fluorescence minus one (FMO) controls, which assist in identifying gating boundaries.

Note: These methods provide general procedures that should always be used in conjunction with the product- and batch-specific information provided by the supplier. A certain level of technical skill and immunology knowledge is required for the successful design and implementation of these techniques. These are guidelines only and may need to be adjusted for particular applications.

Preparation of tissue culture cells stored in liquid nitrogen This method provides a general procedure for use with tissue culture cells stored in liquid nitrogen.

Reagents ■ Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)

Method 1. Prepare PBS/BSA.

2. Carefully remove cells from liquid nitrogen storage.

3. Thaw cells rapidly in a 37°C water bath.

4. Resuspend cells in cold PBS/BSA and transfer them to a 15 ml conical centrifuge tube.

5. Centrifuge at 300–400 g for 5 min at 4°C.

6. Discard supernatant and resuspend pellet in an appropriate amount of cold (4°C) PBS/ BSA, such as 107 cells/ml. Note: higher viability can be obtained by allowing the cells to recover in culture media overnight.

FC2. Preparation of tissue culture cells in suspension This method provides a general procedure for use with tissue culture cells in suspension.

Reagents ■ Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)

■ PBS

Method 1. Prepare PBS/BSA.

2. Decant cells from tissue culture flask into 15 ml conical centrifuge tube(s).

3. Centrifuge at 300–400 g for 5 min at room temperature.

4. Discard supernatant and resuspend pellet in 10 ml room temperature PBS/BSA.

5. Centrifuge at 300–400 g for 5 min at room temperature.

6. Discard supernatant and resuspend to a minimum concentration of 1 x 107 cells/ml in cold (4°C) PBS/BS

FC3. Preparation of adherent tissue culture cells This method provides a general procedure for use with adherent tissue culture cells.

Reagents ■ Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)

■ PBS

■ 1x Accutase solution

■ 0 .25% trypsin

Method 1. Prepare PBS/BSA.

2. Harvest cells by enzymatic release using a solution containing 1x Accutase or 0 .25% trypsin, followed by quenching with media containing serum. (Note: epitopes may be cleaved when using the enzymatic digestion method. Cells can also be harvested by gently scraping them into culture media.)

i. Remove the culture medium and eliminate residual serum by rinsing cell monolayers with sterile, room temperature PBS.

ii. Slowly add 1x Accutase Solution or 0 .25% trypsin to cover the cell monolayer.

iii. Incubate at 37°C for up to 10 min.

iv. After incubation gently tap the flask and the cells will detach and slide off in one sheet to the bottom of the flask.

v. Add growth medium and resuspend the cells by gently pipetting.

3. Centrifuge at 300–400 g for 5 min at room temperature.

4. Discard supernatant and resuspend pellet in fresh, room temperature PBS/BSA to wash off any remaining cell debris and proteins.

5. Centrifuge at 300–400 g for 5 min at room temperature.

6. Discard supernatant and resuspend pellet in an appropriate amount of room temperature PBS/BSA.

7. Count cells using a hemocytometer or an automated cell counter such as the TC20™ Automated Cell Counter (Bio-Rad product code 1450102)

8. Once counted, dilute the cells with cold (4°C) PBS/BSA to a minimum concentration of 1 x 107 cells/ml.

Preparation of Cells for Flow Cytometry

FC4. Preparation of human peripheral blood mononuclear cells This method provides a general procedure for use with peripheral blood mononuclear cells.

Reagents Phosphate buffered saline (AbD Serotec product code BUF036A) containing 1% bovine serum albumin (PBS/BSA)

Histopaque or Ficoll

Method 1. Allow separation media such as Histopaque or Ficoll to equilibrate to room temperature.

2. Dilute blood in equal volumes of room temperature PBS/BSA (for example, add 3 ml of PBS/BSA to 3 ml of blood).

3. Carefully overlay whole blood onto an equal volume of separation media in a 15 ml conical centrifuge tube.

4. Centrifuge at 300–400 g for 30 min in a 20°C temperature controlled centrifuge with no brake.

Note: Centrifugation at 4°C or with brake reduces efficiency of cell recovery.

5. Harvest cells from the serum/separation media interface using a pipe.

6. Place harvested cells in a 15 ml conical centrifuge tube.

7. Adjust the volume to 10 ml with room temperature PBS/BSA.

8. Centrifuge at 300–400 g for 5 min at room temperature.

9. Discard supernatant and resuspend pellet to a final concentration of at least 1 x 107 cells/ml with cold (4°C) PBS/BSA.

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Accuracy of lymphocyte counts from UniCel DxH 800 in β-thalassemia/HbE patients having various numbers of nucleated red blood cells.

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Abstract

BACKGROUND: The UniCel® DxH-800 is an automated cell counter widely used in laboratories. However, the effects of increased nucleated red blood cells (NRBCs) on the lymphocyte counts obtained using the UniCel DxH-800 have not been fully elucidated.

OBJECTIVE: The study's objective was to compare lymphocyte counts obtained using the DxH-800 and those obtained using flow cytometry in various ranges of NRBCs.

METHODS: This cross-sectional study analyzed 25 healthy volunteers and 69 β-thalassemia/HbE patients. The numbers of lymphocytes were determined using a UniCel DxH-800 and a standard flow cytometer using counting beads.

RESULTS: In healthy volunteers, regression analysis of the lymphocyte counts using the two approaches showed an r2 0.85 and a p < 0.0001, and a Bland-Altman plot showed mean bias of +264 cells/µL. In β-thalassemia/HbE patients, regression analysis of the lymphocyte counts obtained using an automated cell counter and a flow cytometer showed an r2 of 0.06, a p = 0.028, and a Bland-Altman plot showed the mean bias of +1,509 cells/µL. In addition, a high degree of discrepancy in the lymphocyte counts was

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