Tayyibe Gerçek IMMUNOMAGNETIC SEPARATION AND IMMOBILIZATON OF LEUKEMIA CELLS USING CELL SURFACE MARKERS AGU2017

IMMUNOMAGNETIC SEPARATION AND IMMOBILIZATION OF LEUKEMIA CELLS USING CELL SURFACE MARKERS

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
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SCIENTIFIC ETHICS COMPLIANCE

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M.Sc. thesis titled Immunomagnetic Seperation and Immobilization of Leukemia Cells Using Cell Surface Markers has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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ACCEPTANCE AND APPROVAL

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ABSTRACT

IMMUNOMAGNETIC SEPARATION AND IMMOBILIZATION OF LEUKEMIA CELLS USING CELL SURFACE MARKERS

Acute Lympoblastic Leukemia, shortly ALL, especially B-precursor Acute Lymphoblastic Leukemia is the most common blood malignancies among childhood cancers. There are different kinds of leukemia treatments but after those therapies, there is likelihood of relapsing the cancer in years because of remained cancer cells in the patient's body. Nevertheless remained cancer cells in the body after therapy cannot be seen in the routine clinical follow-up tests. Those kind of diseases like leukemia are named as Minimal Residual Disease (MRD). Currently, there are only two ways to detect Minimal Residual Disease. These are flow cytometry and real-time quantified polymerase chain reaction (RQ-PCR). However many different labs have these Flow Cytometry and RQ-PCR, both devices should be calibrated to detect MRD cells. Nowadays there is a consensus about requiring MRD detection but still it is discussed by the authorities about when and how it should be done. The ultimate aim of this project is to produce a chip that can detect MRD cells. With these work we try to capture leukemic cells by using magnetic beads in nano and microsize. These magnetic beads are coated with CD19 and CD45 antibody markers that are in the membrane of leukemia cells. Next step after capturing them with magnetic beads is to immobilize them onto a surface. Golden surfaces are used and functionalized by antibodies. Thus an immunosandwich structure is occured and the cells are immobilized.

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MSc. in Bioengineering Department

Supervisor: Assist. Prof. Kutay İÇÖZ

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Keywords: immunomagnetic separation, immobilization of leukemia cells, minimal residuel disease

ÖZET

LÖSEMİ HÜCRELERİNİN HÜCRE YÜZEY AYIRAÇLARI İLE İMMUNOMANYETİK AYRIŞTIRILMASI VE SABİTLENMESİ

Akut Limfoblastik Lösemi, kısaca ALL, özellikle B öncüllü Akut Limfoblastik Lösemi çocukluk kanserleri arasında en yaygın olan kan malignitesidir. Löseminin farklı çeşitlerde tedavileri bulunmaktadır ancak terapiden sonra hastanın vücudunda kalan kanser hücrelerinin yüzünden yıllar içinde hastalığın tekrarlama ihtimali vardır. Fakat terapiden sonra kalan bu kanser hücreleri rutin klinik takip testlerinde görünmemektedir. Bu tarz lösemi gibi hastalıklar Minimal Kalıntı Hastalığı (Minimal Residual Disease-MRD) olarak adlandırılır. Günümüzde MRD tayini için yalnızca iki yol bulunmaktadır. Bunlar akım sitometrisi ve eş zamanlı polimeraz zincir reaksiyonudur. Birçok farklı laboratuvarda bu cihazlardan bulunmasına rağmen, cihazlar MRD tayini için kalibre olmak zorundadır. Bugünlerde MRD tayininin gerekli olduğu konusunda bir görüş birliği vardır ancak nasıl ve ne zaman yapılması gerektiği konusu yetkililer tarafından hala tartışılmaktadır. Bu projenin nihai hedefi MRD tayin edebilen bir çip üretmektir. Bu çalışmayla ise nano ve mikro boyutlarda manyetik boncuklar kullanarak lösemi hücrelerini yakalamaya çalışıyoruz. Bu manyetik boncuklar, lösemi hücrelerinin membranında bulunan CD19 ve CD45 işaretleyicileriyle kaplanmıştır. Manyetik boncuklarla hücreleri yakaladıktan sonraki adım onları yüzeye sabitlemektir. Altın yüzeyler kullanılmakta ve gerekli antikorlarla işlevsel hale getirilmektedir. Böylelikle bir immunosandviç yapısı oluşmakta ve hücreler yüzeye sabitlenmektedir.

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Anahtar kelimeler: immunomanyetik ayrıştırma, lösemi hücrelerinin sabitlenmesi, minimal residual hastalık

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To my sister

You are my other half

Chapter 1

Introduction

1.1 Blood Malignancies

Hemato-oncology is the collective term that refers to all blood cell malignancies [1].. Blood stem cells transform and develop into three types of blood cells: red blood cells, white blood cells and platelets in bone marrow where the blood cells are produced. During this process if a problem occurs blood cells are overproduced without maturing properly. It results in blood malignancies because of extraordinary production and insufficient function of blood cells [2].

Haematologic malignancies are traditionally divided into four main categories: these are leukaemia, Hodgkin lymphoma, non-Hodgkin lymphoma, and myeloma. In developed countries blood malignancies exist around 9% of all cancers. For men (after prostate, lung and colorectum) and for women (after breast, lung and colorectum) blood cancers are fourth common malignances according to the Haematological Malignancy Research Network's datas [3].

Most well-known malignancie is leukemia which divided into two section; myeloma and lymphoma. These cancers are characterized by white blood cells' failure in their job. Our interest is leukemia. Leukemia's initial focus is bone marrow. When marrow's ability to produce white cells decreases, immature leukaemic cells are produced. These cells cannot attack and destroy microbial pathogens as mature leukocytes and cannot defense body against infections [1].

1.2 Leukemia

Most known white blood malignancy is leukemia. Leukemia means white blood. It was coined by Virchow in 1800s. During that time Erlich improved stains in order to distinguish atypical white cells. Its different types according to cell lineage are shown in the subsequent studies [1].

Leukemia is cancer of blood cells in bone marrow where begins cancer. When a marrow cell transforms into leukemic cell, it grows and survives rather than other cells and it is overproduced without proper growth. These leukemic cells blocks development of normal cells [4].

When health of the patient gets worse fastly, it may be determined as acute leukemia. In contrast, health of the patient deteriorates slowly, it may be chronic leukemia. Also leukemia types are classified by the origin of the cells [1].

Blood stem cells convert two type of blood cells which are called “lymphoblastic cell” and “myeloid cell” as shown in Figure 1.3.1. The type of leukemia depens on where to start either lymphoblastic or myeloid [5].

CML: Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML), also known as chronic myelogenous leukemia, is a type of cancer that starts in certain blood-forming cells of the bone marrow. In CML, a genetic change takes place in an early (immature) version of myeloid cells - the cells that make red blood cells, platelets, and most types of white blood cells (except lymphocytes). This change forms an abnormal gene called BCR-ABL, which turns the cell into a CML cell. The leukemia cells grow and divide, building up in the bone marrow and spilling over into the blood. In time, the cells can also settle in other parts of the body, including the spleen. CML is a fairly slow growing leukemia, but it can also change into a fast-growing acute leukemia that is hard to treat [4].

AML: Acute Myeloid Leukemia

Acute myeloid leukemia (AML) which also called acute myelocytic leukemia, acute myelogenous leukemia, acute granulocytic leukemia, and acute non-lymphocytic leukemia. “Acute” means that this leukemia can progress quickly if not treated, and would

probably be fatal in a few months. Most cases of AML develop from cells that would turn into white blood cells (other than lymphocytes), but some cases of AML develop in other types of blood-forming cells.

AML starts in the bone marrow but in most cases it quickly moves into the blood. It can sometimes spread to other parts of the body including the lymph nodes, liver, spleen, central nervous system (brain and spinal cord), and testicles.

CLL: Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is due to the accumulation of mature B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs. The leukemic cells demonstrate atypical phenotype, which is essential to reach exact diagnosis. Despite the phenotypic homogeneity, the clinical outcome may be significantly different. Some patients have an indolent leukemia, with long survival while others experience an aggressive disease, with early and frequent need of treatment [6].

ALL: Acute Lymphoblastic Leukemia

Acute Lymphocytic Leukemia (ALL), is a cancer that starts from the immature version of lymphocytes a type of white blood cell in the bone marrow (the soft inner part of the bones). The term “acute” refers that the leukemia can progress quickly, and if not treated, would probably be fatal within a few months. Acute leukemia needs to be treated aggressively and timely [4].

We are interested in ALL in our research. It is important that ALL stands for Acute Lymphoblastic Leukemia, not lymphocytic. Because it is related to number of blasts [1].

1.3 Acute Lymphoblastic Leukaemia

Cancer means that uncontrolled proliferation and growing of cells. Acute Lymphoblastic Leukemia means a cancer initiating in the early lymphocytes in the bone marrow. Invasion of leukemia cells happens in a hurry and it may spread to other parts of the body. To understand leukemia, it can be better to elaborate bone marrow where the place the blood cells are produced and lymphocytes [1].

Soft inner part of the bones is called bone marrow. It is made up of a few blood stem cells, blood-forming cells, fat cells and other elements for growing cells. Red blood cells, platelets and white blood cells are produced in bone marrow. White blood cells are responsible for protecting body against infections.

Lymphocytes build up lymphoid tissue that are important part of the immune system. They are also originated from lymphoblasts. They are divided into 2 main kinds of cells called B and T cell lymphocytes according to their lineage stem cells.

B lymphocytes are producing antibodies in order to defense the body against the germs such as bacteria, viruses etc.

T lymphocytes have various jobs like destroying infections or boosting or slowing the action of immune system.

Early blood-forming cells can be converted into leukemic cell in either B or T cells at distinct levels of maturity. After this change, leukemic cells won't mature normally. Leucemic cells are reproduced fastly but they are being continued to survive and build up instead of dying [7].

In some cases, leukemia and lymphoma or acute and chronic or myeloid and lymphoblastic leukemia may be confused. The ways to distinguish them are discussed below.

They can be distinguished by starting points. Leukemia starts from bone marrows whilst lymphoma starts from lymph nodes. They both may spread the other organs. Also if there is 25% of cancerous lymphocytes in bone marrow, it can be considered leukemia. If lymph nodes' sizes are larger than normal, it is considered to be lymphoma [8].

Differences between acute and chronic leukemia are cells' maturity and progression of disease and patients' lifespan. Bone marrow cells cannot be grown up in acute leukemias although cells mature partially in chronic leukemia. While patients with acute leukemia may survive only few months without a cure, people with chronic leukemia may live for years. Also in chronic leukemia cells may be seemed normal but they do not destroy infections. The progress of the chronic leukemia is longer and the treatment is more diffucult than acute leukemia [8].

Myeloid and lymphoblastic leukemia distinguish each other from the start point. While myeloid leukemia begins from early myeloid cells, lymphoblastic leukemia initiates in lymphocytes. Both myeloid cells and lymphocytes produce white blood cells [8].

1.4 Classification of Acute Lymphoblastic Leukemia

The classification of haematological malignancies was based on the relationship between the bone marrow, and the immune system, and the genetic variations in the early of twentieth century while the lymphoid classification is considered in the late of 1900s [9]. Hodgkin Disease (HD) and non-Hodgkin lypmphoma (NHL). These two disease is respectively 5% and 6% of all malignancies. Also lymphoma is fifth common cancer type in United States [10].

The first attempt at classifying ALL was the French American British (FAB) morphological criteria that divided ALL into 3 subtypes (L1, L2 and L3) depend on cell size, cytoplasm, nucleoli, nuclear chromatin, vacuolation and basophilia [5]. In 1997, the World Health Organization suggested a classification in attempt to account for morphology and cytogenetic profile of the leukemic blasts and identified three types of ALL: B lymphoblastic, T lymphoblastic and Burkitt-cell Leukemia [11]. In 2008, Burkitt-cell Leukemia was eliminated as it is no longer seen as a separate entity from Burkitt Lymphoma, and B-lymphoblastic leukemia was divided into two subtypes: B-ALL with recurrent genetic abnormalities and B-ALL not otherwise specified [12], [13].

1.4.1 The FAB-French-American-British Classification

In the 1970s, scientists made a classification according to microscope images of cells after staining. They classified leukemia as L1, L2 and L3. Those are separated according to sizes of blasts or nuclolus or homogenous-heterogenous structures of nuclear chromatin [14]. L1 represents lymphoblastic leukemia with homogeneous structure. In this type leukemia blasts are homogeneous, nucleus is regular, chromatin is homogeneous, small or no nucleoli, small cytoplasm and mild to moderate basophilia. L2 type is lymphoblastic leukemia with varied structure. In this type, nucleus is irregular, heterogeneous chromatin

structure and nucleoli is large. L3 type is Burkitt's leukemia. In this type, blasts are large, nucleoli is prominent. Also it has stippled homogeneous chromatin structure, abundant cytoplasm and cytoplasmic vacuolation (bubble type) covering nucleus [15].

1.4.2 Immunophenotype Classification

Following some developments of tests such as cytogenetics, flow cytometry the FAB classification has exchanged with these immunophenotype based tests. In this classification, types of leukemia are separated into two groups. Firstly origin of lymphoblasts whether it is B cell or T cell. The other is maturity of leukemic cells [16].
Immunological Characterization of Leukemias classification was developed by the WHO classification of 2008. Immunophenotyping became a major diagnostic tool in addition to morphology, because of its accuracy and speed [12]. Markers on the cell membrane which detected through immunophenotyping allow a proper definition of hematological malignancies' lineage and differentiation [17]
To detect immunophenotypic features are also helpful for later investigation of MRD, such as co-expression patterns and potential aberrant expressions [17]
It has been recognized with the two major groups of leukemias, which is the lymphoid or myeloid lineages, the following entities can be identified through proper immunophenotyping: [18]
Acute lymphoblastic leukemia (ALL) B lineage: B-I, B-II, B-III, B-IV,2,10 where the degree of differentiation of B-lineage lymphoblastic acute leukemias
T lineage: T-I, T-II, T-III, T-IV [17].

1.5 Detection of Acute Lymphoblastic Leukemia

1.5.1 Detection Techniques in Heamatology-oncology From Peripheral Blood and Bone Marrow

Extraordinary amount of white cells and existence of unusual cells (generally blasts) characterize any kind of leukemias. In the initial stages of malignancies, it is diffucult to see unusual peripheral blood leukocyte pictures, but in the advanced levels it is easy to

recognize different cells in the blood. For further investigation 4 types of analysis are used [1].

Bone marrow is the initial organ for the leukemia. Aspirate and biopsy methods are used to detect leukemic cells from bone marrow [1].

Aspiration means to extract a bit of bone marrow fluid and cells by putting a needle into bone in order to investigate under microscope [19]

Morphology

Morphology is used for finding out what type of precursor in the leukemia, conventional Romanowsky staining is used and it is observed under the microscope [1]. Stains using a combination of methylene blue and eosin have been widely applied for the routine dyeing of blood films in order to study cellular morphology. These stains now include a variety of complex combinations of methylene blue and other closely related thiazine dyes with eosins [20].Romanowsky staining is a blood film staining made of two different components as azure B and eosin Y. It makes shades and to see differences is subtle [21]. A fairly simple approach of Romanowsky staining is wherever a biological substrate is coloured purple [22].

Flow Cytometry

Flow cytometry is a device based on scattering lights on the flowing sample [23]. It is used to analyse the physical and chemical characteristics of particles in a fluid as it passes through one laser or more lasers. Cell components are fluorescently labelled first and then excited by the laser to send light at different wavelengths [24]. It can be used in small molecules such as DNA, nuclei or chromosomos or cell surface markers like CD molecules [1]. Technological developments in multiparameter flow cytometry enable for both more extensive immunophenotypic investigations on small samples and the characterization of small contingencies of aberrant cells together with useful studies of co-expressed markers [17].

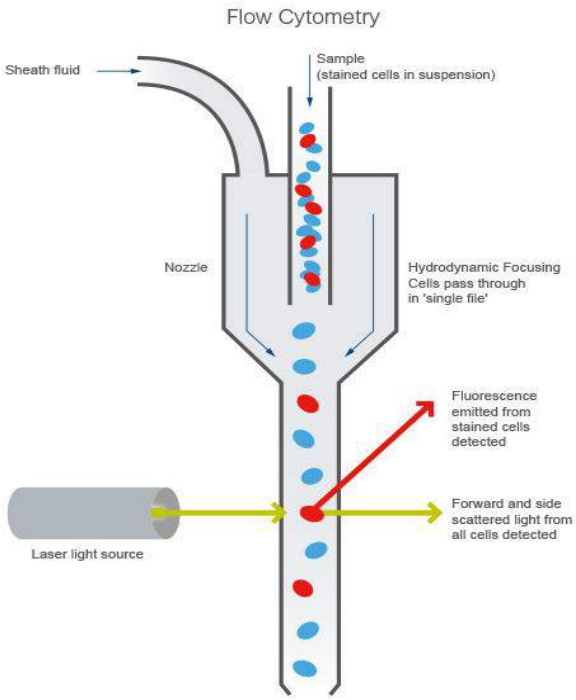


Figure 1.6.1.2: Basic shematic of flow chamber of cytometry [25].

Cytochemistry

It is a method based on staining of enzyme which related to myeloid lineage [1]. It is not only investigation for enzymes but also certain unusual substances released by organs into blood [26]. For instance; Cytochemically, leukemic blasts have negative peroxidase reactions and variable periodic acid-Schiff (PAS) positivity; Sudan black B is weakly positive; ALL with mature cells that are nearly indistinguishable from mature lymphoid neoplasms and require expert observers for accurate morphological identification. Thus, it is required to detect that ALL blasts are negative for myeloperoxidase and other myeloid cytochemical reactions [27].

DNA Analysis and Cytogenetics

This method is used in order to look for abnormality and gene mutations [1]. Conventional chromosome analysis can be useful in the identification of recurrent translocations, besides gain and loss of gross chromosomal material. Aso other genetic techniques to detect and visualize chromosomal abnormalities in ALL can be used such as fluorescence in situ hybridization (FISH) with a sensitivity of around 99%. Additionally array-comparative genomic hybridization (array-CGH, a-CGH) and single nucleotide

polymorphisms (SNP) arrays can allow to detect genetic structure of leukemia [27]. For instance, there may be Philadelphia chromosome in ALL patients. This variation is a translocation between chromosome 9 and chromosome 22 [26]. A segment of the *ABL* gene (9q34) is moved into one of several breakpoint cluster regions of the *BCR* gene (22q11). The chimeric *BCR-ABL* gene is translated into BCR-ABL oncoproteins [28].

1.6 Treatment of Acute Lymphoblastic Leukemia

Children with acute lymphoblastic leukemia are usually treated according to risk groups defined by both clinical and laboratory features. The intensity of treatment required for favorable result changes within children with ALL [29].

There are some standard treatment types such as chemotherapy, radiation therapy, stem cell transplantation and targeted therapy and there is a new therapy approach: Chimeric antigen receptor (CAR) T-cell therapy. These treatments are given the patients according to their origin of lymphocytes, level of risks, age of kid or presence of certain chromosome changes [26].

1.6.1 Chemotherapy

Chemotherapy means giving anticancer drugs to patients in order to kill cancer cells or stop them from proliferating. These anticancer drugs can be taken by mouth or can be injected into vein or muscle or can be placed into cerrebrospinal fluid in the case of spreading to brain or spinal cord. Mixing multiple anticancer drugs are called combination chemotherapy [26]. Therapy program with multiple drugs depends on intensity of dose and time [28]. Types and doses of drugs are determined by doctors according to kid’s risk group [26]. Conventional therapy of ALL previously consisted of four parts-induction, consolidation, maintenance, and CNS prophylaxis. However, many recent treatment protocols have not followed traditional definitions of these approaches.

Induction therapy and postremission therapy are appropriate terms for the stages of treatment in recent trials [30].

Treatment to destroy leukemia cells or prevent the spread of leukemia cells to the brain and spinal cord (central nervous system; CNS) is called CNS-directed therapy. Chemotherapy may be used to treat leukemia cells that may spread, to central nervous system. Since standard doses of chemotherapy may not effect leukemia cells in the CNS, the cells are able to hide in the CNS. Systemic chemotherapy given in high doses or intrathecal chemotherapy (into the cerebrospinal fluid) is able to reach leukemia cells in the CNS [31].

1.6.2 Radiation Therapy

Radiation therapy means sending high-energy X-rays or other radiation types to the cancerous region. Sending radiation may be from outside to the body by a machine which is called external radiation therapy. Also needles, seeds like in Figure 1.7.2.1 or wires or such materials are used to place directly into near the cancerous region [26].

Sometimes external radiation therapy to the brain is also given in order to prevent spreading leukemia cells to central nervous system [31].

1.6.3 Stem Cell Transplantation

This treatment is generally used in relapsed cases. Firstly chemotherapy is given to patient and stem cells of healthy donor is transplanted to the patient. It provides blood forming cells to replace and proliferating patient's blood cells [26]. Immature blood cells or blood stem cells are taken away from the bone marrow of donor. Following given treatment to the patient, the donor's stem cells are infused in the patient. These infused stem cells provides to grow and renew blood cells [32].

1.6.4 Targeted Therapy

To recognize and attack cancer cells without touching normal healthy cells targeted therapy are used. The applications of monoclonal antibodies or some kind of enzyme inhibitors may be examples for targeted therapy [26], [33]. Targeted tharapy has three different types; TKI, Tyrosine Kinase Inhibitors, Monoclonal Antibodies and Proteasome Inhibitors [7].

Targeted drugs have some advantages such as blocking the enzyme which produces more white blood cells than required or killing cancer cells without spreading healthy tissue [26]. A great deal of monoclonal antibodies (MoAbs) have been developed with the aim of specifically targeting cytotoxic agents to leukemia cells while limiting the deleterious effects on healthy tissues [34].

Chapter 2

Minimal Residuel Disease

Most children with acute lymphoblastic leukemia overcome complete remission with current treatments but leukemia relapse, the main cause of treatment failure, still exists in an important proportion of patients [35]. It also can be called Incomplete Remission. After patients with acute leukemia took chemotherapy, disease may be relapsed sooner or later [36]. As MRD reflects its sensitivity to the drug dosages applied, it constitutes the ideal method for surveillance and patient follow-up. The morphological examination of peripheral blood or bone marrow smears, although still an indispensable part of routine follow-up laboratory testing, is clearly insufficient for patient control [37].

Minimal Residuel Disease is term used for explaining presence of remained sequela after cancer therapy but cannot be seen in morhology or routine clinical tests (Figure 2.1). It requires more sensitive tests like flow cytometry or qualitative real time polimerase chain reaction (QR-PCR) [38]. The reason of saying "minimal" is unseen and small amount cells following taking a cure. It is discussed the names of Minimal residual disease (MRD) or incomplete remission [36]. Because detecting MRD or incomplete remission must give us information about remained cancer cells even though the amount of cells are smaller than 0.01 % [36]. MRD is a good prognostic factor which allows to assign risk group and it makes treatment easier. Besides necessity of MRD detection in relapsing cases of ALL, it requires also in patients undergoing stem cell transplantation [39].

Figure 2.1 Minimal Residuel Disease may not be seen in routine follow-up care tests.

MRD monitoring is important to give information about probability of relapsing Acute Lymphoblastic Leukemia (Figure 2.2). It helps to understand how much the patients give

response to therapy [36]. Achivement of monitoring of MRD prevents to be given wrong doses of therapy to the relapsed leukemia [38]. Detection of MRD cells helps the authorities to make a decision about adjusting the drug doses either intensifying or decreasing in the case of relapsing [36], [39].

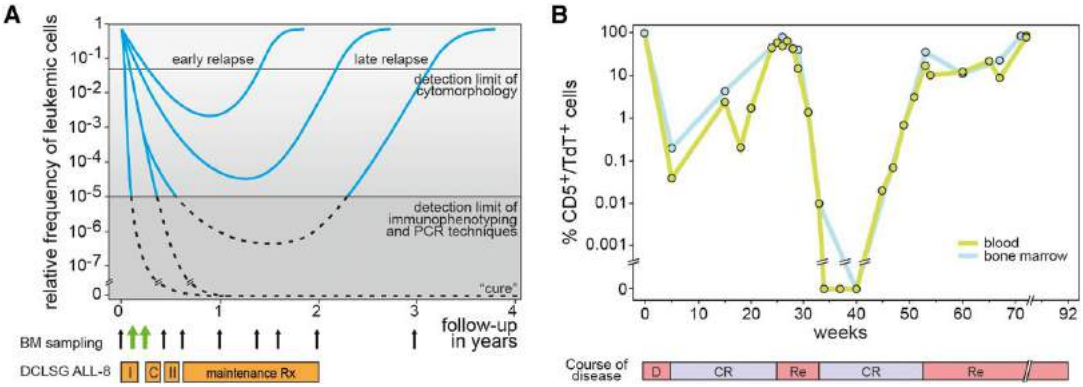


Figure 2.2: Relapsing graphs of leukemia. (A) The graph shows relative frequencies of cancer cells in bone marrow following treatment, I, induction treatment; C, consolidation treatment; II, reinduction treatment. (B)Presence of remained leukemia cells and relapsing graphs both in bone marrow and blood. D, diagnosis; CR, complete remission; Re, relapse [39].

2.1 Detection of Minimal Residuel Disease

The first time detected MRD was in 1980s and utilized by immunoflourescense microscopy.[40] It could show only too specific markers to T-cell precursor ALL (Figure 6) while this technique was not be able to sense B-cell precursor ALL. It requires too highly specific markers in order to distinguish among the normal cells. [39]

Immunofluorescence microscopy was used to detect only T-cell precursor ALL with its special markers such as terminal deoxynucletidyl transferase (TdT) (Figure 2.1.1) but it is not able to make small different markers visual. Because immunofluorescence microscopy is not enough to show small abnormal immunophenotypes. In last decades, PCR based and flow cytometric assays are widely used to detect MRD cells [39]. A group of scientists coins these techniques Immunologic and Molecular Monitoring instead of flow cytometry or polimerase chain reaction (PCR) [36].

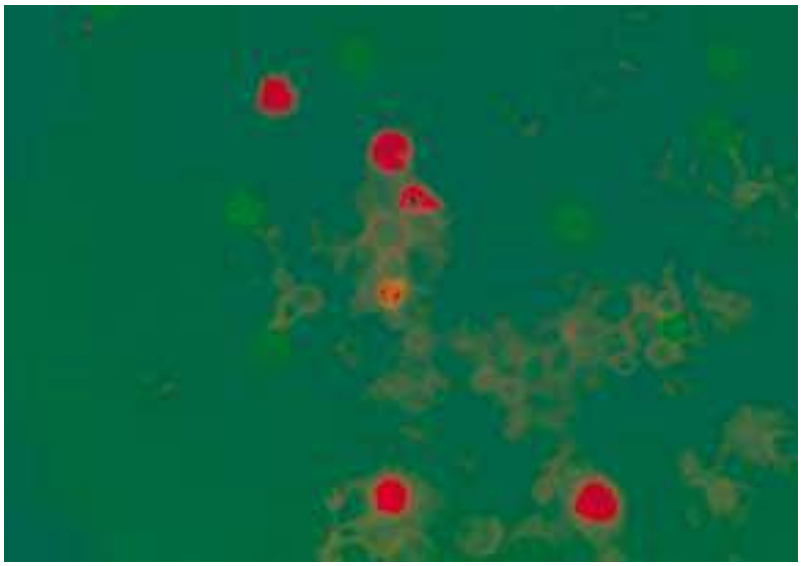


Figure 2.1.1: Two different immunofluorescence represents for different markers of T-cell ALL. Red dots represent for Anti-TdT markers and the green ones represent for Anti-CD3 markers. They both show orange one which indicates presence of T precursor ALL MRD cells [41]**.**

2.1.1 Polimerase Chain Reaction

Polimerase Chain Reaction (PCR) are used with two different kinds of molecular targets. One of them is about V and J junction regions in the Immunoglobulin (IG) and T-cell Receptor (TCR) genes. Those regions of IG an TCR genes are unique to leukemic clone (Figure 2.1.1.1). The other moleucular targets that used to detect MRD are gene fusions like BCR-AML1, MLL-AFF1 and their abnormal mRNA results [38]. Both of measurements are done by real-time quantitative PCR (RQ-PCR) by using fluorescently labeled markers.

Polymerase chain-reaction (PCR) technique is generally used because of the lack of the sensitivity for MRD monitoring on cytogenetic techniques like caryotyping, fluorescence *in-situ* hybridisation (FISH), or comparative genome hybridisation (CGH). chromosomal translocations such as t(8;21) and t(15;17) in acute myeloid leukaemia (AML) are used for DNA-based testing as markers. The allele-specific oligonucleotide PCR (ASOPCR) is based on the fact that B- and T-cell leukaemias exhibit a distinct immunoglobulin and TCR gene rearrangement at the V(D)J junctional region that can be used as a specific marker for that particular leukaemic clone. Although sensitivity of these different PCR-

techniques is high (up to 1 in 105 cells in some studies), they are only applicable in patients with known translocations or other known and specific DNA markers. ASO-PCR requires the development of reagents (patient-specific probes) and assay conditions for each individual patient, which is laborious, expensive and time consuming [37].

Besides real time quantitative PCR technique for conjunctional regions, reverse transcriptase PCR is used to detect leukemia related messanger RNA sequence. Next generetion sequencing is also considered to be improved in order to monitor MRD [37].

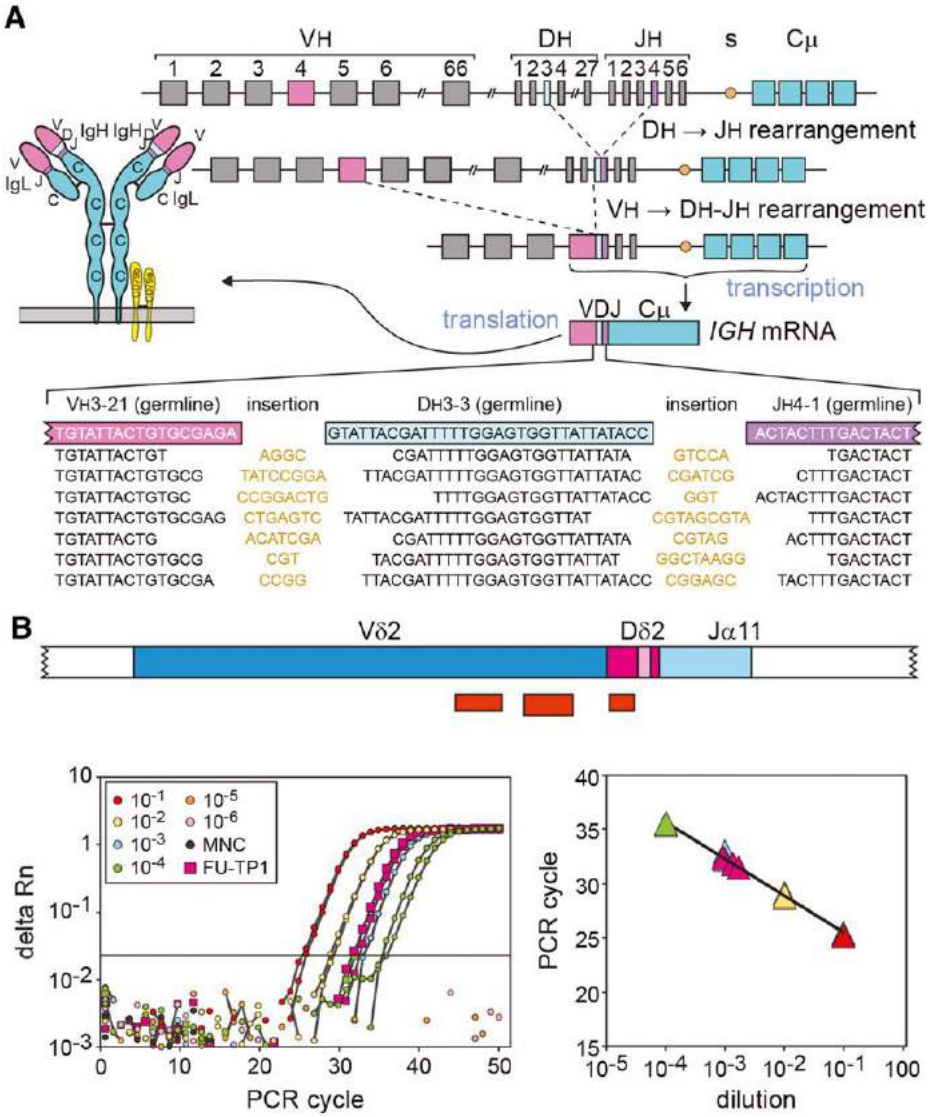


Figure 2.1.1.1: Rearrenged IG and TCR genes' RQ-PCR-based MRD analysis. (A) V-D-J exons, junctional regions. (B) Vd2-Dd2-Ja11 rearrangement MRD analysis [39]**. Presence of these translocations between Ig and TCR genes represents for MRD cells in the sample.**

2.1.2 Flow Cytometry

Flow Cytometry is a technique based on passing through cells and sending laser beam on cells. It gives researchers opportunity to see some properties of cells such as size, granularity according to probes on them [42]. Thus, unique immunophenotypic characteristics of leukemia cells can be used to distinguish leukemic cells among normal cells by flow cytometry [43]. The immunophenotypic proteins can be stained with fluorescent dye-labelled antibodies and detected using MCFC (Multicolor Flow Cytometry) [37].

Leukaemic cells demonstrate quite unusual and unique combinations of the cell surface proteins (LAIP, leukemia-associated immunophenotype) according to normal cells [37]. Combinations of monoclonal antibodies which are unique to B-precursor Acute Lymphoblastic Leukemia are used to detect MRD in flow cytometry (Table 1). [44][45] Most known combination in this detection is CD19, CD10 and CD34 as shown in figure 2.1.2.1 and figure 2.1.2.2 [45].

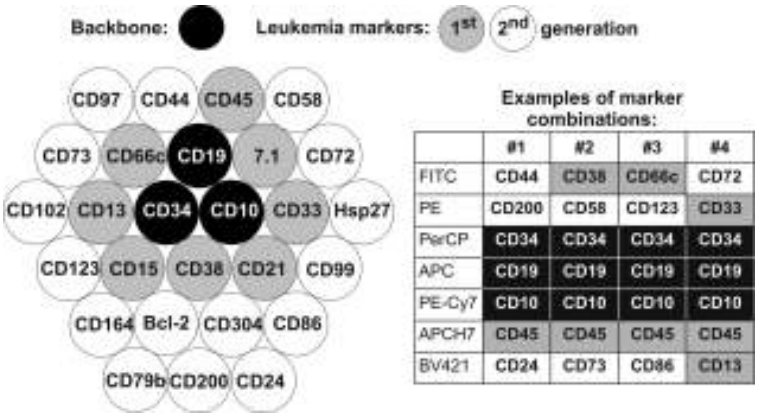
Mab combination	*Frequency of application*[a]
Tdt–CD10–CD19	42 (82%)
CD19–CD34–CD45	26 (51%)
CD10–CD20–CD19	20 (39%)
CD34–CD22–CD45	16 (31%)
Aberrant expression of myeloid markers CD13, 15, 33	22 (43%)
Tdt–CD5–cyt CD3	10 (100%)
Tdt–CD34–cyt CD3	4 (40%)

[a]Percentages of patients with B-precursor ALL and T-ALL, followed using given Mab combination, respectively.

Table 2.1.2.1: Monoclonal Antibody combinations referring to B-cell ALL. [44]

Flow cytometry is the most common method to diagnose and characterise the haematological malignancies. Though this technique is widely used, it is required a high level of expertise to interpret the data proficiently in terms of rare event detection such as MRD [37].

Whilst the flow cytometry technique can detect 1 MRD cells within 10^4 cells, PCR can sense 1 cell among 10^5 cells [37] as the comparison showed in Table 2.1.2.2.



Examples of marker combinations:

	#1	#2	#3	#4
FITC	CD44	CD38	CD66c	CD72
PE	CD200	CD58	CD123	CD33
PerCP	CD34	CD34	CD34	CD34
APC	CD19	CD19	CD19	CD19
PE-Cy7	CD10	CD10	CD10	CD10
APCH7	CD45	CD45	CD45	CD45
BV421	CD24	CD73	CD86	CD13

Figure 2.1.2.1: Combinations of B precursor leukemia markers antibodies. [45]

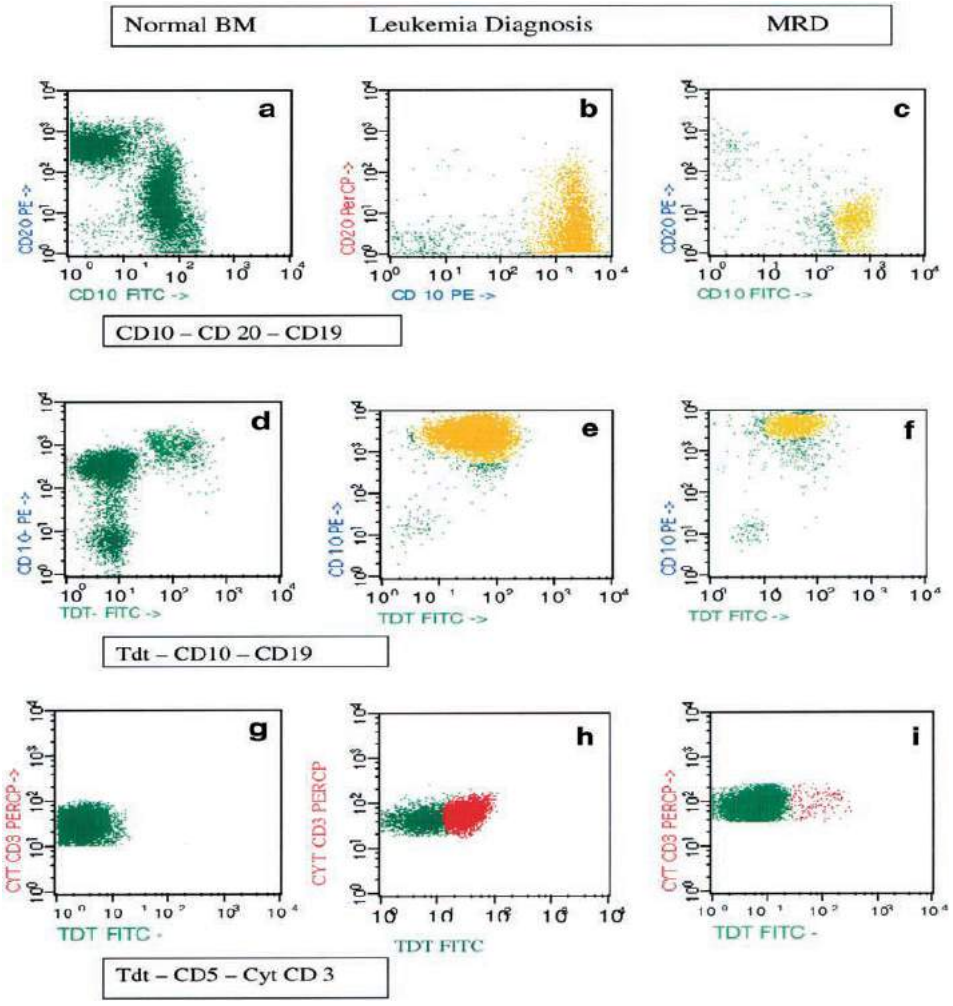


Figure 2.1.2.2: Flow cytometry results of combinations of B precursor ALL. CD10-CD20-CD19, Tdt molecule-CD10-CD19 and Tdt-CD5-Cyt CD3 combinations are in the flow images [44]**.**

Both method have different pros and cons as shown in Table 2. But one of the common disadvantages is necessary an expert to analyse.

MRD technique	Conventional flow cytometry	RQ-PCR of IG/TR genes or breakpoint regions of	RQ-PCR of fusion transcripts and other aberrances
Estimated sensitivity	3-4 colors: 10^{-3}-10^{-4} 6-8 colors: 10^{-4}	10^{-4}-10^{-5}	10^{-4}-10^{-6}
Applicability	BCP-ALL: >90% T-ALL: >90%	BCP-ALL: 95% T-ALL: 90-95%	BCP-ALL: 25-40% (age dependent) T-ALL: 10-15%
Advantages	Fast Analysis at cell population level or single cell level Easy storage of data Information about the whole sample cellularity	Applicable in virtually all BCP-ALL and T-ALL Sensitive Well standardized + regular international QA rounds	Relatively easy Sensitive Applicable for specific leukemia subgroups, such as BCR-ABL or MLL-AF4
Disadvantages	Variable sensitivity, because of similarities between normal (regenerating) cells and malignant cells Limited standardization, no QA results	Time-consuming Expensive Requires extensive experience and knowledge	Limited standardization (only harmonization) Limited QA rounds (with conversion factors) Limited applicability in ALL (absence of targets in >50% of cases) Risk of contamination

Table 2.1.2.2: Comparison of characteristics of current MRD detection techniques[39]. **Like their sensitivity and accuracy are familiar, disadvantages such as being expensive and required expertise are alike, too.**

Chapter 3

Magnetic beads

Magnetic particles are widely used in some biomedical activities because of their attractive features. First of these features is their size. It can vary between nanometers and microns. That means this size can fit different biological entities such as a cell (10–100 μm), a virus (20–450 nm), a protein (5–50 nm) or a gene (2 nm wide and 10–100 nm long). In order to activate magnetic beads biologically, they can be coated with biological molecules. Therefore coated magnetic beads are able to interact interested biological entity in a study (Figure 3.1). It provides to be labeled or addressed. Another important feature is, magnetic beads can be manipulated by applying a magnetic field. Also they can transfer energy because of their resonant respond to time-varying magnetic field. For example by heating magnetic beads. Those kind of properties make magnetic beads useful for some applications like magnetic separation, drug delivery and hyperthermia treatments [46].

Figure 3.1: Functionalized magnetic beads for different biologic molecules [47]

3.1 Application of Magnetic Beads

3.1.1 Magnetic Separation

The main aim of magnetic separation is extracting desired biological material from its environment. It provides better following analyses about the desired material. It is made up of two steps. Firstly labeling or tagging or capturing the interested biological entities with magnetic beads, secondly applying a magnetic field on captured materials by means of magnetic separation device such as separators or fluid-based systems.

In order to achieve labelling magnetic beads must be coated with appropriate complementary material to desired biological entity. It may vary according to the research as shown in Figure 3.1.1.1.

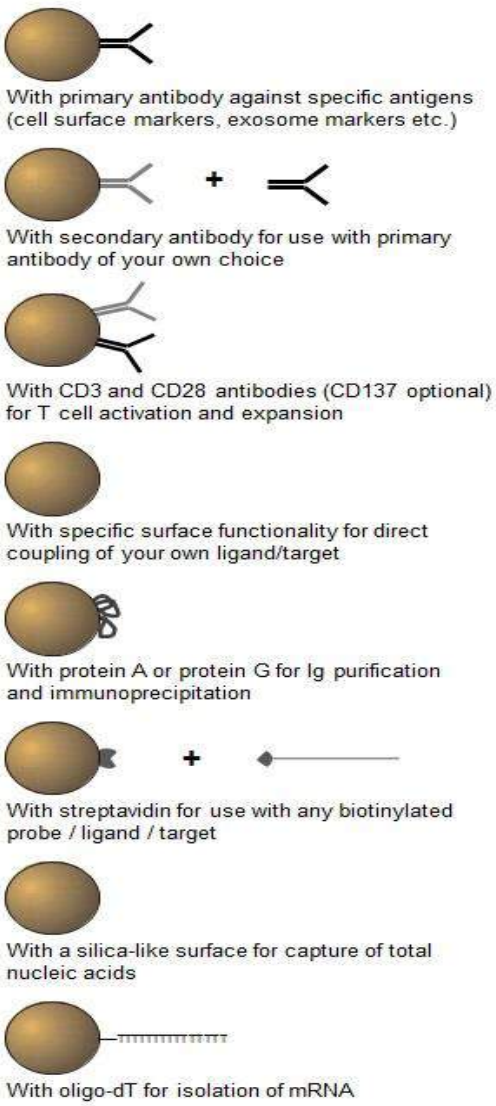


Figure 3.1.1.1: Coated magnetic particles with different biocompatible molecules [48]

For example, matching antibody coated magnetic particles with antigens is very accurate way to label interested entities. Selection of rare tumour cells, detection of malarial parasites from the blood or cell counting techniques are among the applications of magnetic separations [46].

3.1.2 Drug Delivery

Chemotherapy's main disadvantage is to be non-specific. Thus it may spread to the healthy tissue and destroy healthy cells. Drug delivery is to inject drugs especially to targeted entities [49]. Biocompatible magnetic nanoparticles are attached to drugs. This makes them drug carriers. Drug carriers are injected into blood vessel and magnetic field is applied in order to attract carriers on cancerous region in the body (Figure 3.1.2.1). Relasing chemicals from carriers are dependent on enzymatic activity or varying phsiological conditions like pH or temperature [46].

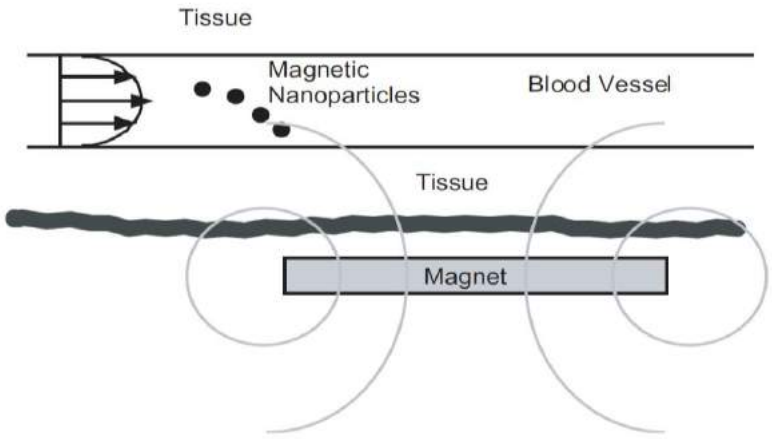


Figure 3.1.2.1: Applying magnetic field to drug carriers in the body [46]**.**

3.1.3 Hyperthermia

Hyperthermia or thermotherapy in cancer shortly means heating the tumorous tissue and killing the cancer cells. Using magnetic particles in hypertermia includes to send particles target tissue and to apply magnetic field in order to heat magnetic beads. 42ºC temperature for 30 minutes or more is sufficient to destroy cancerous tissue.

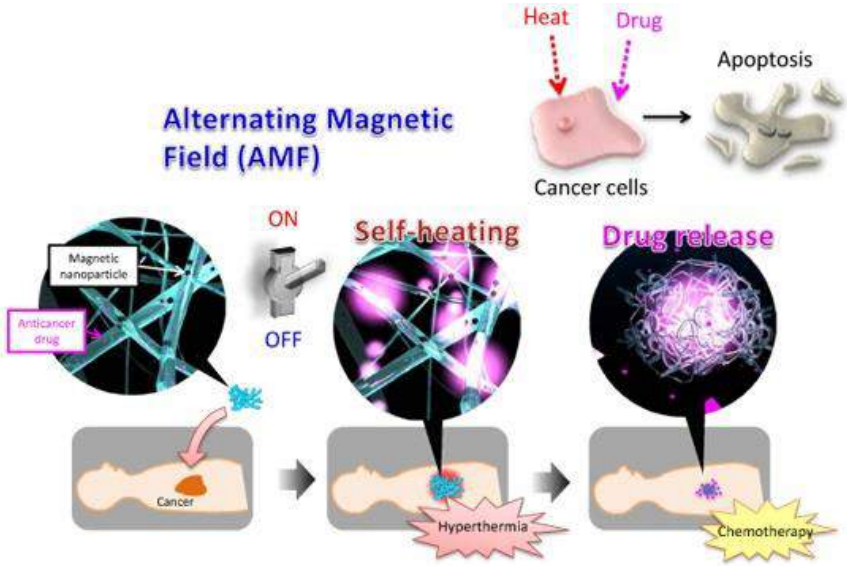


Figure 3.1.3.1: Summary of hyperthermia with magnetic nanoparticle [50]**.**

Chapter 4

Immunomagnetic Separation

Immunomagnetic Separation (IMS) is a technique to isolate specific cells from a heterogenous environment such as blood by using cell marker coated magnetic beads [51], [52].

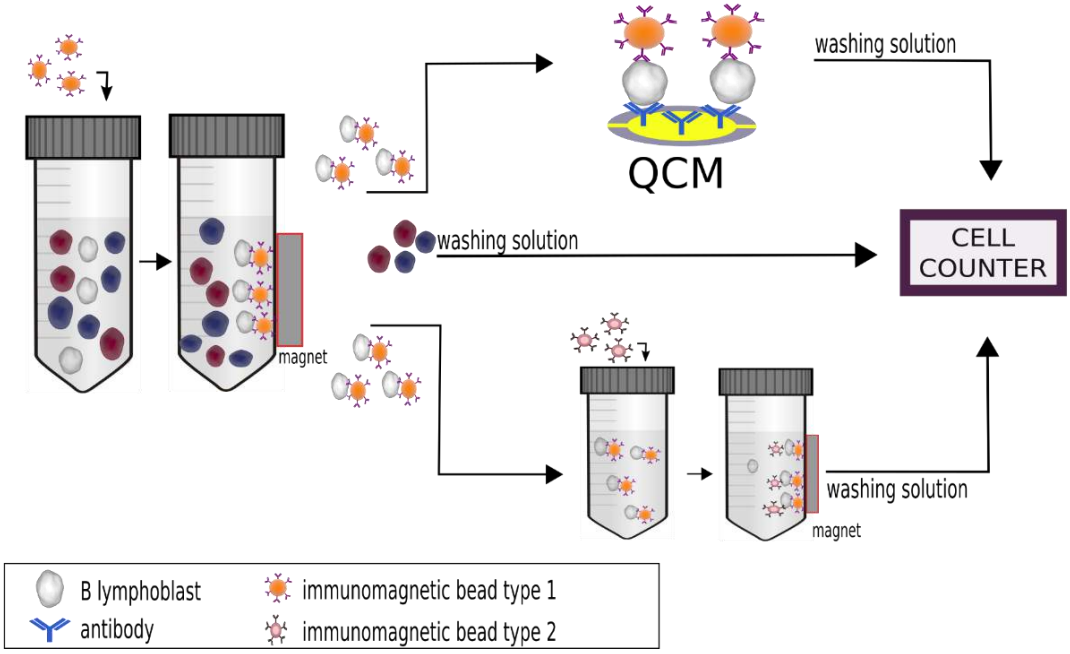


Figure 4.1: Schematic diagram of immunomagnetic seperation. It shows three different type of experiments. The middle one is single sorting which is made by only one antibody type. The bottom one is double sorting which is done with two different kind antibodies and two different cell surface markers. The above is attached cells on QCM device surface by means of different antibodies.

4.1 Method of Nanosize Beads Immunomagnetic Separation

4.1.1 Buffer Preparation

In order to prepare buffer solution, 0,5% BSA (Bovine Serum Albumine) is added into 10 mL of AutoMACS buffer for per labeling. It includes DPBS (Dulbaco's Phosphate Buffered Saline) in 7,2 pH value and 2 mM EDTA.

4.1.2 Cell Preparation

Cell culture is mixed first in order to divide cell clusters by pipette pump and serologic pipette. 5 mL of culture is taken from the flask into a 15 mL type of falcon tube and centrifuge it in 350 xg 5 mins. After aspirating the supernatant, 5 mL of DPBS (Dulbaco's Phosphate Buffered Saline) is added for washing the cells and counting cell numbers by MUSE device and Thoma Glass.

4.1.3 Labeling by Beads

$5x10^5$ cells/mL is taken from the cell-DPBS solution after counting and is centrifuged in 300 xg 10 mins. Supernatant is aspirated. Pellet is resuspended with 80 uL of buffer. 20 uL of CD19 Microbeads or CD45 Microbeads is added and mixed by rotating in 4 C in 15 mins. The brand of Miltenyl Microbeads is used for separate cells by magnetic beads. After 15 mins incubation cells are washed by adding 1-2 mL of buffer and centrifuging 300 xg 10 mins and aspirating supernatant. Pellet is resuspended with 500 uL of buffer (Figure 4.1).

4.1.4 Magnetic Separation

MiniMACS separator is placed onto the MACS stand. MiniMACS column is placed in the separator. The column is prepared by rinsing with 500 uL of buffer. This cell suspension is applied into the column. Unlabeled cells (shown in Figure 4.1.4.1) that passing through are collected into each well of 6 well plate. Column is washed 4 times with 500 uL of buffer. Column is removed from the separator and placed on a suitable collection tube and 1 mL of buffer is pipetted onto the column. Magnetically labeled cells are flushed out by pushing the plunger into column(Figure 4.1).

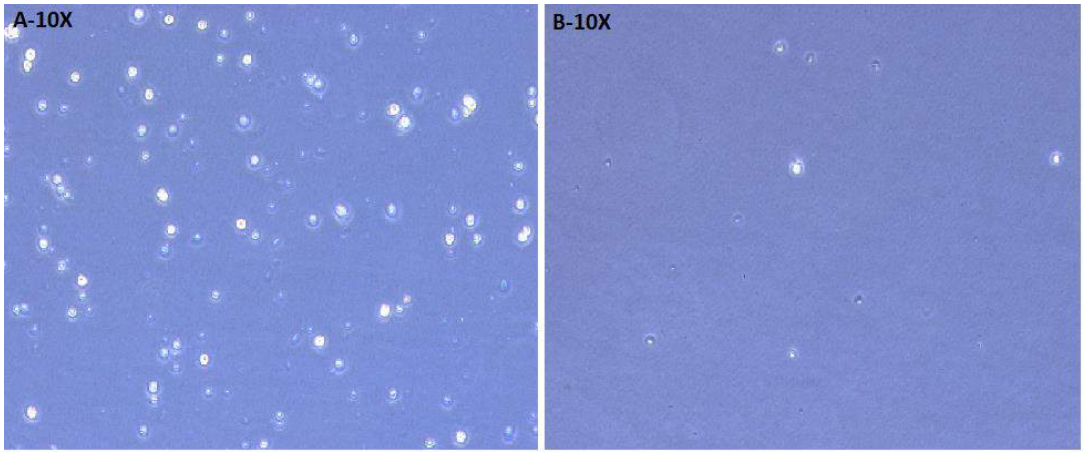


Figure 4.1.4.1: Unlabeled or missed from CD19 (A) and CD45 (B) Microbeads cells after separation. Washing cells in 10X zoom under microscope.

4.1.5 Visualization

Because these beads are approximately 8 nm, they cannot be seen under light microscopy. After collection of unlabeled cells into 6 well plate, it is placed under invert microscope. Images are taken by LAS software (Leica software). 4 unlabeled cells' plates are taken once in 10X. The well plate of labeled cells as shown in Figure 4.1.5.1 and Figure 4.1.5.2 is taken once in 4X and 10X, twice or more in 20X and 40X. All unlabeled cells are collected into one well and it is counted by MUSE and Thoma glass. Unlabeled cells number is taken away from initiate number of cells. It gives us labeled cell number. Graph 4.1.5.1 shows CD19 and CD45 missing percentages.

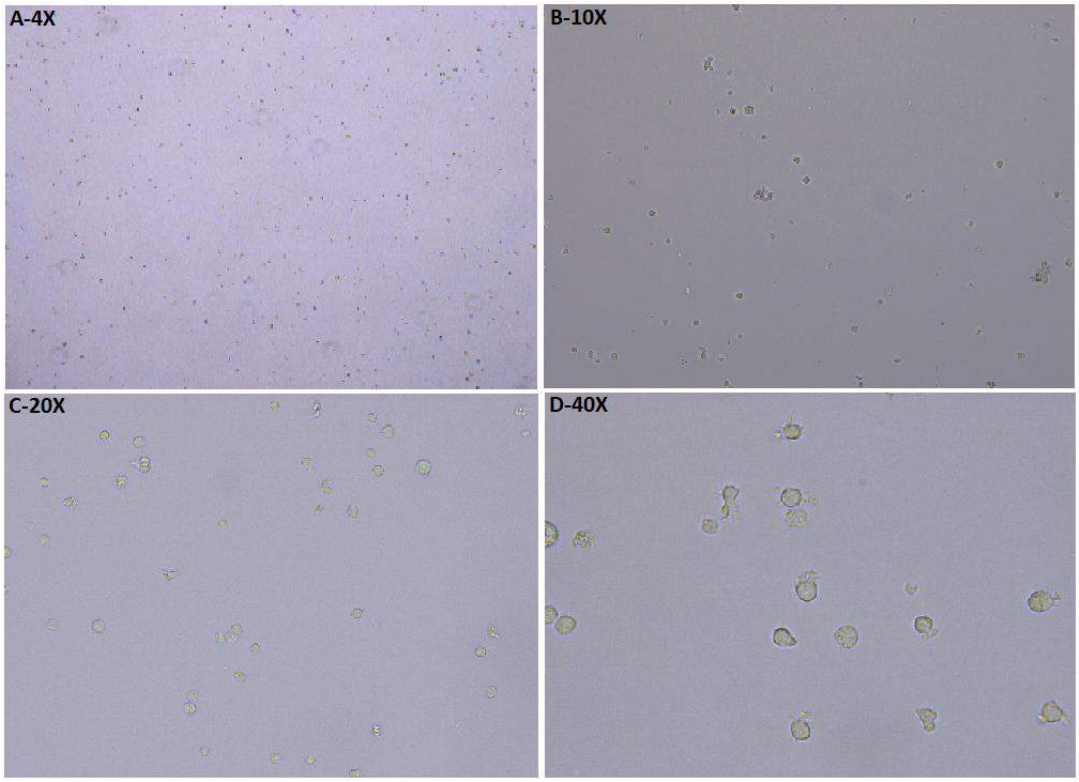


Figure 4.1.5.1: Captured leukemia cells by CD19 nanosize magnetic beads in different zooms.

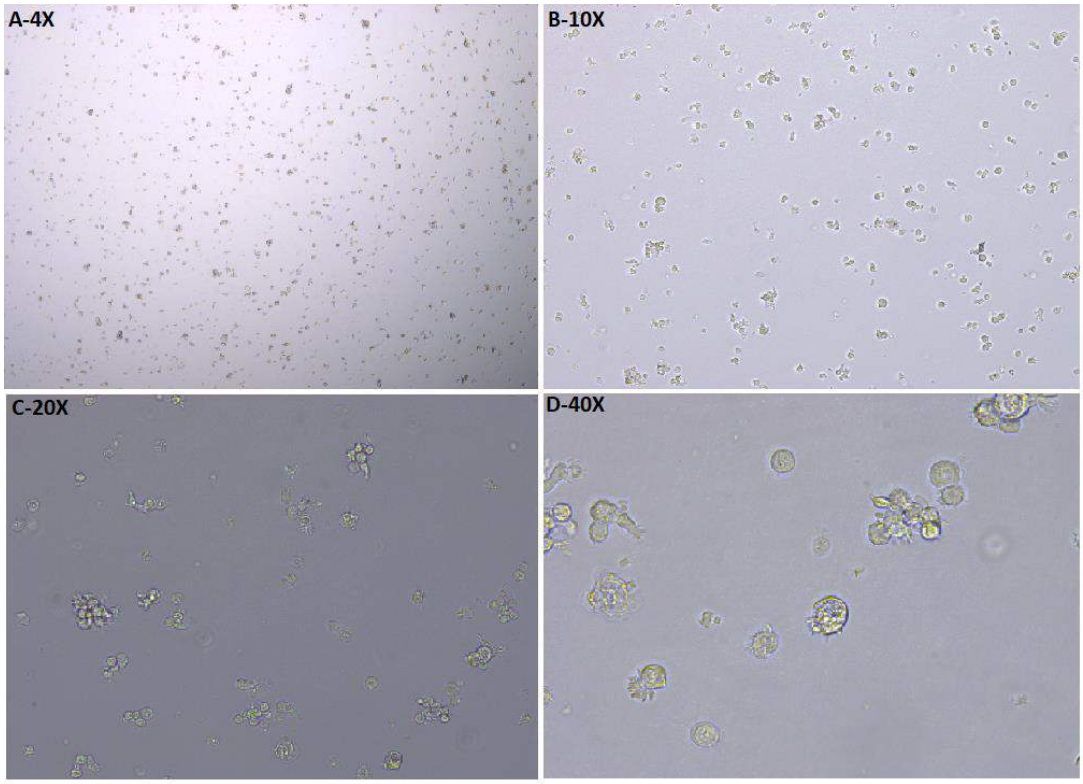


Figure 4.1.5.2: Captured leukemia cells by CD45 nanosize magnetic beads in different zooms.

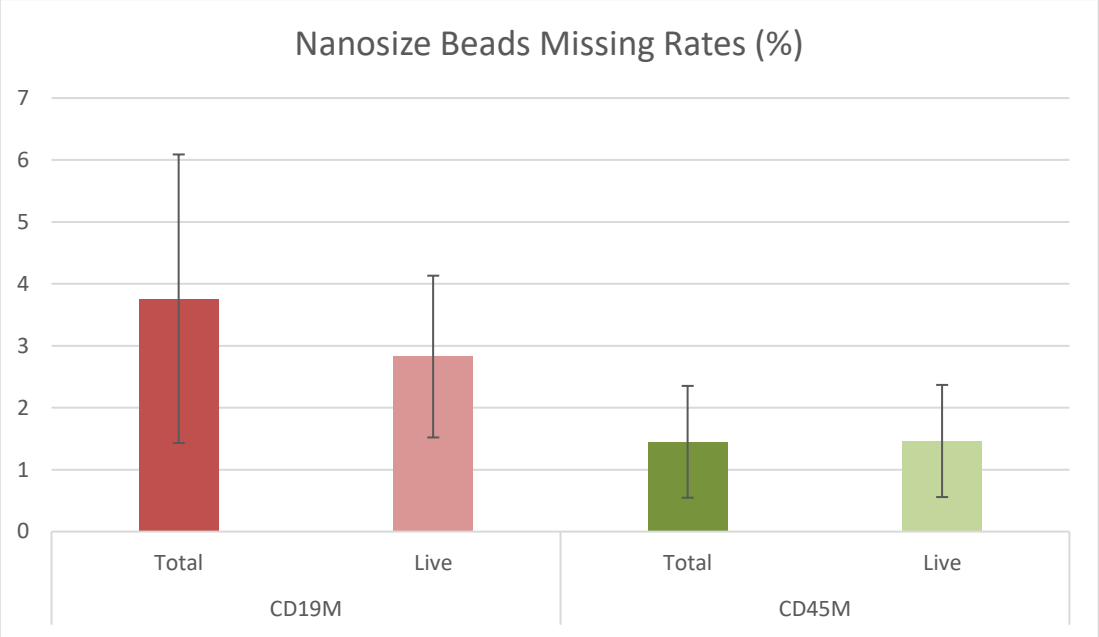


Figure 4.1.5.3: CD19 and CD45 Microbeads missing percentages.

4.2 Method of Microsize beads immunomagnetic separation

4.2.1 Buffer preparation

This buffer called Buffer-I and it includes 1x DPBS (Dulbaco's Phosphate Buffered Saline) without Calcium and Magnesium in 7,4 pH value and 2 mM EDTA and BSA is added in 0,1% percentage.

4.2.2 Preparing(Washing) Beads

These kind of beads are 4,5 µm. All beads are stored in 4 C. Dynabeads are mixed by rotater first in room temperature for 5 mins inside of 50 mL of a falcon in order to homojenize the beads. 1 mL of buffer-I is put into 2 mL of ependorf tube. 25 uL of CD19 Dynabeads or 40 uL of CD45 Dynabeads is added into the tube and mixed gently. This

bead solution is put into a magnetic separation rack as opened. After 1 or 1.30 mins aspirate the supernatant. And tube is taken from magnet and 1 mL of buffer-I is added and mixed again. One more time it is waited in 1 or 1.30 mins and aspirated supernatant. It is ready to incubate in next step.

4.2.3 Cell Preparation

Cell culture is mixed first in order to divide cell clusters by pipette pump and serologic pipette. 5 mL of culture is taken from the flask into a 15 mL type of falcon tube and centrifuge it in 350 xg 5 mins. After aspirating the supernatant, 5 mL of DPBS (Dulbaco's Phosphate Buffered Saline) is added for washing the cells and counting cell numbers by MUSE device and Thoma Glass.

4.2.4 Labeling by Beads

$5x10^5$ cells/mL is taken from the cell-DPBS solution after counting and is centrifuged in 300 xg 10 mins. Supernatant is aspirated. Pellet is resuspended with 1 mL of buffer. These cells are added into prewashed 25 uL of CD19 Dynabeads or 40 uL of CD45 Dynabeads and mixed by rotating in 4 C in 20 mins. The brand of Invitrogen Dynabeads is used for separate cells by magnetic beads. After 20 mins incubation cells are ready to separate into unlabeled and labeled ones (Figure 4.1).

4.2.5 Magnetic Separation

This cell suspension is placed into the magnetic separator in order to manipulate cells stick togetether magnetic beads. The magnet attracts magnetic beads towards itself. After 2 mins waiting 1 mL of buffer solution is aspirated and collected into one well of 6 well plate. Those are unlabeled cells. The tube is removed from the separator and 1 mL of buffer is added and mixed again gently. This washing steps are done 3 times and after

third one 1 mL of buffer is added and this labeled solution is collected into one plate of the 6 well plate. Figure 4.2.5.1 shows missing cells from seperator.

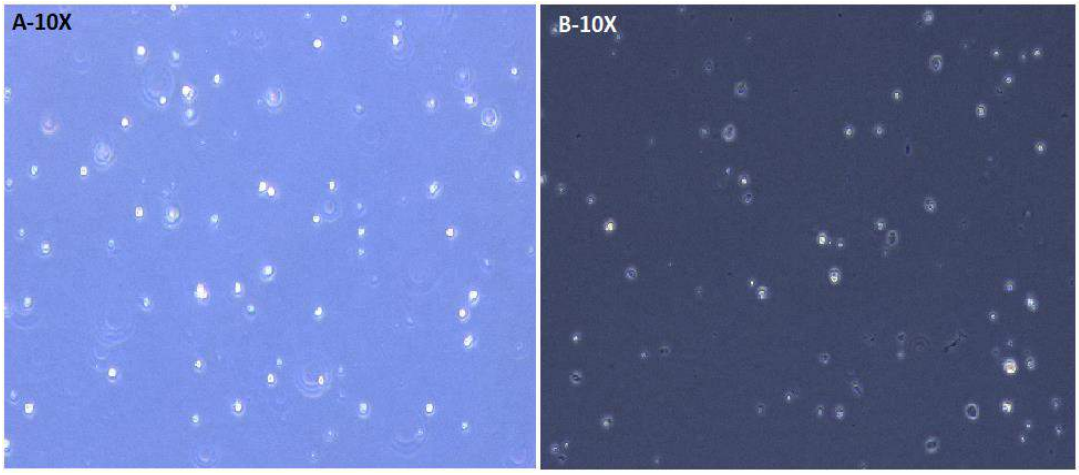


Figure 4.2.5.1: Missing cells from separator. A represents unlabeled cells by CD19 antibodies while B represents for unlabeled cells by CD45 antibodies.

4.2.6 Visualization

After collection of unlabeled cells into 6 well plate, it is placed under invert microscope. Images are taken by LAS software (Leica software). 3 unlabeled cells' plates are taken once in 10X. The well plate of labeled cells is taken once in 4X and 10X, twice or more in 20X and 40X as shown in Figure 4.2.6.1 and Figure 4.2.6.2. All unlabeled cells are collected into one well and it is counted by MUSE and Thoma glass. Unlabeled cells number is taken away from initiate number of cells. It gives us labeled cell number. Graph 4.2.6.1 shows missing percentages of unlabeled cells.

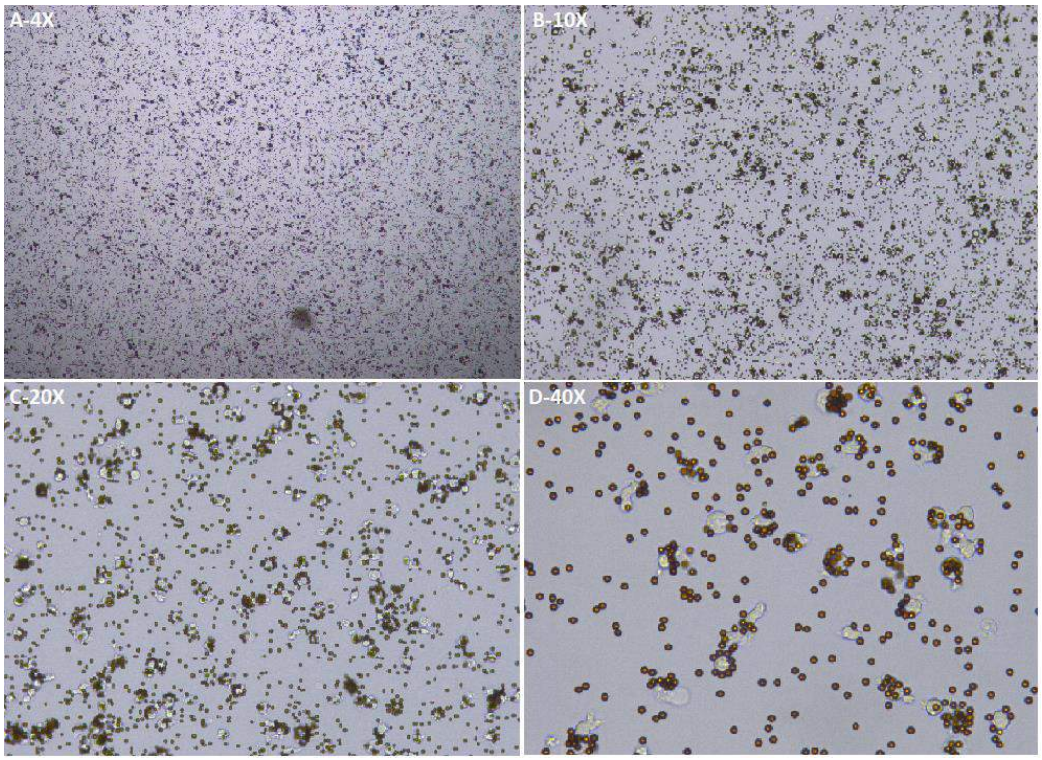


Figure 4.2.6.1: Captured leukemia cells by CD19 microsize magnetic beads in different zooms.

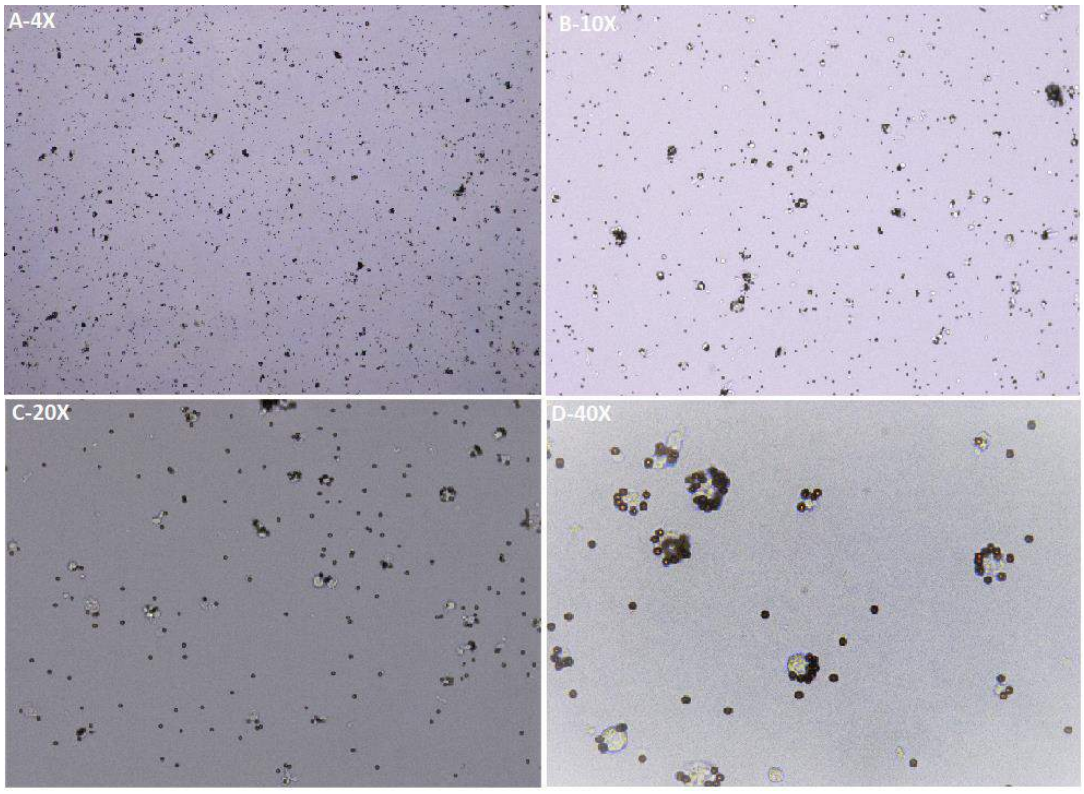


Figure 4.2.6.2: Captured leukemia cells by CD19 microsize magnetic beads in different zooms.

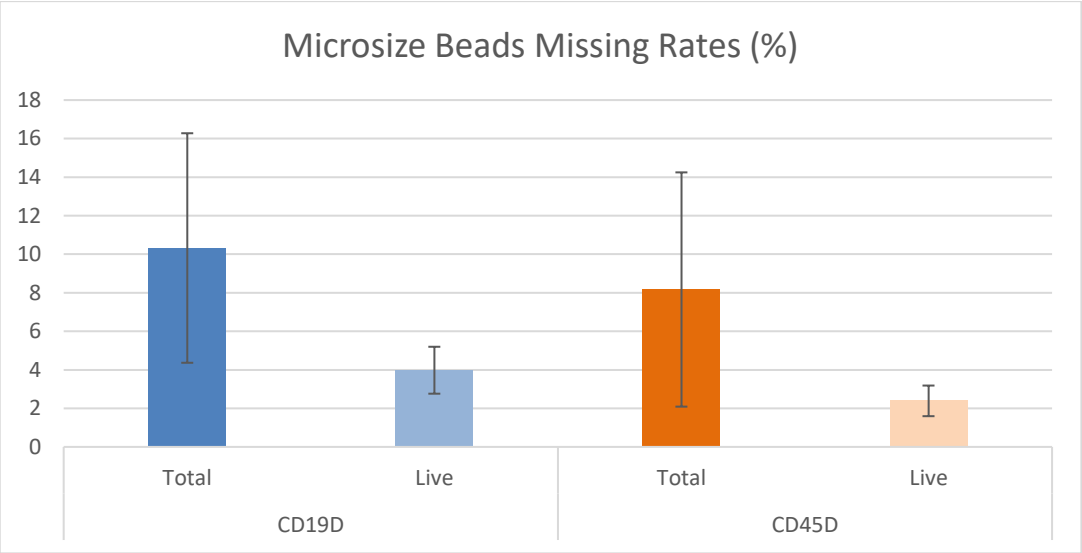


Figure 4.2.6.3: CD19 and CD45 Dynabeads missing percentages.

4.3 Double Sorting

This process involves to be captured cells that labeled with nanobeads by Dynabeads. After cells captured by nanosize beads, bead positive cells are taken and dynabeads protocol is applied. Following that, unlabeled cells (by dynabeads) are counted and missing rates calculated. Dynabeads negative but nanobeads positive cells are counted and missing rates are calculated (Graph 4.3.1). Those experiments are made in two parts. One of them after labeled cells CD19 Microbeads (Figure 4.3.1) capturing them with CD45 Dynabeads (Figure 4.3.2). The other is labeling cells with CD45 Microbeads (Figure 4.3.3) then capturing with CD19 Dynabeads (Figure 4.3.4). As shown in figure 4.1.

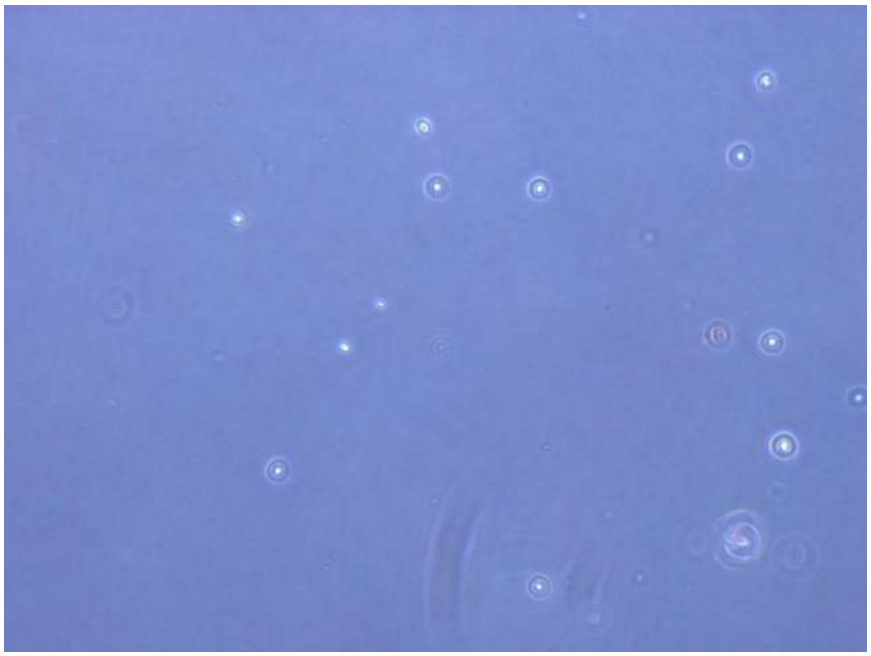


Figure 4.3.1: CD19 Microbeads positive but CD45 Dynabeads negative cells. Washed cells after magnetic rack separator.

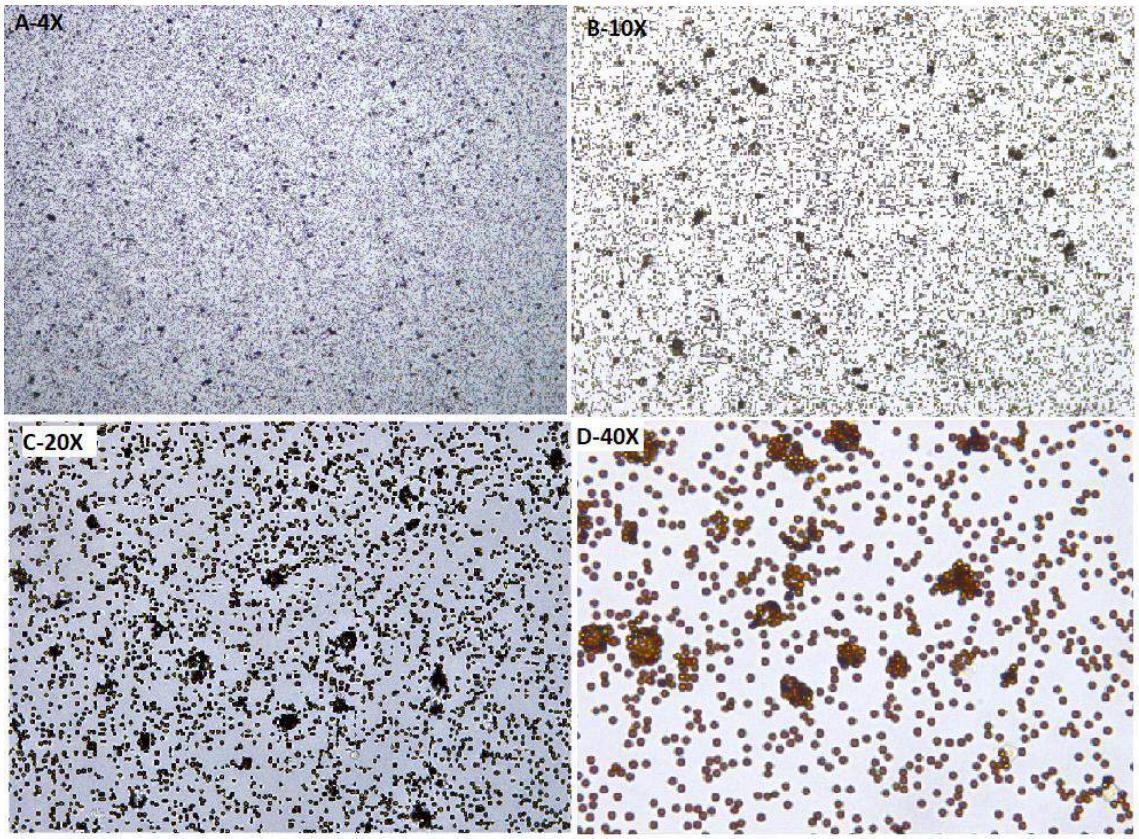


Figure 4.3.2: Labeled cells with CD19 Microbeads and CD45 Dynabeads. Captured cells after separation.

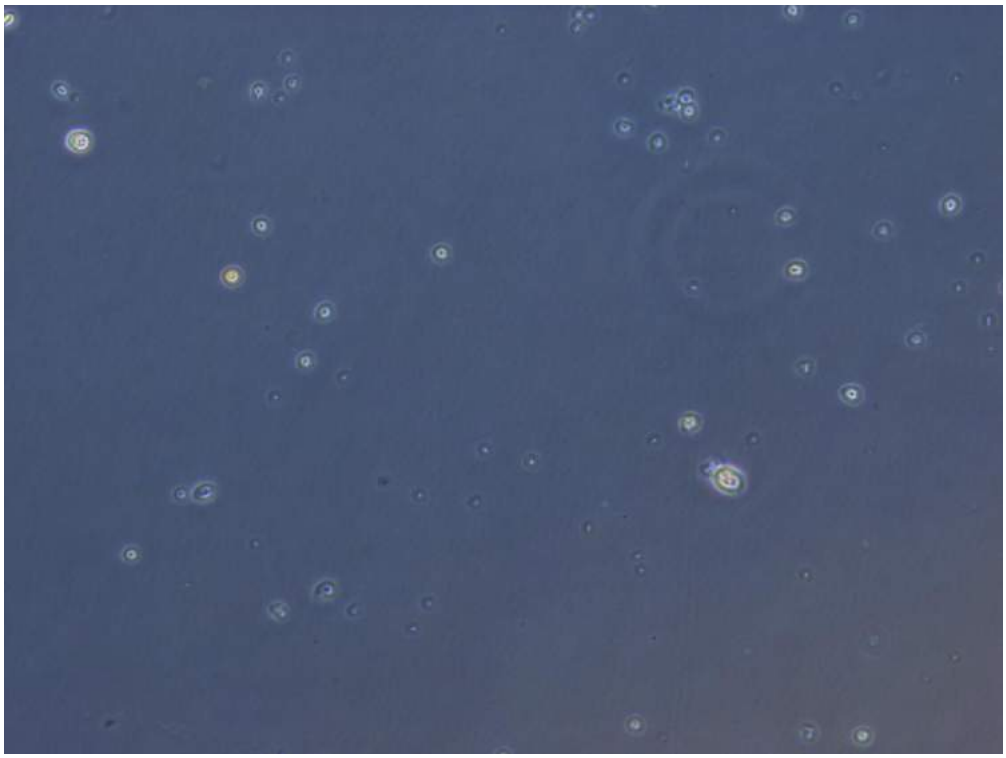


Figure 4.3.3: CD45 Microbeads positive but CD19 Dynabeads negative cells. Washed cells after magnetic rack separation.

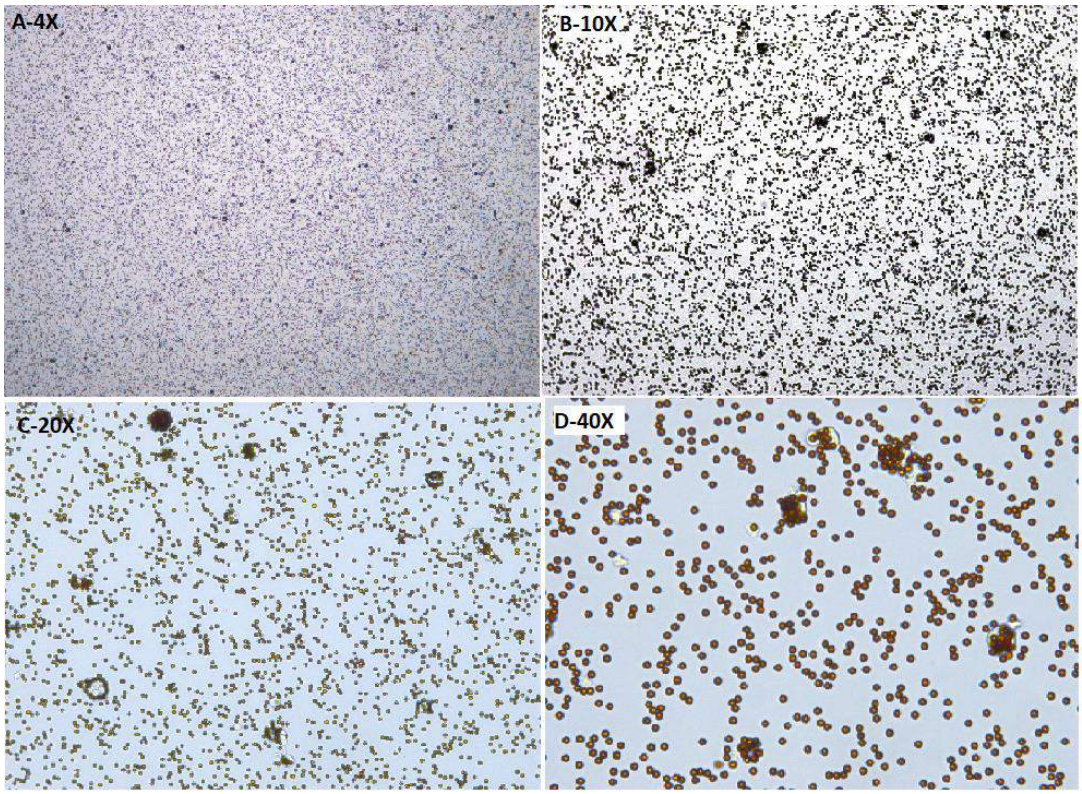


Figure 4.3.4: Labeled cells with CD19 Microbeads and CD45 Dynabeads. Captured cells after separation.

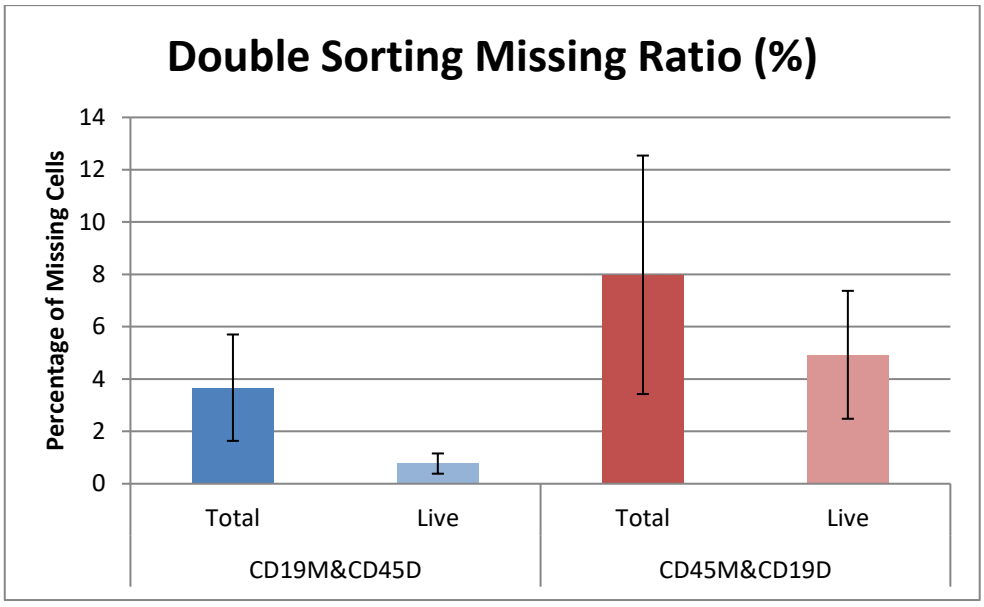


Figure 4.3.5: Washing cells after labeled with both kind of magnetic beads.

Chapter 5

Immobilization of Immunomagnetic Labeled Leukemia Cells

5.1 Quartz Crystal Microbalance (QCM)

Before immobilization studies, to see whether cells could bind to gold surface, binding ratio of leukemia cells is measured by Quartz Crystal Microbalance device. QCM is a vehicle showing decreased frequency when a molecule binds to its surface [53]. It has the quartz disc which has electrodes on both sides. This feature allows biologic molecules to be immobilized more sensitively [54]. Appropriate liquids, which activate the surface of disc to catch biomolecules, are sent to the quartz disc plate by a peristaltic pump as shown in Figure 5.1.1 [54]. It converts mass of binding cells into frequency signals [55]. Decreasing frequency signal shows that mass increases on the quartz disc [55].

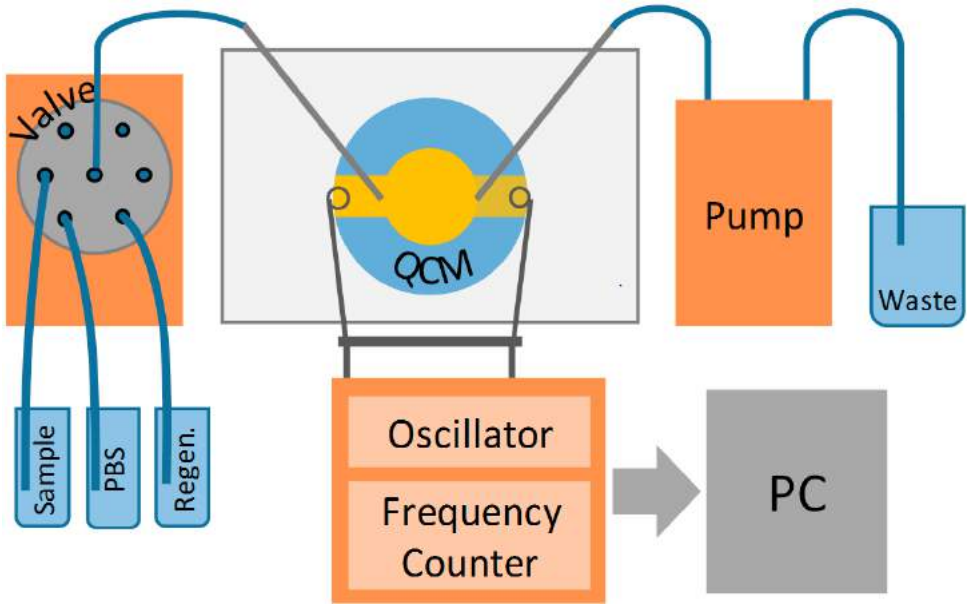


Figure 5.1.1: Experimental set-up for QCM [54]**.**

5.2 Capturing Cells on the Quartz Disc

All liquids are sent to system and are kept there with close loop untill frequency changing reaches to 1 Hz. When the frequency graphs show 1 Hz, it means the surface saturated.

To make quartz disc plate of QCM device able to bind molecules, Mercapto Undecanoic Acid (MUA) is sent to system first (Figure 5.1.1). MUA solution is prepared by adding 2,18 mg of lyophilised MUA into 10 ml of ethanol. The carboxyl group (-COOH) of MUA prepares to bind 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) molecules whilst the Sulfure group of MUA binds to gold surface (Figure 5.1.1 B and C) [56]. Following this step ethanol and deionized water (DIW) is passed through the system in order to wash non-specific bound molecules. Then EDC/NHS coupling is flowed through the system. This two chemical together activates the -COOH group [57]. Again to remove non-specific bindings DIW and Posphate Buffer Saline (PBS) is passed through. Next step is to bind Protein G to NHS molecule. N-terminal region of Protein G binds to previous active region of NHS whilst C-terminus of ProG is reactive for the next antibody [58]. is Protein G is prepared as stock by adding in 5% of Protein G into DIW. Required protein G is taken 800 µL from this stock and added into 5,4 mL PBS. Then washing step is to send PBS to the system.

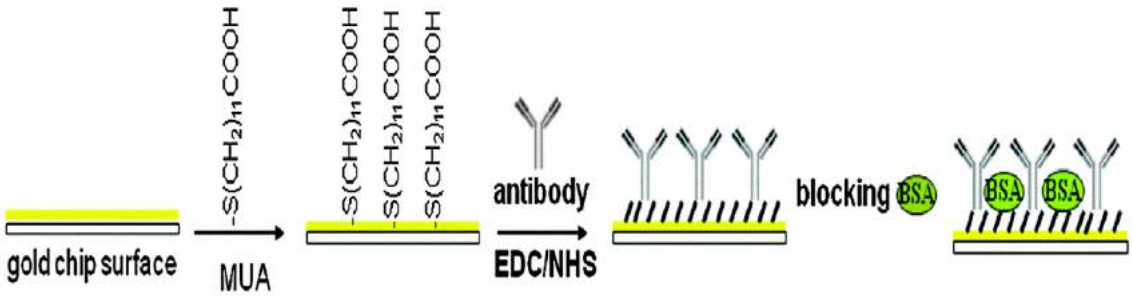


Figure 5.2.1: Illustration of immobilization biomolecules to golden surface. Binding to surface of MUA and EDC/NHS. BSA blocks open non-binding sites are shown [59]**.**

Following that CD19 are flowed the system like in Figure 5.1.1). CD19 BioLegends antibodies are also preparead as stock by adding 20 µL of CD19 in 1 mL PBS and from

this stock 500 µL is taken and joint into 5,4 mL PBS. Again PBS is sent in order to wash unbound molecules. Next step is to send BSA solution which is prepared in 5% percent in PBS solution. BSA closes the unbound molecules to antibodies (Figure 5.1.1). After washing with PBS, labeled cells with CD45 antibody coated nanobeads are added to system and frequencies are calculated. Figure 5.2.2 shows QCM disc image taken by microscopy in 20x zoom.

Figure 5.2.2: QCM disc image under the microscope.

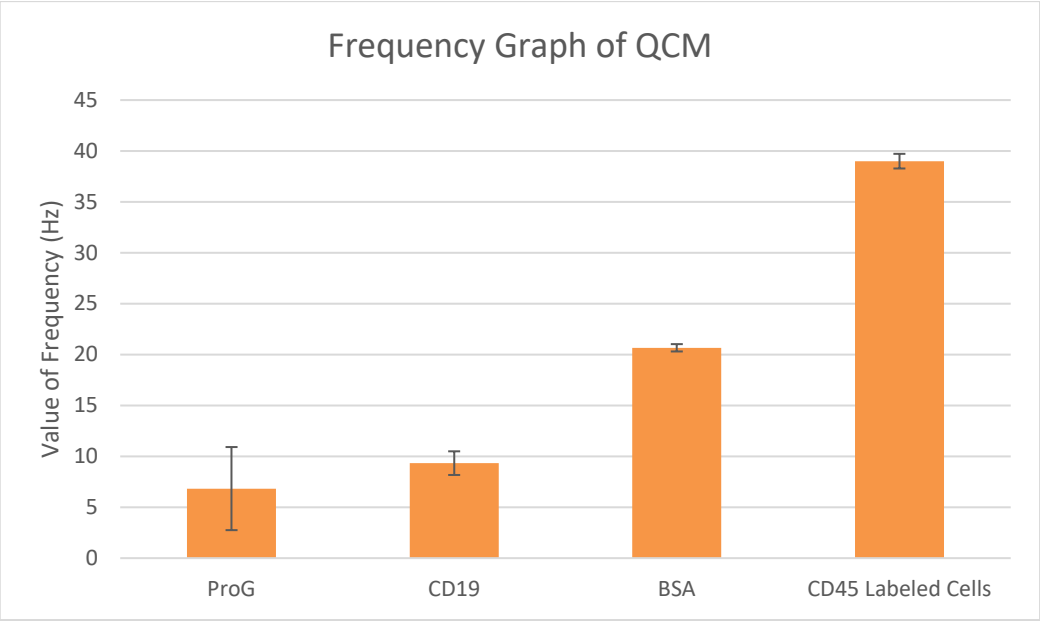


Figure 5.2.3: Frequency values of QCM for labeled cells.

5.3 Capturing Cells on the Square Areas

Same procedure with same amount of chemicals is applied not flowing but dipping method with small difference. After cleaning Square Surfaces by exposing to UV light and dipping them into boiled in the piranha solution. Piranha is made of 30 mL of DIW, 20 mL of ammonia and 20 mL of Hydrogenperoxide (H_2SO_4).

After drying cleaned surfaces, MUA solution is added first for overnight. The next day surface is removed from MUA solution and dipped into ethanol and deionized water alternately to wash non-binding MUA molecules. The gold square surface is dipped into EDC/NHS solution. After a while, the surface is taken and put into DIW and PBS alternately for a short time. Following step is to dipp surface into Protein G solution and to put into PBS. Then CD19, PBS and BSA are the solutions for surface to be dipped. Lastly surface is taken into solution of labeled cells with CD45 immunomagnetic beads like shown in the Figure 5.3.1.

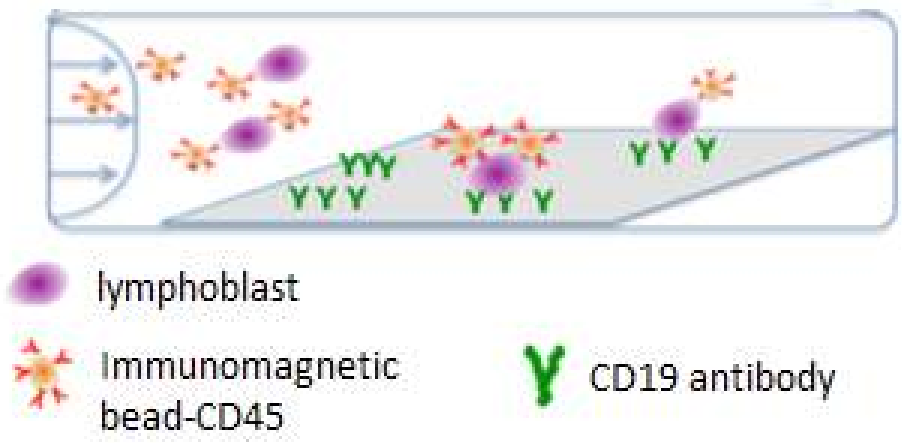


Figure 5.3.1: Shematic of binding immunomagnetic labeled cells to surface activated by antibody.

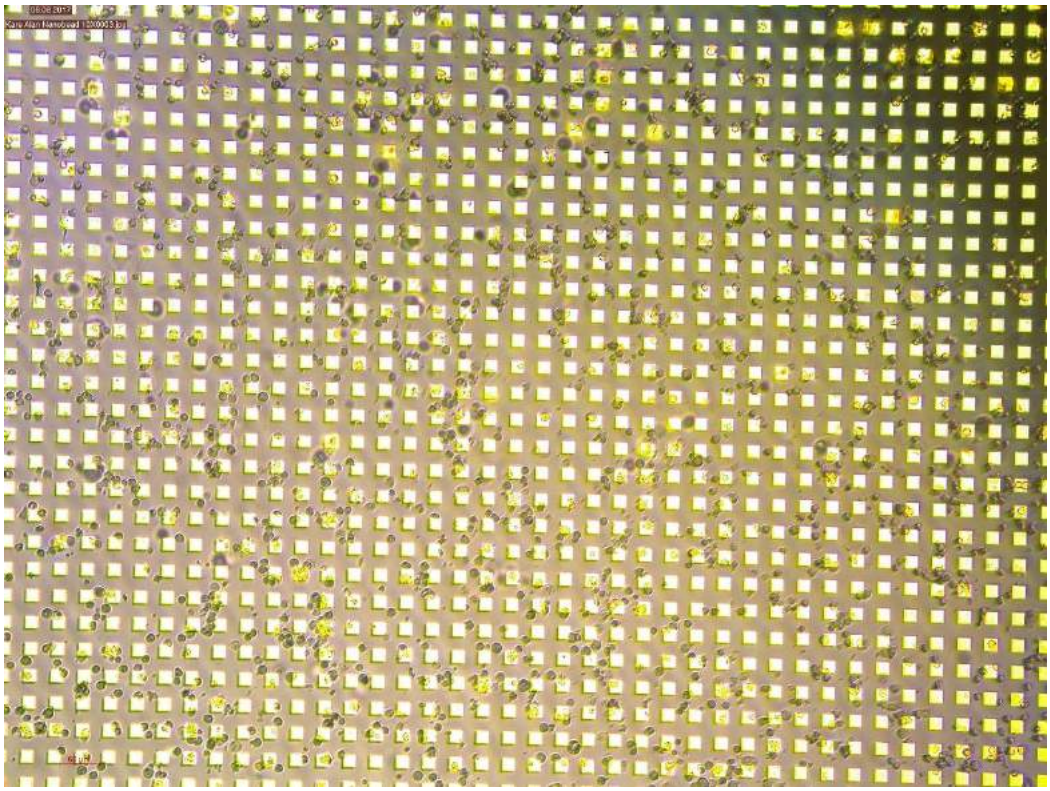
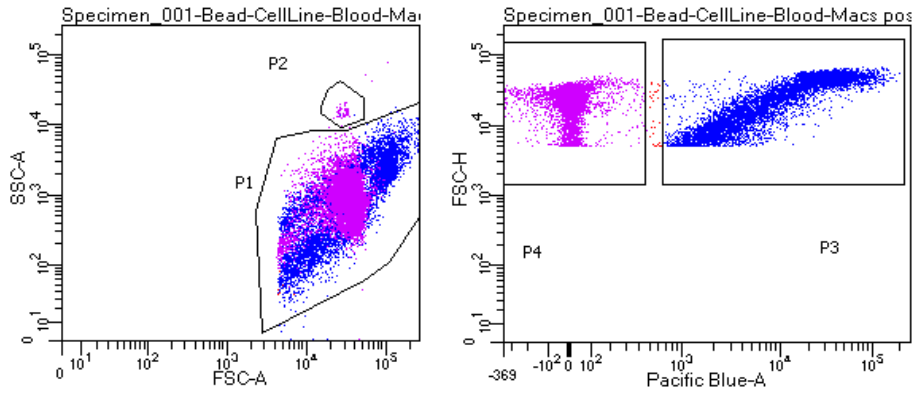


Figure 5.3.2: Microscope image of captured cells on golden square surface.

Chapter 6

Conclusion

For further investigation of separation, CD19 Nanobeads are applied to whole blood in order to extract white blood cells which have interested marker. Unlabeled and labeled cells are measured by XN-3000-L blood analyse device in Erciyes University Fevzi Mercan Child Hospital. In those works we measured blood only, mixed blood with CCRF-SB cell line, and their bead positive version and separated versions magnetically. Also, same samples are measured by Flow Cytometry device. Results are logical according to graphs for both devices and certain numbers in XN-3000 blood analyse device (Figure 6.1).



Tube: Bead-CellLine-Blood-Macs positive

Population	#Events	%Parent	%Total
All Events	13,208	####	100.0
P1	13,134	99.4	99.4
P2	57	0.4	0.4
P3	6,247	47.3	47.3
P4	6,930	52.5	52.5

Figure 6.1: Flow Cytometry results of bead positive cells of CCRF-SB cell line and lympocytes of whole blood. Blue color shows cell line, the cells are painted cell line by Pacific Blue dye. Pink color shows remained blood cells after magnetic separation.

But those experiments still require optimization about leukocytes in the whole blood and cell numbers after magnetic separations.

One of the further works of this study is to design a microfluidic chip that can be easily manipulated the immunomagnetic cells by applying optimized amount of magnetic field.

It includes pumping system to send liquids into inlets of chip. Also in another chip design gold surfaces will be integrated with microfluidics.

The next aim about immobilization work is to image captured labeled cells on gold square surfaces in Scanning Electron Microscopy (SEM). There are partially successful work but still it needs to be improved (Figure 6.2).

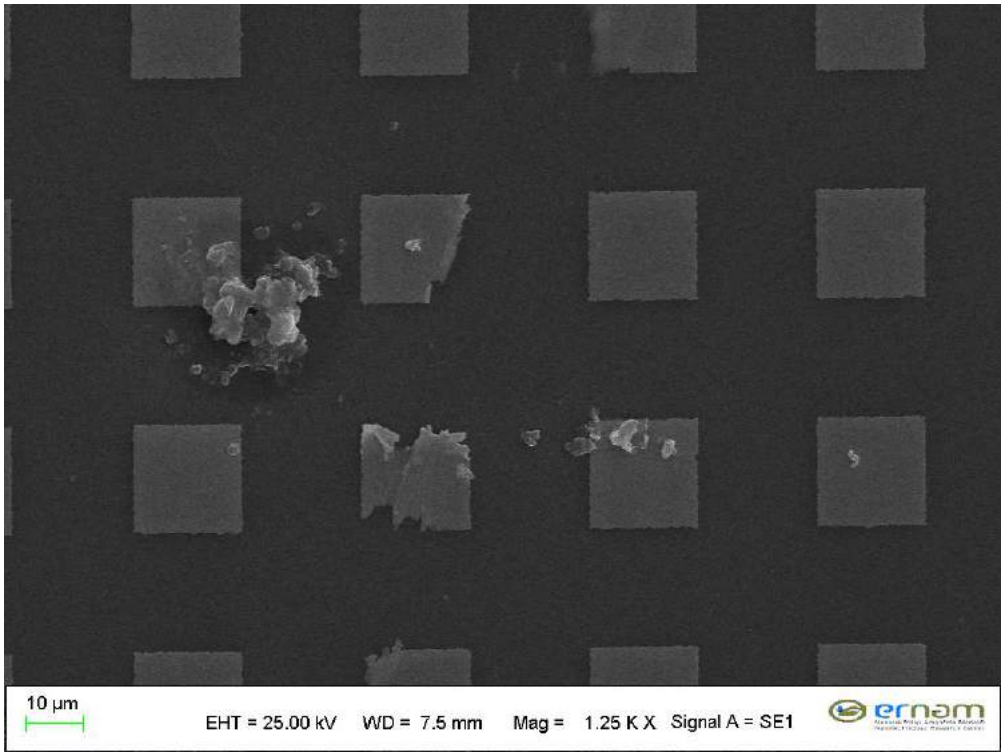


Figure 6.2: SEM image of the piece of nanobead labeled cell.

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