UNIVERSITY OF RWANDA

ASSESSMENT OF HUMAN LEUKOCYTE VIABILITY IN VITRO CELL CULTURE CONDITIONS AT THE UNIVERSITY TEACHING HOSPITAL OF BUTARE (CHUB)

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By

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Co-Supervisor: Dr. TOLESSA MULETA DABA (PhD)

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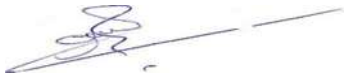
DECLARATION OF INDEPENDENT WORK

I, **Jean Pierre GASORE** hereby declare that this research project submitted to the University of Rwanda, for the degree Master of Science in Biotechnology is my own original work and has not been submitted before to any Institution by myself or any other person in fulfilment of the requirements to the award of any degree or any other qualification.

GASORE Jean Pierre 

DEDICATION

First, I thank my Almighty God for the gift of life and for sustaining me throughout this journey. This work is dedicated to my beloved wife for granting me the chance to pursue the master's studies, her constant encouragement; her help made my journey possible. My beloved children who are patiently staying at home in my absence for this work also deserve dedication. My lecturers who shared knowledge and worked closely with me throughout my academic journey also share this dedication.

Many Thanks to my supervisors for guidance and support, especially in providing the necessary resources to complete the study.

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To my colleagues, thank you for the cooperation, teamwork, and shared commitment to learning. Lastly, to my dear sisters and brothers, thank you for your prayers, love, and moral support.

May God bless you.

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LIST OF ABREVIATIONS

RPMI: Roosevelt Park Memorial Institute (RPMI 1640)

DMEM: Dulbelco`s Modified Eagle Medium

MTT: 2-(4,5-dimethyl-2-thiazolyl)-3,-5-diphenyl-2H tetrazolium Bromide

XTT: (2,3-bis((2-Methox-4Nitrxy-4Nitr0-5Sulfophenyl(-2H-Tetrazolium-5-Carbxanilide)

PHA: Phytohemaglutinine

FBS: Foetal Bovine Serum

WTS-1: (Water-Soluble Tetrazolium Salt-1)

PHA: Phytohemaglutinine

RT: Room temperature **KCl:**

Potassium Chloride

ATP: Adenosine Triphosphate

CHUB: University teaching hospital of Butare

PBMCs: Peripheral Blood Mononuclear Cells

Penstrep: Penicillin/Streptomycin

KCl: Potassium Chloride

PBS: Phosphate Buffered Saline

PBLC: Peripheral Blood lymphocytes culture

IMDM: Iscove's modified DMEM

ABSTRACT

Cytogenetic is the study of chromosomal number and structural observable under a microscopy. It is a valuable tool to diagnose genetic disorders caused by chromosomal abnormalities, as well as acquired conditions such as leukemia. One common cytogenetic technique, karyotyping, involves culturing blood leukocytes in special culture media, usually Roswell Park Memorial Institute (RPMI) 1640, which contains hormones and growth factors that stimulate cell division and proliferation. After three to four days, cells are harvested at the metaphase stage, when chromosomes are most condensed and readily visualized under microscope.The success of karyotyping depends on cell viability and proliferation. Factors such as nuclear alteration in dying cells, low mitotic index, and poorly defined chromosomal pattern can hinder accurate analysis. Moreover, since cytogenetic laboratories are usually located in major facilities, delays in sample processing can impair cell viability. A successful karyotyping is achieved when blood samples are cultured within 24 hours of collection; aging samples greatly reduce mitotic activity, sometimes resulting in complete absence of mitosis. Despite this limitation, there is lack of studies on cell viability during cell culturing for karyotyping at CHUB.

This study aimed to assess leucocytes viability prior to culturing and to evaluate the viability of cells cultured on RPMI by using Trypan Blue exclusion method among patients attending the genetic service at CHUB.

A cross-sectional experimental study wa conducted on 50 heparinized blood samples from adult males and females. Leukocyte count was done by using the Sysmex XN-3500.Cell viability test was assessed at three time points: after 24 hours at room temperature, 72 hours of refrigeration and finally at 72 hours of culturing on RPMI. Data were analyzed using SPSS Version 25 .RPMI 1640 significantly preserved leucocytes viability after 72 hours of culture

:13,34% higher than 24 hrours at room temperature after blood collection. Prolonged Refrigeration impacted leukocyte viability: 4.2% reduction of cell viability . The leucocyte count from fresh samples at 24 hours did not reliably predict the cell viability of leukocytes isolated after RPMI 1640 culture.

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CHAPTER 1. INTRODUCTION

1.1 Background

The percentage of healthy, living cells in a sample population is known as cell viability. It is an essential metric for assessing the physiological condition of cells in experimental settings, such as Karyotyping (Tamar A, 2020). Viable cell density and cell viability are both core indicators of a successful culture in biomanufacturing and pharmaceutical industries (Lomont & Smith, 2024). It is a key and fundamental measurement in manufacturing and release of cellular therapeutic products (CTPs) which contain cells and cellular products as the active products (Pierce et al., 2021). The determination of cell proliferation and viability serves as a cornerstone tool for numerous in vitro analysis that test cellular responses to the surrounding factor (Khalef *et al*., 2024)**.** The process of successful production of live viral vaccines (LVV) in bioreactors relay on the analytical characterization of cell culture. Both cell viability and viable cell density are among important parameters leading the outcome of cell culture in the production of biopharmaceuticals (Lomont & Smith, 2024). Cell viability monitoring is crucial in various applications such as dosage tests for antitumor drugs, virology, and stem cell research. Cell monitoring is the perfect tool to identify diagnostic markers of the diseases and facilitate the designing of target therapies (Cutuli et al., 2025). Many environmental factors affect cell viability; Literatures highlight the culture conditions, exposure to pharmacological agents, growth factors, nutrients content, infections and diseases (Ude *et al*., 2023). The transit time of the sample is another factor that will affect the viability of cells . The development of knowledge in the study of human uncommon diseases depends on the ability to store and transfer human cells over vast distances in a stable medium. Cell viability may be reduced by the common cryopreservation technique, which is regarded as the gold standard. Due to the evaporation of liquid nitrogen or dry ice, transportation delays may cause total cell lysis, leaving samples in cryoprotective chemicals that are cytotoxic at ambient temperature. Researches suffer greatly when patient samples are lost in this way, and this is especially true when the samples come from individuals with rare diseases. (Dewhurst *et al*., 2021). In karyotyping one of cytogenetic techniques, peripheral blood cells are taken from patients and grown in culture usually RPMI 1640 in older to have enough cells to analyze. (Santos et al., 2019). With karyotyping, clinicians

can screen and diagnose inherited genetic disease and cancer (El-Khateeb *et al*., 2019). In all laboratory scales including karyotyping, too few living cells can hinder the effectiveness of the products (Pierce *et al*., 2021). Lymphocyte viability is negatively impacted by sample culturing delays longer than 24 hours, which might result in a marked decrease in the mitotic index in comparison to the freshly cultured sample. In human karyotyping, samples with live cells that can divide to enter the cell cycle's metaphase are examined (El-Khateeb *et al*., 2019). A successful karyotyping is achieved when samples are cultured within 24hrs. Chances of a success in karyotyping of aged samples are very rare as it will affect the viability of cells. Quality of leukocyte culture is challenged by storage, delay in processing, and varying culture media. Understanding the challenges and optimisation of these conditions is verry important to improve diagnostic outcome (Martinovic *et al*., 2020).

1.2 Problem Statement

Karyotyping remains a critical diagnostic tool for detecting chromosomal abnormalities in patients with genetic disorders, infertility, cancers and congenital abnormalities in clinical and research settings. Accuracy and success of karyotype analysis largely depend on the viability and quality of cultured human peripheral blood leukocytes on RPMI. Factors such as delayed blood processing, poor storage conditions, and suboptimal culture media compromise cell viability. This result in poor quality metaphase spreads and ultimately affect the karyotyping results. In many hospitals, especially in low-resource settings like Rwanda, karyotyping tests are performed only in large centers where some samples can reach laboratory beyond 24 to 72 hours while samples are supposed to be cultured in less than 24 hours (Martinovic *et al*., 2020). Without proper evaluation of pre and post-culture viability, failed or inconclusive karyotype results may occur, leading to diagnostic delays, false positive or negative result or the need for repeat sampling. This can burden both the laboratory and patients. In the University Teaching Hospital of Butare (CHUB), karyotyping is based on leukocyte culture on RPMI 1640. Despite the importance of this test, there is no records of study done on cell viability and factors associated with it at CHUB. This discrepancy may hinder cell culture process optimization and lower the caliber of the outcomes. To enhance clinical laboratory procedures, this study sought to evaluate leukocyte viability under culturing on RPMI and refrigeration conditions at CHUB.

1.3 Significance

This study assessed the importance of the human leukocyte culture in RPMI 1640 to improve cell viability. The findings are relevant to the cytogenetic laboratory of CHUB and other research settings where karyotyping relay on culturing human peripheral blood. This work fulfils the knowledge gap in Rwanda where no studies have assessed leukocyte viability on RPMI in local conditions and the information obtained from these study findings can contribute to the advancement in biotechnology industries in Rwanda, biopharmaceutical companies especially those, which use cell culture techniques in their research and development. This study finding may also provide insight on the possibility to build a Biobank of leukocytes for various research purposes in Rwanda.

1.4 Hypothesis

There is a significant difference in the viability of blood leukocytes before and after culturing for karyotype preparation.

1.4.1 Research Questions

- How does the viability of leukocytes in whole blood differ from that of leukocytes from isolated cultures in RPMI medium?
- Is there difference between viability of fresh samples and refrigerated samples?
- Is there a relationship between cellularity and cell viability of human leukocyte?

1.5 Objectives

1.5.1 General objective

The main goal of this study was to assess the vitality of the blood cells before culture by using Trypan Blue exclusion and to examine the cell viability when grown on RPMI after they were harvested.

1.5.2 Specific Objectives

The specific objective of this study were:

- To determine the difference of leukocyte viability on whole blood samples after 24 hours vs. Isolated culture on RPMI after 72 hours of culture.
- To evaluate the impact of refrigeration storage on leukocytes viability after 72 hours of refrigeration.
- To correlate the cellularity of leukocytes with their viability at 72 hours of culture on RPMI.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

The percentage of healthy, living cells in a sample population is known as cell viability. It is among the most crucial markers for biological assessment in in vitro research. Cell viability and proliferation rates are reliable markers of metabolism and cell health (Sukumaran *et al*., 2023). In all laboratory scales, including cytogenetics, cell viability is a crucial metric. The success of karyotyping is influenced by the cells' capacity to endure and continue to function in vitro. Aged samples, low and high cell counts, and technical errors can have an impact on cultures (Ude *et al*., 2023) A successful karyotyping is achieved when samples are cultured within 24 hours and chances of a success in karyotyping of aged samples are very rare as it will affect the viability of cells. Recently, quality of leukocyte culture is challenged by storage, delay in processing, and varying culture media (Martinovic *et al.*, 2020). Understanding the local and challenges and optimisation of these conditions is verry important to improve diagnostic outcome.

Good slides with adequate and acceptable metaphase spreads are essential for conducting successful cytogenetics investigations. It is accomplished by employing particular chemicals to cultivate cells and stopping them at metaphase (Yahaya *et al*., 2023). According to a study conducted at Liuzhou Maternity and Child Healthcare Hospital in China, the success rate was marginally impacted by refrigeration time of less than 10 days, significantly impacted by refrigeration time of more than 11 days, and marginally improved by an increase in blood volume (Wei, 2024).

Several factors can affect the reliability and quality of cell culture and karyotyping results as well. These include sample handling procedures, the quality of mitogens such as phytohemagglutinin, contamination, incubation time, and the exposure of cells to chemical agents whether environmental, therapeutic, or laboratory-derived. Such factors can negatively impact cell health, viability, mitotic index, and chromosomal integrity, thereby compromising the clarity and accuracy of the karyotype analysis (Weiskirchen *et al*., 2023). Cell cultures produce good metaphase spreads depending on certain conditions such as transit time, type of culture media, availability of Carbon Dioxide (CO_2), agitation, time of introduction of Colcemid,

the time of harvest and other related factors. (Yahaya *et al.,* 2023). The delay in sample dispatching, clotted samples and insufficient volume shown the association with the failure of cell culture for karyotyping and cytogenetic in general, (Fauzia Khan *et al*, 2024). The transit time of the sample will affect the viability of cells and the success in karyotyping of aged sample is almost impossible (Martinovic e*t al.*, 2020). During cell culturing, if these factors are not efficiently controlled, they affect the cell viability and results in delayed diagnosis or give false results.

While karyotyping is done on viable cells, dying cells undergo morphological changes such as fragmentation of the nucleus into small pieces, complete dissolution of the nucleus and irreversible chromatin condensation. These nuclear changes highlight the importance of assessing cell viability prior Karyotyping (Medicus, 2025).Thus, accurately distinguishing between live and dead cells is crucial for ensuring high-quality results and refining laboratory protocols (Kumar, 2023). Different culture media have shown various advantages over the others. In long-term culture, blood cells grown on RPMI have a greater viability rate than cells grown on DMEM and IMDM (Gong *et al*., 2023).

There are numerous ways to evaluate cell viability. Each of the five classes colorimetric, fluorometric, luminometric,cytometric, and dye exclusion assays has advantages and disadvantages over the others. The ideal assays for cell viability should be quick, safe, effective, and economical. It shouldn't obstruct the ongoing experiment (Aslantürk, 2018). Literatures show that fluorescent nuclear staining is the most used viability assay; 83% of respondents and trypan blue exclusion method ;36% (Pierce *et al*., 2021). Trypan blue (C34H28N6O14S4) is a toluidine-derived 960 Dalton molecule. Azidine, Benzamine Blue, Chlorazol and Diamine Blue are synonym for trypan Blue. At Mulago Hospital in Kampala, Cell viability using trypan blue was used to diagnose and manage patients with cryptococal menengitis. During this clinical trial participants were HIV Positive patients with a positive cryptococal antigen test, Their Celebrospinal fluid were stained with 0.04% trypan blue ,using hemocytometer counting , Trypan blue exclusion methode showed a sensitivity of 98% while celebrospinal fluid culture had a sensitivity of 95% (Kwizera *et al*., 2017). Over all trypan exclusion methode have shown to be quick and affordable technique, it can generate consistant data as the widly used lactate deshydrogenase (LDH) assay (Lebeau *et al*., 2019).

2.2. The Cell cycle

Interphase (preparation phase)

G_1 (Gap 1): Before dividing, the cell develops, makes proteins and organelles, and assesses its surroundings.

S (synthesis): DNA replication: two sister chromatids are created from each chromosome.

G_2 (Gap 2): The cell verifies the integrity of its DNA and produces spindle proteins in preparation for mitosis.

Phase M of mitosis: nuclear division

1. Prophase: Spindle fiber formation starts; chromosomes condense.

2. Prometaphase: Spindle fibers cling to kinetochores while the nuclear membrane disintegrates.

3. Metaphase: Chromosome alignment occurs in the metaphase plate, or cell equator, during metaphase, a crucial stage. Attached to spindle fibers from opposing poles are sister chromatids.

Metaphase step is important for karyotyping because at this point, the chromosomes are most compact, distinct, and aligned, which makes it simple to see, take pictures of, and organize them into a karyogram (chromosome chart). Mitotic inhibitors, such as colchicine and colcemid, are frequently used in laboratory karyotyping procedures to stop cells in metaphase.

4. Anaphase: Sister Chromatids split apart and go to opposing poles during anaphase.
5. Telophase: Nuclear envelope reforms and chromosomes decondense.

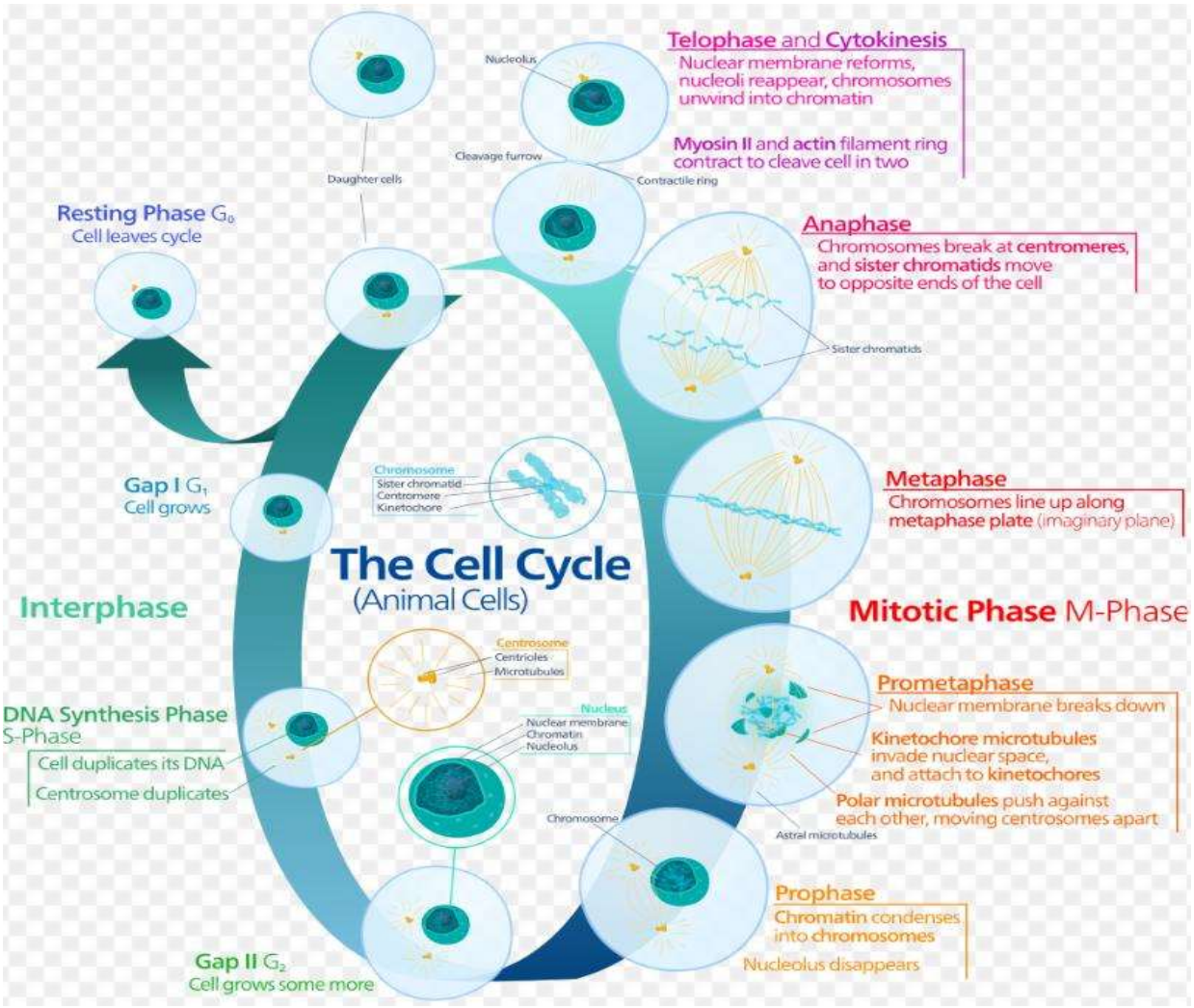


Figure 1: Illustration of the cell cycle

https://en.wikipedia.org/wiki/Mitosis

2.3. Karyotype

2.3.1. History

For almost 40 years, clinical cytogenetic laboratories have employed the cytogenetic procedure known as karyotyping. (Smith *et al*., 2023). It was created in response to the late 19th-century discoveries of chromosomes and their connection to heredity. In 1956, Tjio and Levan utilized Giemsa staining to view the chromosomes in a human cell and generate the first karyotype human chromosome number, which was 46 instead of 48. The primary technique for karyotyping in clinical settings, Giemsa banding, was created Karyotype gives

details on a person's chromosomal complement (de Chadarevian, 2020). These days, human karyotyping is a potent instrument of the human genome that offers fresh perspectives on hereditary illnesses, including cancer. One of its special benefits is that it is one of the few methods that preserves the genome (i.e., it does not necessitate fragmentation and remapping to the reference) and enables the identification of both structural and numerical genomic alterations. It is one of the few methods that allows for an actual count of chromosomes in each metaphase and directly evaluates the ploidy of the nucleus.(Smith *et al*., 2023). After culturing, different banding techniques like Quinacrine or GIEMSA banding are applied, by staining whole chromosomes enbling the identification of particular chromosomal segments and the recognition of chromosomes. A chromosomal formula that encapsulates and characterizes an individual's karyotype is provided by Karyotyipng (Campos-galindo, 2020) In karyotyping, cells are taken from the patients (blood cells, chronic villus cells or amniocentesis cell samples) and grown in culture usually RPMI 1640 in older to have enough cells to analyze. Cells are stimulated to divide treated with colchicine to accumulate mitotic cells. Cells are then treated with hypotonic solutions, fixed, transferred to a slide, and then stained to reveal banding.Low mitotic index, sample type and time elapsed for sample processing and culture conditions affect karyotyping results (Santos *et al.*, 2019)

2.3.2. Principle of Karyotyping

Karyotyping is defined as the microscopic examination of metaphase chromosomes for structural and numerical anomalies linked to disease (Lee, 2025). The banded chromosomes are examined under a microscope by a skilled cytogenetic technician. Representative cells are digitally photographed, and the chromosomes are organized in a karyogram standard pattern. The most often referred specimen type for analysis is peripheral blood (Khan; *et al*., 2024)

2.3.3. Procedure

Karyotyping requires a cell sample, which can be extracted from tissue, amniotic fluid, or blood, among other sources. In culture media, Leukocyte cells are stimulated to proliferate by Phytohemagglutinine. The stage of cell division known as metaphase is when the chromosomes are most compact and visible. The chromosomes are then stained with Giemsa, quinacrine, or

other dyes, producing a characteristic pattern of dark and light bands that allows the chromosomes to be identified and arranged based on size and shape.

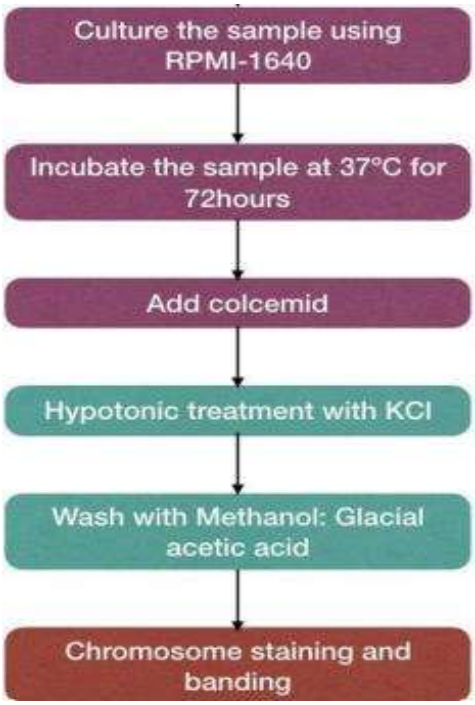


Figure 2: Overview of the steps in PBLC karyotyping process.(Chauhan, 2019)

2.3.4 Limitation of Karyotyping

This test may detect only a tiny section of the genome or the number or shape of chromosomes visible during metaphase. It cannot detect abnormalities that affect the expression of genes, such as mutations or epigenetic modifications. Additionally, karyotyping requires a sufficient number of cells for analysis, which may not be feasible in certain situations, such as when utilizing fetal tissues or early embryos. The limitations of karyotyping in determining an individual's sex are also noteworthy. It can only determine whether or not a Y chromosome is present, which isn't always definitive. In rare cases, karyotyping alone can identify individuals with ambiguous genitalia or variations in the composition of the sex chromosomes. Furthermore, karyotyping has a number of disadvantages for analyzing interspecies evolutionary relationships. It is limited by the resolution of the chromosomal bands, which may not be able to discern more subtle

differences in gene content or chromosome shape. Furthermore, it cannot account for parallel or convergent evolution, where related traits arise separately in many lineages (Lee, 2025)

2.4 Cell Culture

Cell culture is the practice of cultivating prokaryotic, eukaryotic, or plant cells under carefully monitored conditions. Practically refers to the process of cultivating cells that are produced from animal cells. Cells are extracted from an organism and then grown in a controlled, artificially favorable environment. Biomedical research, regenerative medicine, and biotechnological production all make extensive use of cell culture studies. Because animal protection laws prohibit the use of laboratory animals and because the 3Rs (Replacement, Reduction, and Refinement) are strictly enforced, Over the coming years, it is anticipated that the widespread usage of cell lines to replace animal-based research will continue to grow. However, those cell culture procedures are prone to errors if they are not carried out correctly. Therefore, to ensure the repeatability of in vitro experiments, cell culture investigations must be carried out using good cell culture practices (GCCP). Literatures estimate that Approximately 16.1% of published publications employed problematic cell lines. In its most recent registry, which was made public in June 2021, the International Cell Line Authentication Committee (ICLAC) identifies 576 cell lines that have been misdiagnosed or cross-contaminated (Weiskirchen *et al.*, 2023).

2.5 Types of Cell Cultures

There are three categories for cell cultures: initial cell cultures give rise to finite cell lines. The growth rate of these cell lines is sluggish. They undergo senescence after being cultivated in a limited number of cell generations. Transformed and malignant cells are used to create cell lines that develop continuously. Compared to finite cell lines, they divide quickly and reach a far higher density in cell culture. (Weiskirchen *et al*., 2023). Stem cells are pluripotent, undifferentiated cells that come from multicellular organisms. These cells can be activated to generate cells with specific roles under the correct circumstances, or they can be expanded to produce additional cells of the same type indefinitely. In all case, the development of these cells from diverse sources necessitates a synthetic yet regulated environment (media), wherein

occasionally highly specialized media, supplements, and growth factors are required for appropriate cell growth (Weiskirchen *et al.*, 2023).

2.6 Culture Media

2.6.1 Overview of Culture media for animal tissue culture

Using the right cell culture media is essential for maintaining cell culture development and improving the reproducibility of experimental findings. Some cells have special needs like amino acids depending on purposes. These days, several chemically specified formulations have been created to promote the development of numerous well-established cell lines.

The main examples are Dulbecco's Modified Essential Media (DMEM) and Eagle's Minimum Essential Media (MEM) and Roosevelt Park Memorial Institute (RPMI 1640). The vitality of peripheral blood mononuclear cells' (PBMCs') cultured in RPMI or IMDM have shown to be is greater than that of DMEM-cultured cells (Gong *et al*., 2023). Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) media are frequently used to maintain the development of a wide variety of mammalian cells (Weiskirchen *et al*., 2023). The typical medium for isolating and cultivating PBMCs is RPMI. DMEM is regarded as a richer medium than RPMI and serves a variety of functions. Iscove's modified DMEM, a DMEM derivative, is commonly utilized for T cell tests assays (Gong *et al.*, 2023).

2.6.2. Culture media formulation (RPMI)

Table 1: Procedure of culture media preparation

Names	**Manufacturer/ Firm**	**Storage**	**Reagents Preparation procedure**
Culture media (RPMI 1640)	Gibco	Refrigerator(4°-8°C)	RPMI+20%FBS+30% PHA+1%Penstrep
Diluted Colcemide 4x(2.5µl/ml)	Karyomax	Refrigerator 4°-8°C)	10ml Colcemide +30ml RPMI
KCl 0.075M	Gibco	Incubator 37°C	5.6gram+1000ml H2O Distilled
Fixative	Merk	Room Temperature (RT)	3Volumes of Methanol +1 Volume of Acetic Acid

2.7 Application cell Viability

There are numerous uses for cell viability in drug development and scientific researches.

Evaluation of effects of drug candidates on the cells

It can be used to assess a novel drug candidate's cytotoxicity, provide safety information, and choose which candidate to pursue further development. It aids in assessing these items' efficacy and safety to make sure that contact with living cells won't result in damage. Cell viability is a measure of how a chemical affects the health of cells (Sukumaran et al., 2023). By evaluating cell viability, researchers can learn more about the possible harmful effects of medications and medical devices. This allows them to optimize the formulation and design in order to reduce the possibility of negative consequences. Additionally, it contributes significantly to the creation of innovative therapeutic approaches.(Sukumaran *et al*., 2023)

Optimizing cell culture conditions

With the use of cell viability evaluation, researchers can maintain the health and functionality of cells during in vitro investigations, by optimizing the culture conditions for different cell types Cell viability is an indispensable aspect of biocompatibility testing for medical devices and pharmaceutical products. (Sukumaran *et al.*, 2023)

Screening for Potential Therapeutic agents

In order to find new therapeutic medicines, cell viability assays are used to evaluate vast libraries of compounds for their capacity to either promote cell survival or cause cell death. (Sukumaran et *al*., 2023)

Investigating the Mechanisms of cell Death

Assays for cell viability are used to investigate the molecular processes that lead to cell death, giving insight into the biological processes underlying a variety of illnesses and facilitating the creation of focused therapies (Sukumaran *et al.*, 2023). Numerous techniques for evaluating cell viability have been developed during the past century, and they can be categorized in a variety of ways.

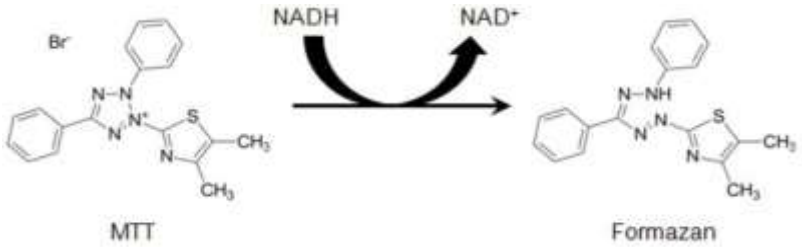
2.8 Classes Cell viability assays

Cell viability assays are grouped under five classes which are; Assays that are colorimetric, fluorometric, luminometric, and cytometric and Dye exclusion assays, each having merit and demerits over the others . Researchers choose the method that best fits their experimental design.(Madorran *et al.*, 2025)

2.8.1 Colorimetric Assays

The 3-(4,5-dimethylthiazol-2-yl)-,5-diphenyltetrazolium bromide assay (MTT)

The MTT experiment is a popular colorimetric technique for determining cell viability is. It measures the cellular metabolic changes using colorimetric shifts. In this test, The enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase transforms soluble MTT tetrazolium dye into insoluble formazan dye, cell viability is evaluated by measuring the reduction activity that takes place in live cells' mitochondria. The quantity of formazan dye crystals is correlated with mitochondrial activity. While dead cells have a lower metabolism or lower MTT reduction levels, proliferating cells exhibit a high rate of MTT conversion. Formazan crystals are dissolved in a solution containing sodium dodecyl sulfate or dimethyl sulfoxide following MTT conversion. A spectrophotometer is used to quantify the concentration of formazan at wavelengths between 540 and 720 nm.



Structures of MTT and colored formazan product.

The 2,3-bis-(-2-Methoxy-4-nitro-5-sulphenyl)-H-tetrazolium-5-carboxanilide) assay (XTT)

Although it depends on the reduction of XTT into a soluble orange formazan product, it is comparable to the MTT test.

Cell viability is shown by the colorimetric changes. The formazan dye's intensity is measured by an ELISA reader. Cell proliferation in response to growth hormones, cytokines, or nutrients is measured by the XTT assay.

MTS (2,3-bis-(2-methox-4-nitro-5-sulphenyl)2H-tetrazolium-5-carboxanilide) assay

Also referred to as the MTS assay, it is used to assess the viability, proliferation, and cytotoxicity of cells. It is employed to evaluate the viability of cells following exposure to cytokines, hormones, medications, and anti-cancer agents. The basic idea behind the test is that tetrazolium reduces living cells to create colorful formazan dye. When phenazine methosulfate is present, living cells' mitochondrial reductase enzymes transform MTS into formazan crystals. Water dissolves the reduced formazan crystals, which are measured by a spectrophotometer at 490-500nm. MTS reagents are stable than that of MTT or XTT

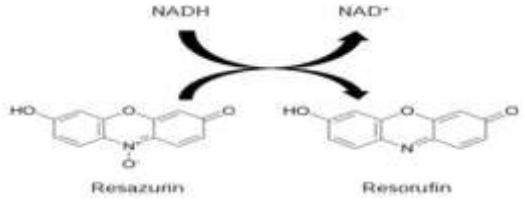
LDH (Lactate Dehydrogenase) Release Assay

An intracellular enzyme called LDH is a commonly utilized technique that is released into the environment when cell membrane disruption occurs. The principle relies on the fact that lactate is converted to pyruvate by LDH when NAD+ (nicotinamide adenine dinucleotide) is present. NAD+ is converted to NADH during this process, and NADH then combines with Tetrazolium salt to form a colorful Formazan product, measured by a spectrophotometer.

2.8.2 Fluorometric Assays

Resazurin reduction /sometimes called Alamar Blue assay.

This technique detects metabolic activity in cells using a fluorometric methodologyResazurin is a cell-permeable, non-toxic phenoxazin-3-one dye. It's non-fluorescent and blue. Resazurin is converted into the red, luminous chemical resorufin once it enters cells. Resazurin in culture media is continuously reduced to Resorfin by mitochondrial NADPH dehydrogenase in living cells.The amount of resolfin directly correlates with the amount of color generated. The quantification on live cells if done by using a microplate reader fluorometer at 560nm (Aslantürk, 2018)



Resazurin substrate structure and the pink fluorescent resorufin product that is produced when live cells are reduced

2.8.3 Luminometric Assays

ATP Assay

Adenosine triphosphate (ATP) measurement is a sensitive technique used in many research domains to assess cell viability, metabolism, and cytotoxicity. Living cells' main source of energy. The principle is the quantification of intracellular ATP a sign of metabolic activities in active cells. It uses the bioluminescent enzyme, Luciferazes which catalyse oxidation Luciferin when ATP is present.

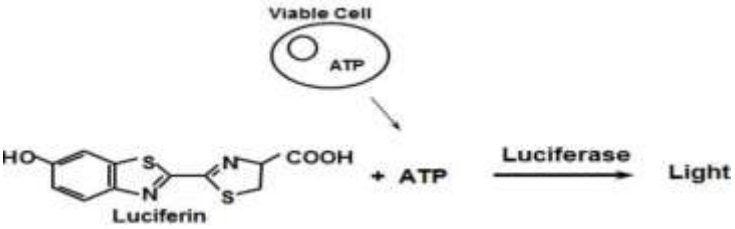


Illustration of how luciferase uses ATP and luciferin as substrates to produce light

Real-Time Assay for Viable cells

A tiny chemical pro-substrate and an engineered luciferase from a marine shrimp are used in the real-time test. As reagents, the pro-substrate and luciferase are introduced straight to the culture medium. Luciferase does not use the pro-substrate as a substrate. The pro-substrate is converted into a substrate by viable cells with an active metabolism, and this substrate diffuses into the culture medium where luciferase consumes it to produce a luminous signal (Promega, 2021).

2.8.4 Flow cytometric Assay

Characterizing or phenotyping the cells in a liquid flow using a laser beam is the basic idea behind flow cytometry. Cells are classified based on their size, granularity, and whether or not they contain a certain fluorescent chemical. (Kamiloglu et al., 2020) .A good example is **Propidium iodide** staining with Annexin V. This technique stain cells with damaged membranes (Propidium iodide) or undergoing apoptosis (Annexin V) using fluorescent dyes. Researchers can ascertain the percentage of living, necrotic, and apoptotic cells by examining the fluorescence of each dye (Sukumaran et al., 2023).

2.8.5 Dye Exclusion Assay

Trypan Blue Exclusion assay

Trypan Blue dye is used in this commonly used assay. Trypan blue (C34H28N6O14S4) is a 960 Dalton molecule that is produced from toluidine. It is a big molecule that is negatively charged. Trypan Blue is interchangeable with Azidine, Benzamine Blue, Chlorazole, and Diamine Blue. The idea is that whereas dead cells allow this dye to enter the cell, living cells' intact cell membranes prevent it from doing so. After mixing the dye with the suspended cells, they are incubated. Researchers use a hemocytometer or automated cell counter to count the stained (dead) and unstained (living) cells after the cells have been incubated with trypan blue dye. This approach is easy to use and fast (Campos-galindo, 2020)

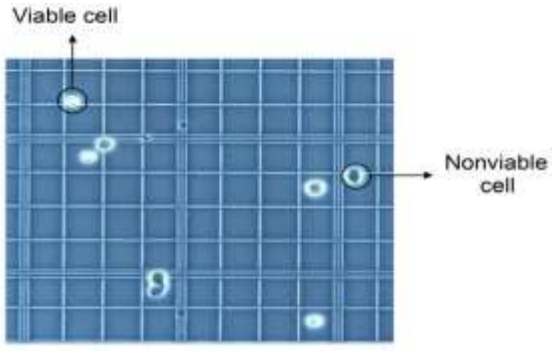


Figure 3: Microscopic illustration of cell viability assessment using the trypan blue assay with Newbauer hemocytometer (Kamiloglu et al., 2020)

.

Advantages: Dead cells turn blue in a matter of seconds after being exposed to the dye, making this technique easy, affordable, and a reliable measure of membrane integrity. The practicality of this technique makes it a commonly used choice in many laboratories. (Kamiloglu *et al*., 2020)

Disadvantages: A hemacytometer is typically used for cell counts. As a result, human counting errors might cause discrepancies (~10%) in the technique. Poor cell dispersion, cell loss during cell dispersion, incorrect cell dilution, incorrect chamber filling, and the presence of air bubbles in the chamber are all reasons for counting inaccuracies (Kamiloglu *et al*., 2020)

CHAPTER 3. METHODOLOGY

3.1 Study Area

This investigation was carried out in the genetic laboratory at the University Teaching Hospital of Butare (CHUB).

3.2 Study Design

The experimental design used in this study is cross-sectional and quantitative. Clinical blood samples from consenting participants referred for karyotyping was collected. Each samples were split into two aliquots, one for viability assessment, and cell count with Sysmex XN-3500 , another for culturing and viability studies after refrigeration. SPSS version 25.0 software was used to analyze the captured Data. Correlation Analysis was done by using Pearson correlation of Cell viability prior and after Cell Culturing on RPMI.

3.3 Sample Size

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 \sigma_d^2}{\Delta^2}$$

n = sample size

$\mathbf{Z_{1-\alpha/2}}$ = Z-score for the desired confidence level (1.96 for 95%)

$\mathbf{Z_{1-\beta}}$ = Z-score for the desired statistical power (0.84 for 80% power)

$\boldsymbol{\sigma_d}$ = standard deviation of the differences between paired observations

$\boldsymbol{\Delta}$ = minimum detectable mean difference

$\mathbf{Z_{1-\alpha/2}}$=1.96

$\mathbf{Z_{1-\beta}}$= 0.84

$\boldsymbol{\sigma_d}$= 6

$\boldsymbol{\Delta}$= 2.4

N= 49 samples which is rounded to 50 samples.

Clinical samples from 25 adult females and 25 males collected in lithium heparin tubes were analyzed for cell viability at 24 hours and leukocyte cell count simultaneously. Results were recorded by excel sheet for further analysis.

3.4 Sampling Method

Clinical samples from 25 adult females and 25 males (16 -60 years old) collected in lithium heparin tubes were selected for the research.

In fact that patients seeking the service of karyotyping are very few, due to scarcity of clients convenient sampling strategy was used. All samples which were referred to the genetic Lab for karyotyping which fulfil the requirements were considered during the period of the study.

3.5. Inclusion criteria

Healthy adult female and females samples from genetic unit within 16 -60 years

3.6 Exclusion Criteria.

Participants with chronic underlying chronic diseases were rejected from the study. The participants with history of recent transfusion were excluded from the study. Children were not part of the study. Patients out of 16 -60 years were excluded.

3.7. Blood sample collection and analysis

3.7.1 Blood sample collection and processing

At the University Teaching Hospital of Butare, 80% of samples for karyotyping are coming from other clinical centers all over the country, this prone the samples to be processed at around 24 hours after sample collection. ml of peripheral blood collected on heparin tubes was used, sysmex XN-3500 hematology analyzer was used to count leukocyte cells according to manufacturer`s instructions. To facilitate cell counting, an aliquot of 100 µl of whole blood cells was simultaneously lysed using ammonium chloride potassium lysis buffer (ACK lysis buffer), which destroyed all red blood cells while leaving only leukocytes intact .

3.7.2 Cell viability assessment procedure

After discarding the supernatant, 50 µl of the suspended leukocytes and 50 µl of 0.4% trypan blue dye were combined for three minutes.10µl of stained cells was transferred onto the counting chamber of improved newbauer cell then counted under Zeiss microcope. The viable cells were counted against the dead ones and recorded. The remaining sample was refrigerated at 4-8°C, 72 hours after refrigeration, the viability of leukocytes was again performed according the procedure

After discarding the supernatant, 50 µl of the suspended leukocytes and 50 µl of 0.4% trypan blue dye were combined for three minutes. 10 µl of stained cells was transferred onto the counting chamber of improved Neubauer cell then counted under Zeiss microscope. The viable cells were counted against the dead ones and recorded. The remaining sample was refrigerated at 4-8°C, 72 hours after refrigeration, the viability of leukocytes was again performed according the procedure

At the same time, Aliquots of 450µl of whole blood was cultured on 5ml of RPMI Gibco, 20% fetal bovine serum (FBS), 30% phytohemagglutinin (PHA), and 1% penicillin and streptomycin (Penstrep) were added to this medium. The culture tubes were then incubated for 72 hours at 37°C. Following a 72-hour incubation period, before adding stop solution, 200µl of cultured cells were centrifuged at 1200 rate per minutes for 10 minutes, the supernatant was discarded. For three minutes, 50 µl of resuspended leukocytes and 50 µl of 0.4% trypan blue were combined. From the mixture 10µl were transferred onto Neubauer hemocytometer for cell counting. Viable cells were counted against the dead ones and recorded by using Zeiss Microscope. The following formula was applied to determine the leukocyte viability.

$$\text{Viable cells (\%)} = \frac{\text{total number of viable cells}}{\text{total number of cells}} \times 100$$

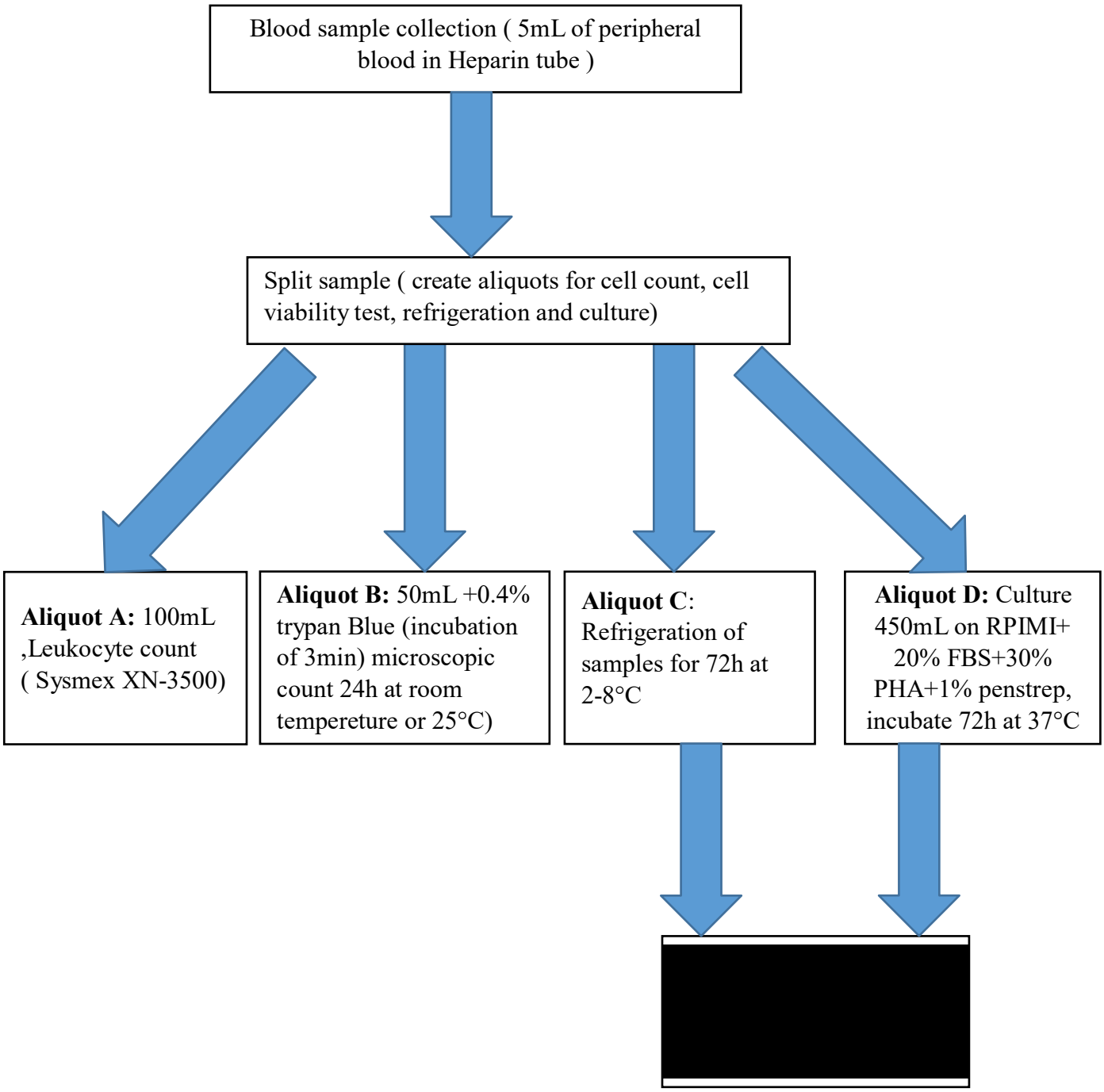


Figure 4: Sample handling & processing workflow

Figure 5: Zeiss Microscope (left side), Trypan blue (in the middle) and Hemocytometer (Improved new Bauer on the right side)

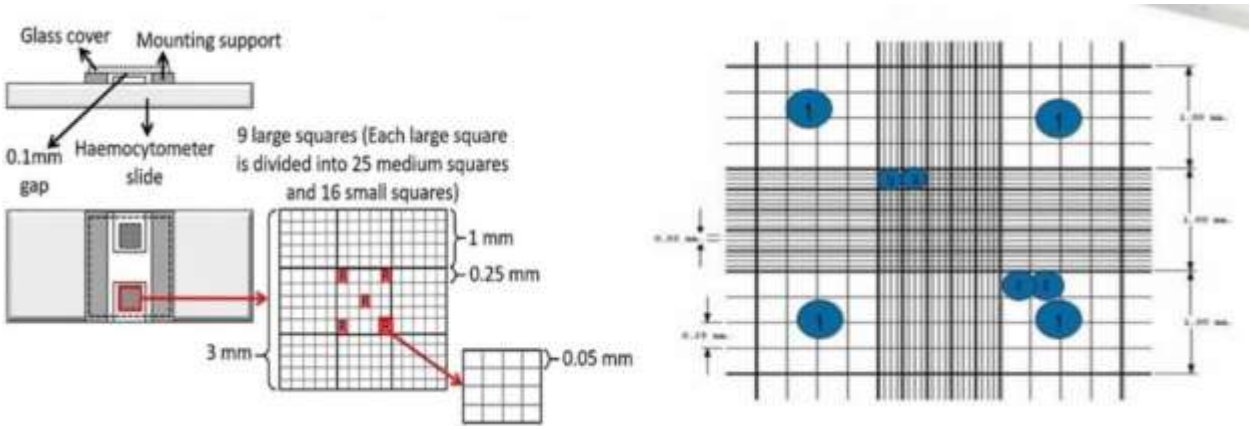


Figure 6: Cell counting guideline

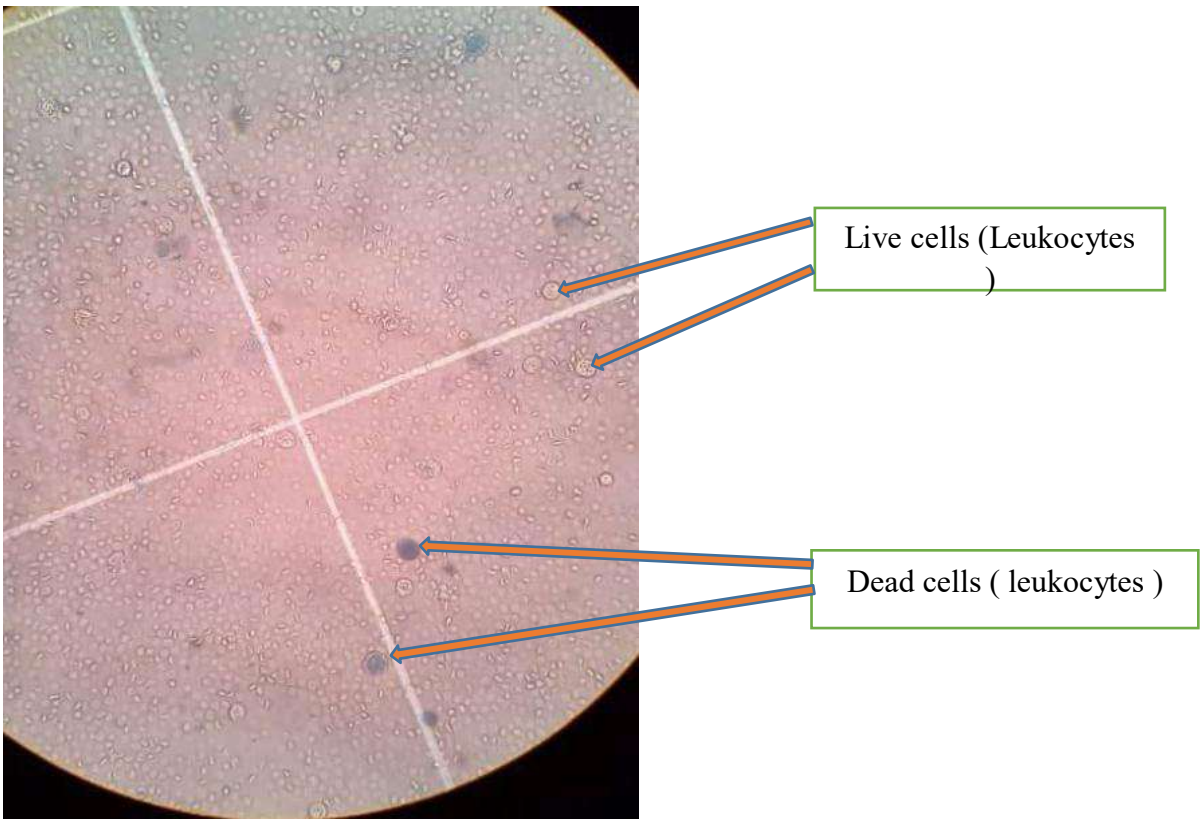


Figure 7: Illustration of live and dead leukcytes under light microcope

CHAPTER 4. PRESENTTION OF THE RESULTS

4.1 Comparison of cell viability after 24 h of blood collection and 72 hours of Culture on RPMI

Leukocytes viability changes after culturing on RPMI with the mean of viability of 86.50 and 99.84 at 24 and after 72hrs respectively. This shows a high viability of cell harvested after 72 hrs of culture on RPMI. The degree to which the value deviates from the mean is indicated by the standard deviation. There is more viability among samples (6.450) culture at room temperature for 24h on while there is a tight consistent value near the mean (0.510) for cell cultured on RPMI 72h culture. Sample kept at room temperature for 24hrs, standard error mean = 0.912, samples at 72hrs on RPMI, standard error mean = 0.072, this is more precise error mean suggest that leukocytes viability is improved when cultured on RPMI.

Table 2: Comparison of Cell viability at 24 hours and 72 hours of Culture on RPMI

	Mean	Std. Deviation	Std. Error Mean
Cell viability at 24 hours of blood collection in %	86.5	6.45	0.912
cell Viability harvested at 72 hours on RPMI (%)	99.84	0.51	0.072

4.2. Paired samples t-Test for cell viability at 24 hour room temperature and 72hours of culture on RPMI

After 72 hours in RPMI culture, the mean leukocyte viability was 13.34% greater than it was at room temperature for 24 hours (SD = 6.41, SEM = 0.906). The significance of the difference is confirmed by an extremely small p-value ($p < 0.001$) and a big t-value (14.716).

Table 3: Paired samples t-Test

	Mean	Std. Deviation	t	Sig. (2-tailed)
Cell viability at 24 hours of blood collection in % - cell Viability (%) at 72 hours on RPMI	13.34	6.41	14.72	0

4.3 Comparison of viability between 24hours and 72hours of refrigeration (4-8°C)

The table 4 below shows comparison of viability of leukocytes at 24 hours after blood collection and viability after 72hours of refrigeration (4°C-8°C). The average of leukocyte viability is 86.50% at 24hrs after blood draw (fresh sample) and 82.30% after 72 hours of refrigeration at 4-8°C. This highlights a decrease in viability by 4.2% due to refrigeration. There is a remarkable variability in viability after refrigeration marked by standard deviation.

Table 4: Comparison of Leukocyte Viability at 24 and 72 hours of refrigeration using paired sample descriptive statistics

	Mean	N	Std. Deviation	Std.Error Mean
Viability at 24 hours after blood collection in %	86.5	50	6.45	0.912
Viability of cells (%) after 72 hours of refrigeration at 4 °C -8°C	82.3	50	8.069	1.141

4.4 Correlation: of cell Viability at 24 h at room temperature vs. 72 h Refrigeration

Leukocyte Viability at 24 and 72 Hours of Refrigeration Shows a Statistically Significant Positive Association. The degree and direction of the association between the two variables are shown by this Pearson correlation coefficient, which goes from -1 to + 1. This 0.697 is a strong positive correlation.

This means samples with higher viability at 24hr tend to have a higher viability at refrigeration. As p=000 which is <0.05, the correlation is statistically significant which indicate that Two variables' association is not the result of chance.

Table 5: Correlation of paired samples' leukocyte viability 24 hours after collection and 72 hours after Refrigeration (4 °C–8 °C)

	N	Correlation	Sig.
Viability at 24 hours after blood collection in % & Viability of cells (%) after 72 hours of refrigeration at 4 °C -8°C	50	0.697	0

4.5. Leukocyte viability significantly decreased after 72 hours of refrigeration as compared to 24 hours after collection, according to paired samples t-test results.

The table below shows p<000. This indicate that the viability at 24hrs is higher than at 72hours of refrigeration. Leukocytes decreases slightly but is consistent. This paired sample t-test showed a statistically significant reduction in leukocyte viability after 72 hours of refrigeration compared to 24hrs post-collection, where t(49) is 5.084, and p<0.001. This indicates leukocyte viability decline after 72hours of refrigeration at 4-8°C compared to fresh samples. This suggest that a prolonged refrigeration lead to a reduction in cell viability

Table 6: Paired Differences Confidence Intervals between cell viability at 24 hours

	Mean	Std. dev	t	Sig. (2-tailed
Viability at 24 hours after blood collection in % - Viability of cells (%) after 72 hours of refrigeration at 4 °C -8°C	4.2	5.841	5.084	0

4.6 Significance of Correlation between Cultured Leukocyte Count and Viability After 72

The table 7 shows the correlation between cultured leukocytes and the viability (%) of harvested cells at 72hours of culture at RPMI. This person correlation coefficient (.059) is almost closer to zero and p-value is 0.684 which is greater than 0.05. Since this link is not statistically significant, the quantity of cultivated cells and the leukocyte viability at 72 hours in RPMI do not correlate linearly.

Table 7: Correlation of Cellularity with Cell Viability after 72 hours of culture in RPMI

	N	Correlation	Sig.
The total cultured cells (cells /450µL) & Viability of cells harvested at 72 hours on RPMI) in %	50	0.059	0.684

CHAPTER 5. DISCUSSIONS

This study aimed to assess the differences in leukocyte viability between the whole blood at room temperature for 24 hours and leukocytes cultured on RPMI 1640 medium for 72hours. The findings show that culturing leukocytes in RPMI improve their viability. These results are in line with previous research that highlights the function of enriched medium, such as RPMI 1640, in preserving cell viability in vitro. According to Gong *et al*., 2023, at Roswell Park Memorial Institute (RPMI), human peripheral blood mononuclear cells (PBMCs) from five healthy individuals were cultivated in Dulbecco's minimum essential medium (DMEM) and Iscove's modified DMEM (IMDM) without additional stimulation. On the first day, they found that PBMC viability was greater than 80% across all culture media, with only slight variations depending on the medium. The viability declined with time across all mediums. PBMCs cultivated in DMEM showed a more noticeable decline in viability on days 7 and 10 compared to cells cultured in RPMI and IMDM. Of the five donors, all four exhibited this tendency. Based on their research, they concluded that RPMI is the industry standard for PBMC isolation and culture. DMEM serves several functions and is thought to be a richer medium than RPMI. A common T cell assay product is IMDM, a variant of DMEM.(Gong *et al.*, 2023).

After the comparison of viability of leukocytes at 24 hours after blood collection and viability after 72hours of refrigeration (4°C-8°C), the results showed that the average of leukocyte viability is 86.50% at 24hrs after blood draw (fresh sample) and 82.30% after 72 hours of refrigeration at 4-8°C. This highlights a decrease in viability by 4.2% due to refrigeration (p=000°.This means that samples with higher initial viability tend to maintain the higher viability even after being refrigerated at 72hrs. This was supported by (Jerram et al., 2021) during the study; *Effects of storage time and temperature on highly multiparametric flow analysis of peripheral blood samples; implications for clinical trial samples* .They discovered that the viability of recovered PBMC subsets is compromised and their composition is changed when blood is refrigerated before processing.

A prolonged refrigeration lead to a reduction in leukocytes viability: The Similar literature was published by (Tantikositruj *et al*., 2021). In his research: Comparison of freshly extracted chicken peripheral blood mononuclear cells with blood that has been refrigerated for 24 hours,

Determining the quality and quantity of chicken peripheral blood mononuclear cells (PBMCs) isolated from freshly collected blood and blood that had been refrigerated for 24 hours was the goal of his investigation. Furthermore, the ability of PBMCs grown in medium to survive was examined. PBMCs isolated from fresh blood had a 95% viability rate, which was significantly higher than that of blood that had been chilled for 24 hours (90–92%). Additionally, the viability of PBMCs recovered from both blood samples declined from 90–95% to 60–65% over time. He came to the conclusion that blood-derived PBMCs were far more viable than blood PBMCs that were refrigerated for 24 hours. Additionally, PBMC viability declined dramatically over time.

The analysis show no correlation between the cellularity and the cell viability of leukocytes after culture 72 hours of culturing on RPMI, meaning that the leukocyte count of a sample will not predict the leukocyte viability after culture on RPMI. Martinovic et al have found the different results in their study: The Fundamentals to Minimize the Culture Failure in Hematological Malignancies, 23,8% of samples, had high cell count and culture failure as the majority of cells cannot divide and it will inhibit the other cell to divide(Martinovic *et al*., 2020)

CHAP 6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

RPMI improves significantly the viability of leukocytes at 72 hours of culture. This improved viability is due to the hi nutrient content in RPMI like Fetal Bovine Serum, Glutamine or other aminoacids with the controlled pH. Long term Refrigeration of leukocytes at 4-8°C affect leukocytes viability for karyotyping. This could be due to loss of blood composition
According to the results obtained, cellularity of leukocytes of fresh samples at 24 hours of blood collection cannot predict the cell viability of leukocytes isolated from RPMI 1640 cultures

6.2 Recommendations

Research on the impact of prolonged refrigeration (15–15 days) on leukocyte viability as well as additional research to examine the relationship between the cellularity of prediluted samples (serial dilutions) and the leukocyte viability of cells following isolation from prolonged RPMI Culture should be conducted to check for local line production

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APPENDICES

1.APPROVAL

This is to certify that this thesis has been developed under our supervision and that it is bearing Submitted with supervisor's approval.

Supervisor 1:

Dr. Jeanne P. UYISENGA

SIGNATURE… Date 18/09/2025

Supervisor 2:

Dr. TOLESSA MULETA DABA (PhD)

SIGNATURE…….…….. Date 18/09/2025

2. Ethical Clearence

chub.rw

CHUB

RESEARCH

CLINICAL EDUCATION AND RESEARCH DIVISION
DIRECTORATE: RESEARCH -ETHICS COMMITTEE

Huye,23rd, July,2025

Approval Notice: No: REC/CHUB/090/2025
GASORE Jean Pierre
Email: gasorepi@gmail.com

Reference is made to your letter requesting ethical clearance for **"Assessment of human leukocyte viability in vitro cell culture conditions at CHUB"** Having reviewed your application and been satisfied with your protocol, your study is hereby granted ethical clearance and should be conducted within the University Teaching Hospital of Butare. Please note that approval of the protocol and consent form is valid for one year starting on the issue date and shall be renewed on request. You are responsible for fulfilling the following requirements:

- Changes, amendments and addenda to the protocol or consent form must be submitted to the committee for review and approval before activation of the changes
- Only approved consent forms are to be used in the enrollment of participants
- All consent forms signed by subjects should be retained on file.
- The committee may conduct audits of all study records. Consent documentation may be part of such audits
- A continuing review application must be submitted to the committee in a timely fashion and before the expiry of this approval
- Failure to submit a continuing review application results in termination of the study
- Notify the committee once the study is finished
- Identification of participants must be kept confidential for the duration of the study

Sincerely

Dr. HABIMANA Emmanuel
Chairperson of Ethics Committee/CHUB

RESEARCH ETHICS COMMITTEE (CNUBREC) UNIVERSITY TEACHING HOSPITAL OF BUTARE

Cc: - Director General
- Head of Clinical Education and Research Division
- Head of Clinical Service Division
- Director of DTS
- Head of Pathology Department

3. Data collection tool

SN	Cell count (Leukocytes/µL)	Number of Live Cells at 24h	Number of dead Cells at 24 h	Number of live cells at 72(refrigerated cells)	Number of live cells(haversted at 72)	Number of live cells(haversted at 72	Comments
1							
2							
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ASSESSMENT OF HUMAN LEUKOCYTE VIABILITY IN VITRO CELL CULTURE CONDITIONS AT THE UNIVERSITY TEACHING HOSPITAL OF BUTARE (CHUB)

by Jean Pierre Gasore

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